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Oleoylethanolamide in the gut-brain axis

A Dissertation in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmacology and Toxicology

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To all our students,
thank you.

Try to realize it's all within yourself,
no one else can make a change.
(*"Within you without you"*, The Beatles)

Now, here, you see, it takes all the running you can do to keep you in the same place.
If you want to get somewhere else, you must run at least twice as fast!
(*"Alice through the looking glass"*, Lewis Carroll)

For certain, you have to be lost to find a place that can't be found, elseways everyone would know where it was.
(*"Pirates of the Caribbean: at world's end"*, Captain Barbossa)

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Chapter 1:
General introduction

1.1 Obesity

According to the World Health Organization (WHO), obesity is the world's most widespread chronic pathological conditions, to the point that it has been defined as a global epidemic[1]. In fact, in the past years, overweight and obesity have reached epidemic proportions, with 1,9 billion overweight adults, 600 million obese adults and over 100 milion obese children[2]. Obesity is a complex condition that leads to the impairment of the quality of life, and acts as a risk factor for the development of other diseases, such as cardiovascular diseases, diabetes, and hypertension[3,4].

Obesity is characterized by the excessive accumulation of body fat in various districts of the body. The most used parameter to define the severity of obesity is the body mass index (BMI), obtained by dividing the weight of the person expressed in kilograms by the squared height expressed in meters. The value obtained will fall in a category defining a pathological condition, as follows:

- BMI < 16,5: severely underweight
- 16,5 < BMI > 18,4: underweight
- 18,5 < BMI > 24,9: normal weight
- 25 < BMI > 29,9: overweight
- 30 < BMI > 34,9: obese I class (moderate obesity)
- 35 < BMI > 39,8: obese II class (severe obesity)
- BMI > 40: obese III class (very severely obese)

However, even though it is the most used index to describe body mass and obesity, BMI does not take into account other factors that influence weight, such as gender, age, and the percentage of lean/fat mass. Therefore, this index is usually accompained by the measurment of abdominal circumference, since the accumulation of fat in the visceral area is correlated to cardiovascular and metabolic disorders[5].

Many factors contribute to the development of obesity, such as the uncontrolled consumption of foods rich in fats and sugars, sedentary lifestyle,

and genetic background[6]. Hence, obesity is considered a multifactorial pathological condition, that can be linked to a chronic disruption of energy balance, defined as the ratio between the energy assumed through food consumption and the energy expenditure (basal metabolism, body temperature maintenance, physical activity). Therefore, when energy intake exceeds the energy expenditure, the excessive energy can be stored as fat, laying the basis for the development of obesity[7,8]. Energy balance is controlled by multiple physiological mechanisms, that involve a plethora of signals that, from the periphery, communicate with the brain, and viceversa[9]. Many organs partake in this intricated interplay, such as adipose tissue (that acts as storage), liver (the center for lipid and glucose metabolism)[10], and central nervous system (CNS), that acts as an integration center for all the signals conveyed from the periphery, that will result in a behavioral response[11] (Fig. 1.1). Therefore, when these mechanisms are altered they may result in the onset of a pathological condition, for instance anorexia (with a low intake of energy) or obesity (with an excessive intake of energy)[12,13].

During the past few years, an increasing number of evidence demonstrated that obesity is not only the result of disrupted physiological patterns, but also environmental, social, and behavioral factors play a crucial role in the regulation of energy balance and fat accumulation[14]. For instance, the availability of calorie-dense foods, such as snacks, the reduction of physical activity due to a sedentary lifestyle, together with unhealthy eating habits, are all considered as pivotal factors for the development of obesity[15]. To date, an improvement of lifestyle, the introduction of healthier eating habits, and an increase of physical activity are the most effective ways to prevent the onset of obesity and eating-related disorders[16]. However, since the European Association for the Study of Obesity (EASO) estimated that 3,3 billion people will be overweight by 2030[14], novel pharmacological approaches that would allow to regulate the metabolic alterations associated to this pathology are needed, in particular to control overfeeding. Therefore, in the past years, research has focused on investigating the mechanisms involved in the control of feeding and energy balance, that has led to the discovery of a variety of signaling pathways[17,18], that are organized in a complex network of

heterogeneous molecules[19–24], among which N-acylethanolamines (NAEs)[25–28] and N-acylphosphatidylethanolamides[29] (NAPEs) have gained a great deal of attention.

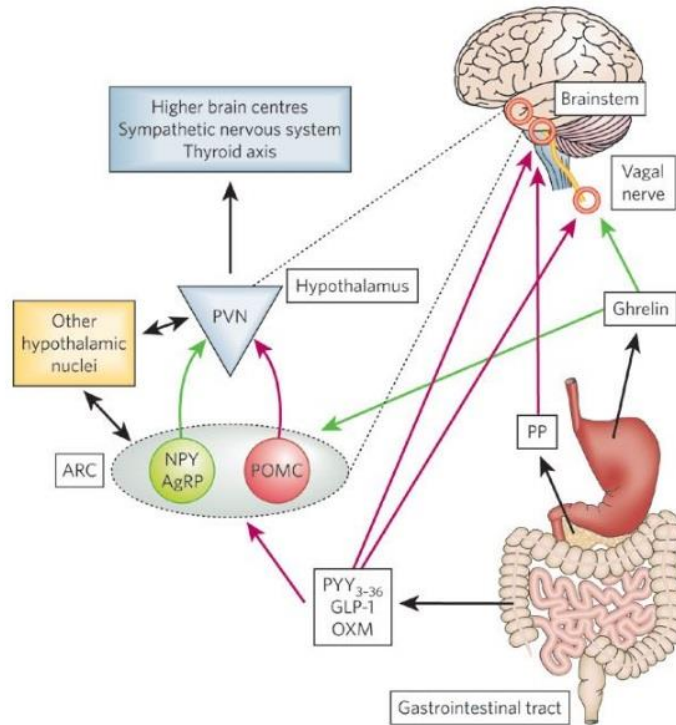

Fig 1.1: The pathways by which gut hormones regulate energy homeostasis. Schematic representation of the main pathways of the gut-brain axis. (Kevin G. Murphy & Stephen R. Bloom, *Gut hormones and the regulation of energy homeostasis*, Nature volume 444, pages 854–859)

1.2 The gut-brain axis

The consumption of a quantity of calories that is sufficient to satisfy the energetic requirements of the body is of extreme importance. In fact, organisms that are able to regulate the intake of food based on the necessities are evolutionary selected[30]. The reduction of the caloric intake or the increase of the energetic consumption lead to the activation of orexiant signals, that stimulate food intake and body weight gain. On the other hand, fat accumulation and the increase of body weight induce the release of anorexiant mediators, that decrease the caloric intake and the exploitation of fat storage[31].

The gastrointestinal (GI) tract is innervated by the autonomic nervous system (ANS), that is divided in the parasympathetic and sympathetic divisions. The main nerves of the former are the vagus and the pelvic nerves, that exert and inhibitory tone on the GI tract; the main nerves of latter are the splanchnic nerves that have an exitatory effect on the digestive system.

Then, a third component of the ANS was added, composed of the net of neurons located in the myenteric plexus[32], called enteric nervous system (ENS)[33]. In general, the parasympathetic efferent nervous fibers of the gut-brain axis, in particular vagal and pelvic efferents, are the main ways through which the activity of the ENS is regulated by the CNS during the digestive phase, whereas the sympathetic (splanchnic) efferent neurons are involved in the regulation of nociception and stress response[34].

The afferent fibers of the gut-brain axis are mainly represented by vagal afferents and spinal nerves, for the parasympathetic and sympathetic components, respectively. They convey to the CNS the stimuli produced in the intestine, that can be mechanical (distention and contraction) or chemical, such as nutrients in the intestinal lumen, hormonal stimuli, neurotransmitters, neuromodulators, citokynes and other mediators of inflammation[35].

The ingestion of food exerts the release of hormones and peptidic mediators, the modulation of the gastrointestinal motility and of bilio-pancreatic secretions[36].

Incretins, such as the glucagone-like peptide-1 (GLP-1), and other hormones, like the peptide YY (PYY) and oxyntomodulin (OXM), secreted in the small intestine, inhibit the cephalic phase of digestion through vagal stimulation[37,38]. Moreover, other intestinal hormones, such as colecystokinin (CCK) and the gastric inhibitory peptide (GIP) inhibit gastric motility both by relaxing the fundus of the stomach and stimulating the contraction of the pylorus. These actions slow down gastric emptiyng, increase the duration of the digestion and of satiety, that, altogether, reduce caloric intake[36,39].

Since the vagus nerve innervates the majority of the GI tract, it plays a pivotal role in the regulation of the energy intake, hunger and satiety[40]. In fact, the pharmacological or surgical lesion of the vagus reduces the amount of food consumed in a meal and increases meal duration[38,41]. Moreover, it has

been demonstrated that a low frequency stimulation of the vagus induces a reduction of food intake[38]. However, the manipulation of the vagus nerve is paricularly difficult due to the anatomic connections between vagal, sympathetic and ENS fibers[38].

Circulating nutrients, that reflect the levels of nutrients in the periphery, are detected by the area postrema (AP), a circumventricular organ that lacks a functional blood-brain barrier (BBB) located on the floor of the fourth ventricle[42], that, in turn, activates other nuclei in the brainstem[41]. In response to these stimuli, the brainstem, thanks to the activity of vagal efferent fibers, plays a crucial role in the control of the ENS, thus modulating many functions of the GI tract, and in the activation of neural circuits in the hypothalamus to reduce food intake. In particular, vagal afferents convey informations from the periphery to the nucleus of the solitary tract (NST)[43], that, in turn, projects to hypothalamic nuclei[43,44]. The hypothalamus comunicates with vagal efferents, whose cell bodies are located in the dorsal motor nucleus of the vagus (DMV), through which it can slow gastric emptiyng[32,45]. Altogether, AP, NST and DMV form the dorsal vagal complex (DVC).

In the hypothalamus, a variety of molecules and receptors involved in the control of appetite are produced, in particular endocannabinoids (ECs)[46], neuropeptide Y (NPY), pro-opiomelanocortin (POMC), alpha melanocyte-stimulating hormone (α-MSH), Agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), CCK, and GLP-1[36,47].

The endocannabinoid system (ECS) plays a crucial role in the regulation of energy homeostasis and feeding behavior. The receptors for ECs are in fact largely expressed in the CNS, in particular in the hypothalamus and brainstem, and in the periphery, in organs that are important for the metabolism, such as liver, pancreas, muscle and adipose tissue[48,49]. Moreover, ECS is involved in many physiological aspects, such as the modulation of appetite and reward, lipid storage, energy consumption and insulin homeostasis[50–52].

1.2.1 The vagus nerve

The vagus nerve is the tenth cranial nerve and innervates mainly the thoracic and abdominal cavities. Vagal fibers are not only composed of nerves but contains also glial and dendritic cells belonging to the immune system[53]. Moreover, paraganglia are often in close contact with its branches[54,55] (Fig 1.2).

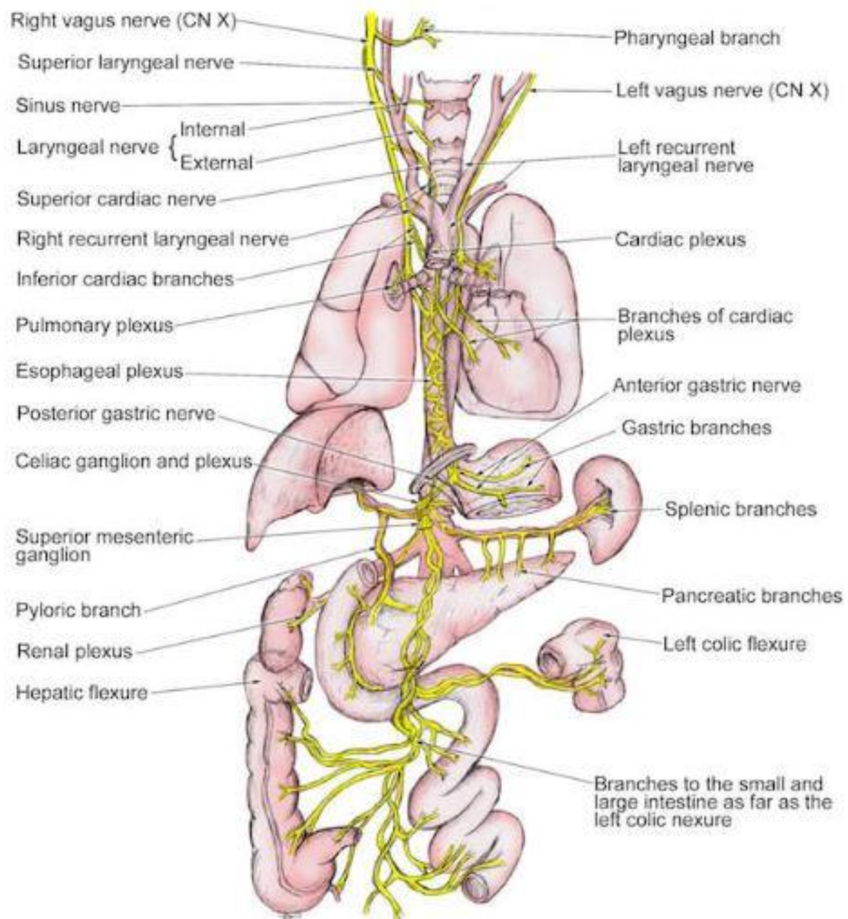

Fig. 1.2: Anatomy of the vagus nerve. Representation of the main branches of the vagus nerve.

Cervical and thoracic vagus

The right and left branches of the cervical portion of the vagus nerve are both made of afferent and efferent fibers, that leave the skull from the jugular

foramen. Outside of the skull, the jugular (proximal) and nodose (distal) ganglions are located, where the cell bodies of sensitive neurons can be found. At this level, the vagus runs along the carotid artery and branches at the jugular ganglion into the auricular and meningeal branches, that provide sensorial innervation to the skin of the external acoustic meatus and to the dura of the posterior cranial fossa, respectively. Then, the pharyngeal branch departs at the nodose ganglion, and from its caudal part the superior laryngeal nerve branches, and runs under the carotid artery to the larynx, providing also smaller branches that innervate the caudal part of the pharynx and the esophagus. A cardiac cervical brach departs either from the cervical portion of the vagus or from the laryngeal nerve. This branch is called aortic[56] and contains a large number of afferent fibers receiveng stimuli from the baroceptors in the aortic arch. The laryngeal nerve departs at the level of the subclavian artery in the right part of the body, and at the level of the aortic arch in the left, then running along and innervating the trachea and the exophagus. Then, the fibres that innervate bronchi, lungs, and heart branch in the superior mediastinum.

In particular, the afferent fibers that depart from the jugular ganglion produce neuroactvie peptides, such as substance P or Calcitonin Gene Related Peptide (CGRP), whereas those that depart from the nodose ganglion do not produce these molecules[57,58].

Moreover, abdominal and thoracic branches contain afferent or efferent fibers that decussate from one side to the other[54,59,60]. In the rat, there are few lines of retrotracing evidences about decussating efferent branches, however, possibly 20% of afferent branches may decussate in the thoracic cavity[61].

Abdominal vagus

The anterior (or ventral) portion, along with the left cervial vagus, branches in the common hepatic, gastric and coeliac portions. In the rat, there are about 11000 nervous fibers in each subdiaphragmatic branch, of which about 8000 are afferent and 3000 efferent. Moreover, less than 1% of all the fibers is myelinated. The common hepatic branch is important for the communication between the immune and nervous systems and for the thermoregulation, and

contains about 3000 fibres, of which 2200 are afferent, 200 efferent and 600 non-vagal[62]. The terminations of vagal afferents end in the connective tissue around the intrahepatic triads, extrahepatic bile ducts, portal vein and paraganglions[63]. Other afferents run along the common hepatic branch and, to a lesser extent, along the periarterial plexus of the common hepatic artery, around the portal vein[63]. Bile ducts are more innervated compared to portal vein: here, vagal afferents are tightly connected to the main branches of bile ducts and are found also in the walls of intra- and extra- biliary ducts[63]. This branch is called hepatic, but it innervates also the pylorus, the pancreas and the proximal duodenum[64,65]. Once the hepatic branch reaches the hepatic proper artery, it moves towards the hepatic common and gastroduodenal arteries, and then it divides in two branches, one following the right gastric artery (to the stomach), an the other following the dorsal and ventral duodeno-pancreatic arteries (to the proximal duodenum and pacreas). The anterior gastric branch innervates the ventral part of the stomach and, through small fibers inside the circular muscle of the pylorus, resches the duodenum.

The dorsal (posterior) part of the gastric branch, on the other side, enters the dorsal part of the stomach, near the cardias, running along the left gastric artery, and innervates the proximal duodenum through trans-pyloric fibers. In the stomach, vagal nerves are in both layers of smooth muscle, in the myenteric plexus and in the lamina propria. The fibers located in the longitudinal and circular layers have been described as long axon bundles parallel to muscles and connected by short branches[64,65].

The most common vagal afferent ending is the intraganglionic laminar ending (IGLE), mainly linked to the neurons of the myenteric plexus[66]. This type of neuron is also observed in the whole intestine, both small and large, where vagal afferents penetrate through the myenteric plexus to the circular smooth muscle and the submucosa, to form a net of axon branches in the lamina propria. Thus, it has been hypothesized may function as distention receptor[67,68]. Then, the ventral celiac branch, after leaving the ventral portion of the esophagus, reaches the dorsla celiac branch near the left gastric artery. Both these branches go down the celiac artery to reach the celiac ganglion,

and then the small and large intestine running along the superior mesenteric artery.

Role of the vagus nerve in the homeostatic control of energy balance

The intestine constantly sends signals about the quantity and quality of the nutrients to the CNS, that is able to process a variety of inputs and to transform them in information of various nature, that will determine a behavioral response. These processes can engage either the somatic or the autonomous nervous systems, the former being mainly controlled by the prefrontal cortex (PFC), hippocampus (Hippo) and nucleus accumbens (NAcc) and being influenced by the emotional cues linked to feeding; the latter, on the other hand, is more involved in the homeostatic control of energy balance. In particular, the parasympathetic nervous system, composed of afferent (sensitive) fibres that convey signals to the CNS, and efferent (motor) fibres, that convey signals from the CNS to the parasympathetic ganglions near target organs, receive stimuli from the brainstem, and in particular from the DVC and pars reticolata[10,69]. This bidirectional pathway, or vago-vagal reflex, is the major extrinsic neural way involved in the control of pancreatic and GI tract functions[70]. The main stimuli received by vagal afferents are:

Mechanic stimuli. The GI tract is rich in mechanoreceptors, in particular located in the mucosa, sensitive to friction[71], and on the outer muscle layer, sentitive to distention, such as IGLE fibres[72].

Chemicals and nutrients. In the rat, the majority of vagal fibers that innervate the duodenum respond, apart from mechanic stimuli, to chemical stimuli, such as pH and osmolality[70]. Moreover, these fibres respond to peptides acting in the intestine, such as CCK[73,74], GLP-1[75], serotonin (5-HT)[76], somatostatin[75] and interleukin-β (IL-β)[77].

Other stimuli. Vagal afferents are also sensitive to temperature, osmotic pressure[74] and pain. For example, a prolonged gastric distention activates vagal afferents and induces Fos in the NST[78].

Vagal afferent teminals are mainly found, in the CNS, in the NST, AP, and, to a lesser extent, in the DMV and in the trigeminus. The terminations of

the cardiac and pulmonar vagal afferents are located in the lateral subnuclei of the NST, whereas the terminations of the alimentary canal are found in the medial subnuclei of the NST[79–81]. Moreover, many of vagal afferents of the jugular ganglion produce peptides such as CGRP and substance P[82,83], whereas glutammate has been found in cardiac afferents. In the same way, it has been demonstrated that pharmacological blockade of NMDA receptors is able to abrogate gustative inputs in the NST[84]. In fact, many studies suggest that ionotropic glutammate receptors, including NMDA, in the NST play a crucial role in the control of eating. For example, the blockade of NMDA receptors with a microinjection of dizocilpine, a non-competitive antagonist of this receptor, leads to a delay of the onset of satiety and to an increase of food consumption[85]. Moreover, the electric stimulation of the NST results in a current (either eccitatory or inhibitory) in the DMV, suggesting a connection between these two areas[86]. Moreover, pharmacological and histological evidences showed the presence of glutammate receptors in the DMV[87], where the cell bodies of the neurons that innervate the GI tract are located[88,89]. In this area, two distinct populations of preganglionic neurons modulate the GI functions: on one hand cholinergic neurons, located in the rostral DMV and increase gastric motility[90]; on the other, non-adrenergic non-cholinergic (NANC) fibres, located in the caudal DMV and that decrease GI motility[90,91]. Therefore, the DMV is characterized by two parallel neuronal pathways that modulate gastric motility: an exitatory cholinergic pathway that increases gastric motility, and an inhibitory NANC pathway that slows gastric functions. Intraganglionic vagal afferent terminals are located in the capsule of connective tissue of the ganglions of the myenteric plexus, between the longitudinal and circular layers of smooth muscle[32], and, therefore, respond to passive distention and active contractions of the muscles[72]. This type of vagal afferent has been found in the esophagus and along the GI tract[92,93].

Intramuscolar bundles are almost exclusively located in the longitudinal and circular layers of the stomach smooth muscle[64], where the presence of food is detected also by vagal afferents found in the gastric mucosa, sensitive to mechanical stimuli[94], whereas the quantity of ingested food is detected by vagal afferents located in the outer muscle layer, sensitive

to distention[95]. Moreover, vagal afferents of the gastric mucosa can respond to hormones released locally, such as leptin and ghrelin[96]. In fact, vagal afferents express leptin receptors[97,98]. In addition, the appetite-stimulating effects of ghrelin are abolished by total subdiaphragmatic vagotomy (TVX) or the treatment with capsaicin in rats[99]. Hence, since vagal neurons located in the nodose ganglia express ghrelin receptor[99,100], it maybe hypothesized that ghrelin, like leptin, acts through the activation of vagal afferents.

Several studies suggest that the inhibitory effects on feeding mediated by CCK[101] involve vagal afferents, that express the CCK receptor A (CCKA)[102,103]. In the periphery, these neuronal terminals are located in the walls of the GI tract, both in the mucosa and in the muscolar layer[104]. Moreover, it has been demonstrated that the inhibition of gastric emptying is mediated by CCKA-expressing vagal afferents[105]. In addition, it has been observed that a total or selective vagotomy abolished or decreased, respectively, the satiety effect of CCK[106]. Furthermore, another important study demonstrated that the treatment with capsaicin, that destroys unmyelinated fibres (including unmyelinated vagal fibres), dampened the satiety effect of CCK[107].

Food intake, and, in particular, the intake of carbohydrates, fats and proteins, induces the release of GLP-1, that seems to be induced indirectly by the stimulation of the nerves of the apical portion of the intestine, or directly by the contact with the lower portion of the intestine[108,109]. Since GLP-1 modulates pancreatic secretions, it plays a crucial role in glucidic homeostasis[110,111]. The peripheral administration of GLP-1 induces satiety in rats and humans[112,113]. This effect may be due to the paracrine stimulation of the gastric mucosa by vagal afferents: in fact, GLP-1 receptor is expressed in the nodose ganglion, and, moreover, in the CNS, in the AP[114,115], NST and lateral parabrachial nucleus (LPB)[116].

1.2.2 Mediators of energy balance

Peripheral signals

The GI tract releases more that 20 different regulators and hormones, that are involved in the regulation of many physiological processes[37]. The release of

hormones such as PYY, GLP-1 and OXM is triggered by gastric distention and by the interaction between nutrients and the intestinal walls[117,118], and, once released, intestinal hormones act on target organs, such as endocrine glands, smooth muscle and peripheral nervous system (PNS)[39,119]. It is well known that hormones and intestinal peptides play a crucial role in the modulation of hunger and satiety[120]: many studies support that signals like CCK, PYY, GLP-1 and OXM, on one side, reduce food intake by reducing the levels of orexigenic signals while increasing anorexigenic signals in the hypothalamus[121,122], and, on the other, by triggering negative feedback mechanisms on the intestinal transit, that contribute to enhance the feeling of satiety during the intermeal interval[123], hence aiding the regulation of the after meal GI motility[110,118].

Ghrelin is a peptide hormone produced by the stomach and released into the bloodstream, initially discovered for the affinity for the growth hormone secretagogue receptor (GHS-R). It is a peptidide chain of 28 aminoacids and undergoes one post-translational modification that involves the addition of one molecule of ottanoic acid on the serine 3[119,124], necessary for the binding to the GHS-R and to cross the BBB[125]. Ghrelin is defined as the "hunger hormone"[125], and its circulating levels increase during fasting and decrease after a meal. Moreover, the administration of ghrelin in the CNS produces an increase of food intake and the release of the growth hormone (GH) in rats, whereas its peripheral administration reduces the use of fat from adipose storage[126]. Several studies show that the disruption of the ghrelin-mediated signaling induce several alterations in the control of energy homeostasis[127,128]. The pharmacological inhibition of the GHS-R was thought to be a valid strategy for the treatment of obesity. In fact, GHS-R antagonists are able to decrease food intake in fasted animals[127], and that the vaccination against ghrelin induces weight loss[129]. However, even though promising results have been obtained from animal models, an irreversible approach, such as vaccination, is not considered safe for human treatment[124]. Ghrelin agonists, on the other hand, may be used for the treatment of anorexia in oncologic patients, that experience appetite loss[130], in patiens with dialysis-induced malnutrition[130], or to improve gastric emptying in diabetic patients with

gastroparesis[131]. Moreover, it has been shown that the gene encoding for ghrelin encodes for another peptide, called obestatin. preliminary studies demonstrate that central administration of obestatin reduce food intake, while the periheral administration reduce body weight[132]. These effects seem to be exerted by the activation of the GPR39, an orphan receptor. However, further studies did not confirm the previous results, suggesting that obestatin does not bind GPR39 and does not control feeding behavior[133,134].

PYY is a peptidic hormone related to the neuropeptide Y (NPY). Both these peptides have a structural feature characteristic of the PP proteins and exert their action by binding to Y receptors. There are two endogenous isoforms of PYY, based on the presence of the N-terminal, PYY_{1-36} e PYY_{3-36}, the latter having hagher affinity to Y2 receptor (Y2R). In fact, the effects of the PYY_{3-36} are dampened by the administration of a Y2R antagonist[135] and abolished in Y2R-KO mice[121]. Preliminary studies demonstrated that the peripheral administration of PYY_{3-36} reduces foof intake in rodents and humans[121]. However, these preliminary findings were controversial, since many laboratories could not reproduce them[136]. Then, further studies demonstrated that the effects of the peripheral administration of PYY_{3-36} on feeding were dampened by stressful conditions, such as the lack of handling or the introduction to a new environment[137,138]. Many subsequent studies then confirmed that the acute administration of PYY_{3-36} reduces food intake[113,139]. The fact that the biological effects of PYY_{3-36} are dampened by the pretreatment with a Y2R antagonist[135] and abolished in Y2R-KO mice[136] further confirm that this peptide acts through the Y2R. Furthermore, an altered control of energy homeostasis is observed in PYY-KP mice, thus validating the role played by this peptide in its regulation[140,141].

The role of the **CCK** on the esocrine pancreas and gallbladder became clear when, in 1973, it was the first intestinal hormone proven to be involved in the control of feeding behavior[142]. CCK is released during the aftermeal period and exerts its effect through the activation of the CCK receptor 1 (CCK1), expressed on vagal fibres. The pretreatment with antagonists of this receptor increase food consumption in rodent and humans[124,143], and Otsuka Long-Evans Tokushima rats, KO for this receptor, are obese and

hyperphagic[143]. However, continuous infusions of CCK failed to reduce food intake, and, although the intermittent administrations reduce short-term food consumption, this effect is dampened by the compensatory increase of food intake during the intra-administration period[144]. Moreover, some studies suggest that physiological concentration of CCK do not activate vagal circuits, suggesting that the action of this peptide on FI is mainly due to its paracrin action more than to an endocrine one[145].

The pancreatic polypetide (**PP**) is synthesized and released by the endocrine pancreatic parenchyma and has a high affinity for Y4 and Y5 receptors. As for PYY_{3-36}, PP levels increase after a meal, and decrease during fasting. Acute PP administration reduce food intake in mice and humans[146,147], while chronic administrations reduce body weight in ob/ob mice[148]. It has been hypothesized that the anorexiant effect of PP is due to the delay of gastric emptying[147,149].

Amylin is a 37 aminoacids-long peptide, belonging to the calcitonin peptides family, that is released, as insulin, by pancreatic β-cells after the ingestion of food. Although its main function seems to be linked to glucose homeostasis, it reduces food intake after peripheral administration[150]. In fact, the administration of Pramlintide, a synthetic analogous of amylin, reduces body weight in patients with type 1 and 2 diabetes[151,152]. The anorexiant effects of amylin may be due to the modulation of the serotonergic, histaminergic and dopaminergic systems, as long as the inhibition of NPY release[153]. Moreover, many studies reported that increased circulating levels of amylin are observed in obese patients[154].

GIP is a polypeptidic chain composed of 42 aminoacids, synthesized and released by the K cells of the duodenum after food consumption. There are no clear studies linking this peptide to the regulation of food intake, although mice with the genetic deletion of the gene encoding for its receptor are resistant to the diet-induced obesity (DIO)[155]. Therefore, further studies are required to investigate the physiological mechanism involved in the mechanism of action of GIP, that may involve adipocytes and neural circuits in the CNS[156].

GLP-1 is co-secreted with PYY by the L cells of the intestinal mucosa upon the ingestion of food. It is produced by the cleavage of a precursor, the preproglucagone, that, after enzymatic cleavage, produces several biologically active peptides, such as glucagone, GLP-1 and -2 and OXM. GLP-1 has two different active forms, $GLP\text{-}1_{7\text{-}37}$, found in the periphery, and $GLP\text{-}1_{7\text{-}36}$ amide, found in the CNS[38]. $GLP\text{-}1_{7\text{-}36}$ amide- positive neurons have been found throughout the CNS, in particular in the paraventricular nucleus (PVN), in the dorsomedial nucleus (DMN), in the DVC, in the hypophysis and in the thalamus[157]. Moreover, GLP-1 is a potent incretine: in fact, its release after food consumption stimulates the release of insulin[38,124]. Furthermore, central administration of GLP-1 drastically reduces food intake in rodents, whereas peripheral administration reduces appetite in both rodents and humans[158]. Exendin-4 is an agonist of the GLP-1R, while its cleaved form, $exendin_{9\text{-}39}$, is an antagonist of the same receptor. It has been demonstrated that the acute central administration of $exendin_{9\text{-}39}$ increase food intake, and its chronic administration increases body weight[124,158]. Although it seems that endogenous GLP-1 is involved in physiological processes, such as the regulation of feeding, in a mouse model knock-out for the GLP-1R feeding and body weight are not altered[158]. Moreover, a phase III clinical trial demonstrated that exendin-4 ameliorates glucose homeostasis in patients with type 2 diabetes, and that it induces a reduction of food intake[159]. Along with exendin-4, the GLP-1R agonist exenatide has already been approved for the treatment of type 2 diabetes in co-treatment with metformin.

GLP-2 shares the same synthesis pathway as GLP-1, glucagone and OXM. High concentrations of this peptide have been found in the brain, and its central administration reduces food intake[160]. However, in the periphery, GLP-2 is involved in the regulation of gastric motility, digestion and absorption of nutrients, and does not seem to influence appetite[161].

OXM is a 37 aminoacids-long plypeptidic chain produced by the cleavage of preproglucagone. Like GLP-1, is released upon the ingestion of food, and, when administered centrally, reduces food intake[162]. Although many studies suggest tha OXM exerts its biological action through the activation of the GLP-1R, supported by the fact that its anorectic effect is

abolished in GLP-1R KO mice, it seems that these effects do not perfectly match those exerted by GLP-1R[38,124]. In fact, it has been demonstrated that the administration of exendin$_{9-39}$ in the arcuate nucleus (Arc) abrogates the OXM-induced anorectc effects, but not thos of GLP-1[162]. Moreover, chronic administration of OXM, either peripheral or central, induce a body weght reduction greater than that of pair-feeding animals, suggesting that OXM may act by increasing energy expenditure[162].

The role played by **glucagone** in the control of glucose homeostasis is well defined. It is produced by α-cells of Langerhans islets in the pancreas and increases glucose levels in response to a hypoglycemic state. Moreover, glucagone improves the stress response by increasing energy expenditure[163]. The administration of glucagone reduces food intake, probably through the modulation of the vagal tone and of the gastric emptying[164]. Furthermore, recent findings demonstrated that the co-administration of antagonists for glucagone receptore and GLP-1R ameliorates insulin sensitivity and DIO[165].

Insulin, produced by the β-cells of the pancreas, is released after the ingestion of food and induces its very well-known hypoglycemic effects[166]. Moreover, it acts on the CNS as a satiety factor: in fact, the central administration of insulin dose-dependently reduces food intake and body weight gain in rodents and baboons[167,168]. Insulin is carried in the CNS by a receptor-mediated transport[169]. Furthermore, insulin receptors are expressed throughout the brain, in particular in the Arc, the DMN and PVN[170].

Neuroactive mediators

Neuropeptides are small proteic molecules, released from the cells of the nervous system in response to a stimulus, able to control the comunication between neurons by binding secific receptors. Hypothalamic peptides may be classified based on the effect they exert on feeding behavior. On the orexigenic side, **NPY** is produced by neurons in the Arc that project to other hypothalamic nuclei[171]. Although NPY can exert several different effects on feeding behavior[172], the most known is in the increase of appetite upon central administration[173]. The synthesis of NPY occurs in Arc and its release in the PVN, and both the processes are negatively regulated by leptin and insulin, and positively regulated by ghrelin[173]. Five receptors for NPY have been

described: Y1, Y2, Y3, Y4, Y5 and Y6; among them, Y5 receptor is the most involved in the modulation of NPY effects on feeding[174,175].

Another orexigenic peptide is the **AgRP**, a 132 aminoacid chain exclusively expressed in the Arc. It has been demonstrated that, upon central administration, either in the PVN or DMN, AgRP increases food consumption[176].

Hypocretins 1 and 2, also known as **orexins** A and B, are neuropeptides produced in the lateral hypothalamus (LH) and linked to the stimulation of appetite by binding their receptors, OX1R and OX2R. OX1R is mainly expressed in the ventromedial hypothalamus (VMH), whereas OX2R in the PVN[177]. Moreover, orexin-secreting neurons are located in the LH and DMH, and project to other hypothlamic nuclei, in particular to the Arc[178,179], and input on NPY secreting neurons expressing OX1R[178].

Apart from orexigenic signals, there are a lot of anorexigenic peptides acting in the hypothalamus: **CART** is a neuropeptide synthesized in the DMH, PVN, LH and Arc[180]; melanocortins, in addition, are bioactive peptides that derive from the pro-opiomelanocortins (**POMC**) after a tissue-specific post-translational cleavage[181]. The gene encoding for POMC is expressed in several tissues, such as hypothalamic neurons, the adenohypophysis and the pars intermedia[182]. Moreover, the intermedial lobe of the hypophysis produces **α-MSH**, an anorexiant peptide that binds to melanocortin receptors 3 and 4 (MC3R and MC4R), expressed by brain areas known to be involved in the control of feeding behavior and in telencephalic structures, such as the cortex[182].

The corticotropin-releasing hormone (**CRH**) is a 41 aminoacids-long hormone and regulates the secretion of the adrenocorticotropic hormone (ACTH) from the hypophysis. CRH is highly expressed in the PVN and, when centrally administered, inhibits food intake and reduces body weight in rats[171]. On the other hand, the periheral administration of CRH in humans increases energy expenditure and fatty acid oxidation (FAO)[171]. Moreover, the infusion of leptin increases the expression of CRH, whereas the pretreatment with a CRH receptor antagonist attenuates the leptin-induced reduction of food intake and body weight[171].

Among neurotransmitters, **histamine** is a hypophagic agent synthesized from the decarboxylation of histidine, exerted by the histidine-decarboxylase (HDC)[183]. In fact, the central infusion of α-fluoromethylhistidine, an inhibitor of HDC that, therefore, leads to a decrese of central histamine, is able to disrupt feeding and hydration patterns, along with deambulation[184]. Moreover, it has been demonstrated that histaminergic system is involved in the anticipatory phase of eating: in fact, the specific activation of the E3 subdivision of the ventral tuberomammillary nucleus (vTMN)[185] is rats with restricted access to food. However, several lines of evidence suggest that histamine is also involved in the consummatory phase of feeding behavior, during which a rapid transitory increase of hypothalamic histamine levels is observed[186].

Histamine binds to 4 GPCR receptors: H1R, H2R, H3R, H4R[187,188].

H1R, coupled with Gq, is expressed in the brain, bronchial epithelium, cardiovascular system, liver and cells of the immune system[189]. In the CNS is expressed in the VMH, that is likely the site where histamine exterts its appetite-suppressing effects[189].

H2R, coupled with Gs, is expressed in the gastric mucosa, in the muscular layer of arteries, in the cells of the immune system and in the brain[189].

H3R, coupled with Gi/o, is highly expressed in the CNS, where it acts as presynaptic autoreceptor, thus inhibiting the release of histamine[190]. Moreover, it acts as heteroreceptor, modulating the release of other neuronsmitters, such as acetycholine, dopamine, noradrenaline, and serotonin.

Lastly, H4R has a primary role in the inflammatory response[189].

Several studies have demontrated that the intra-hypothalamic administration of histamine, where the activation of H1R induces satiety, increases mRNA expression levels of the uncoupling protein 1 (Ucp1), marker of energy expenditure, in the brown adipose tissue (BAT), and, moreover, increases the electrophysiological activity of the sympathetic nerves around that area[191,192]. Furthermore, it is likely that histamine released in the periphery is involved in metabolic and homeostatic processes linked to food consumption[193].

1.2.3 Central circuits involved in the homeostatic control of feeding behavior

Feeding and metabolism are regulated by complex systems in the CNS[194]. The main area of the CNS involved in the control of food intake is the hypothalamus, that is costantly informed by signals secreted in the periphery about the nutritional and energetic statuses of the body[43]. These signals are then integrated and conveyed to other brain areas. Satiety signals are generated in the GI tract during meal consumption and modulate feeding through the release of peptides that reach the NST via the vagal afferent system[195] (Fig. 1.3).

Among all the systems of the gut-brain axis that interplay in the regulation of feeding behavior, an important role is played by the ECS. In the CNS, this system plays a role in the motivation for the research and consumption of food, through the activation of mesolimbic pathways that regulate reward[196]. N-arachidonoylethanolamide (or anadamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most studied ECs[197] and have a hyperphagic effect by acting on CB1 receptors in the PVN[198] and in the lateral hypothalamic area (LHA), or by influencing the action of hormones like ghrelin[197]. The role of this system has gained a great deal of attention, starting from the development of synthetic compounds acting on CB1 receptor, the rimonabant, as a potential target for the treatment of obesity[50]

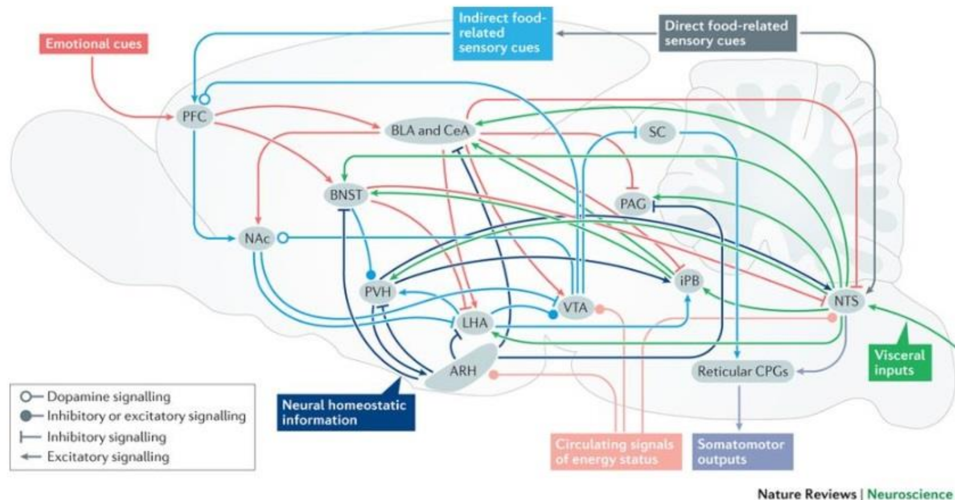

Fig. 1.3: Crosstalk between circuits modulating feeding behavior. Lori M. Zeltser, *Feeding circuit development and early-life influences on future feeding behavior*, Nature Reviews Neuroscience volume 19, pages 302–316.

Brainstem

The brainstem is one of the most important areas for the homeostatic control of feeding behavior, since it acts as an integration center between sensitive inputs and motor outputs. the NST is located in the most caudal portion of the brainstem, and represents the first relay station of vagal afferents, that convey the informations about the quantity of food consumed from the periphery[199]. In particular, it is in contact with the preganglionic vagal neurons, in order to regulate nutrient adsorption in the alimentary canal[79], and sympathetic preganglionic nerves, in order to modulate energy expenditure[10]. The NST is then connected with other brain areas involved in the homeostatic control of feeding behavior: in particular, it has direct inputs towards the hypothalamus, and contacts indrectly telencephalon and the cerebral cortex[10]. Moreover, it is known that part of the NST projections that contact the hypothalamic neurons are the A2 noradrenergic fibres[200], and many studies have demonstrated that the ablation of these fibres dampens the effects of some satiety factors, such as CCK[199].

The NST is composed by different subnuclei that specifically respond to stimuli received[10]. For instance, the gustative fibres of the tongue and the posterior oropharynx input in the rostral part of the NST[79], whereas the

afferents from below the diaphragm project to the caudal part of this nucleus[69]. Neurons of the DMV respond mainly to gastric distention[201], while the neurons below the AP respond mainly to duodenal signals, whereas the neurons located in the medial part of the NST respond both to gastric and duodenal signals[201]. Notably, in this nucleus, there are neurons that express POMC[97], melanocortin[202] and leptin[97] receptors.

The AP, in close contact with the NST, is another important nucleus involved in the homeostatic control of feeding behavior, and expresses receptors for amylin, GLP-1 and ghrelin. Moreover, it is able to convey informations about gastric emptying[42,203]. Studies conducted in rats that underwent the surgical lesion of the AP highlighted the involvement of this area in the regulation of the signals sent from the periphery[204,205]. The AP is a circumventricular organ outside the BBB, that lacks tight junctions and is rich in fenestrated capillaries[206]. Thanks to these features, some peptides and other signaling molecules may have direct access to the AP wich is one of the main integration stations in the CNS that conveys numerous physiological signals from the bloodstream[206]. Moreover, many studies demonstrated that AP projects extensively to other nuclei of the brainstem and to the hypothalamus[207].

In particular, it projects to the NST and LPB, known integrative stations of the brainstem[207,208]. Moreover, neurons of the AP project direclty to the dorsal and medial subnuclei of the NST[207]. Furthermore, the AP projects to a lesser extent to the nucleus ambiguous, to the DMV, the dorsal parts of the tegmental nucleus and to the spinal tract of the trigeminus[207].

The afferent neurons pojecting to the AP come from functionally distinct parts of the brainstem and of the hypothalamus. Apart from the bidirectional connections to and from the NST and LPB, the majority of the hypothalamic connections comes from the PVN and the DMH[207,209]. Moreover, it has been demonstrated that the AP receives direct inputs from vagal afferents[207].

It has been observed that the AP contains the receptors for a variety of hormones that regulate feeding behavior, such as CCK, intestinal vasoactive peptide, NPY, vasopressin (AVP), substance P and insulin[210,211].

Another important brain structure involved in the homeostatic control of feeding behavior is the LPB, located in the pons. Its anatomical position, between the medulla and the prosencephalon, allows it to integrate the signals coming from both ways, hence helping to organize behavioral responses[212]. Moreover, the LPB integrates a variety of sensitive inputs, associated to food consumption or to stress[212,213]. In particular, the LPB receives sensitive informations mainly from the NST[214], and projects to the PVN and Arc[10].

Hypothalamus

The mammalian hypothalamus consists in over 40 nuclei in histologically distinct areas[215]. The medial part of the hypothalamus is mainly composed of large nuclei that receive bloodborne stimuli and pass them down onto other hypothalamic nuclei, controllig endocrine responses. Then, the lateral portion is a large area that bridges the hypothalamic nuclei with extrahypothalamic brain areas, such as the cortex and the limbic system[215].

Since its lesion induces hyperphagia and body weight gain, the VMH has been identified as the satiety center in the hypothalamus[23]. On the other hand, the LH has been identified as the “hunger center” of the hypothalamus, since its lesions induce weight loss[216].

Among hypothalamic nuclei, the PVN interplays with the endocrine and autonomic nervous systems and is involved in many behavioral responses. Moreover, the PVN is able to comunicate with the preganglionic neurons that innervate the pancreas, and with the parasympathetic and sympathetic nervous systems[217]. PVN neurons project towards di- and telencephalic structures[218] and to other hypothalamic areas, such as DMH, VMH and Arc[218,219]. In general, the PVN can be divided in magnocellular and parvocellular. Magnocellular neurons contain oxytocin (OXY) and AVP, that are released in the posterior lobe of the pituitary gland[220]. Many medial parvocellular neurons contain CRH and tireotropin-releasing hormone, that are released in the ME. The dorsal, ventral and lateral parvocellular neurons, on the other hand, project to the periacqueduttal grey, the LPB and the NST, and to both sympathetic and parasymathetic preganglionic neuronal populations[220,221]. The better-known endocrine signals produced by the PVN

are those of the magnocellular projections, that reach the posterior part of the pituitary gland releasing OXY and AVP, and those of the parvocellular projections, that release CRH and TRH. The non-endocrine signals are those coming from the dorsal raphe, LPB, DMV and NST[222]. Therefore, the PVN seems to be in the best position to integrate internal stimuli, such as the availability of nutrients in the GI tract and adipostatic signals, to thus organize endocrine and autonomic responses[10].

The supraoptic nucleus (SON) lies behind the optic chiasma, and is composed, as the PVN, of magnocellular neurons[10]. These neurons contact the posterior part of the hypophysis, through which the SON contributes to the endocrine control of the organism. The major inputs to the SON come from the branstem, in particular from the NST[223].

The Arc is a hypothalamic region in close contact with the ME, a circumventricolar organ rich in fenestrated capillaries that lacks a functional BBB[224]. It contains two major neuronal populations that control appetite and satiety. The former is composed of orexigenic neurons, and express NPY and AgRP[225], while the latter contains anorexigenic neurons, that contain α-MSH (derived from POMC), and CART[10,225].

The TMN is a group of large cells located in the tuberal caudal and mammillary rostral areas and form the median hypothalamic area. In the medial part of the vTMN[226] a complex network of histaminergic neurons is found, that project to several brain areas[193,227]. These neurons are involved in many physiological responses, such as circadian rythm, emotions, learning and memory[228,229].

1.3 Gut microbiota

Microbes are everywhere, and it is known that microbial populations are resident, in humans, on the skin, in the oral cavity, in the urogenital tract and in the gastro-intestinal (GI) tract. In all these areas, the microbial populations partake in the physiological control of the homeostasis by establishing a symbiotic relationship with the host[230]. Human microbiome is involved in many mechanisms that allow the maintenance of the well-being of the host, like metabolic and immunological processes[231]. These lines of evidence, therefore, led to hypothesize the existence of the "olobiome", represented by the symbiotic interaction between the microbiome and the host[232]. On the other hand, this interaction may play a role in the onset of pathological conditions, and, therefore, in the past 20 years many studies were conducted to demonstrate how feeding behavior can alter the gut microbiome[233]. In fact, it has been shown that different host's characteristics, like genetic background, gender, age, and immunological profile, play a pivotal role in shaping the gut microbiome[234], as long as environmental and behavioral factors, such as pharmacological therapies, surgeries, physical activity or stressful conditions[235]. In newborns, the microbiota is spread during delivery and is, then, influenced by a variety of factors, such as the type of delivery (natural or c-section) or breastfeeding[236]. For example, the microbial flora of naturally delivered babies is characterized by the presence of *Lactobacillus*, *Prevotella*, and *Atopobium*, whereas in babies delivered by c-section the flora is rich in *Staphylococci*, as for the mother's skin[236]. Moreover, in the recent years, new lines of evidence show that the intrauterine environment is not sterile, but colonized by *Enterococcus fecalis*, *Staphylococcus epidermis*, e *Escherichia coli*[237]. The newborn's flora, rich in aerobic bacteria, undergoes changes during the post-natal period towards a flora composed mainly by anaerobic bacteria[238]. This first colonization happens together with the activation of the hypothalamus-pituitary-adrenal axis (HPA axis), that has a strong impact on the ENS[231]. Moreover, it has been shown that the metabolites produced by the enteric flora may induce the release, by the enteroendocrine cells of the GI tract, of mediators involved in the control of feeding, lipid storage, and energy homeostasis[7,239]. Among these mediators,

short-chain fatty acids (SCFAs) activate the GPR41 receptor expressed by the enteroendocrine cells[240], involved in the gut microbiota-mediated control of adiposity and inflammatory processes[241].

Then, the population of the gut microbiome becomes more stable during adult life[242], with a last change during the elderly life: in fact, aging is associated to physiological changes tha influese the composition of the gut microbiome[231]. The microbiome is composed by over a 100 billion of microorganisms, the majority belonging to the reign of Bacteria[243]. Moreover, the entire genome of the commensal flora contains over 3 million genes, 100 time more than the human genome[243]. Therefore, the variety of protein products yields a pool of metabolic and biochemical functions that have a high impact on the host's physiological processes[244]. Both the host and the gut microbiota produce a variety of small molecules during the catabolism of food and xenobiotics, that play a crucial role in the communication between the cells of the host and of the microbiome. Moreover, the microbiota composition changes in different portions of the GI tract[236,245], and an interplay between all the different populations along the GI tract has been observed[246]. The exchange of low molecular weight metabolites, like peptides and small proteins, underlies this type of chemical communication, together with pathway mediated by the immune system[230].

The human body eliminates up to 100 mg of volatile phenols per day, in particolar 4-cresol and phenol, either as glucuronate conjugate or solfates[247]. In mammals, the production of these chemical species is due to the activity of different microbial populations: for example, cresol is produced by the species *Clostridium*, *Bifidobacterium*, and *Bacteroides fragilis*[230], whereas *E. coli* has been associated with the production of phenol[230]. Moreover, an alteration of 4-cresol metabolites in urine has been associated with several pathophysiological conditions, that span from weight loss to inflammatory bowel diseases[230]. Moreover, these conditions have been associated to a variation of gut microbiota composition, in particular to a decrease of the populations of *Lactobacillus* and *Bacteroidetes* in inflammatory bowel diseases[248], and to an unbalance between the populations of *Firmicutes* and *Bacteroidetes* in weight loss[249]. The enzymatic

activities of gut microbiota products can directly act on the fermentation of carbohydrates and on the metabolism of bile acids. Indigestible carbohydrates, the so-called functional fibers, are an important energy source for many members of the gut microbiota populations, such as *Bacteroides thetaiotaomicron* and *Bacteroides ovatus*[250]. In fact, the fermentation of these fibers leads to the production of SCFAs, such as acetate, propionate, butyrate and lactate[249]. In particular, butyrate is a crucial substrate for cell metabolism of colon epithelium. In fact, it has been observed that germ-free mice show a severe energetic deficiency[251], characterized by an increased activity of the AMP kinase, that is involved in the monitoring of the energetic state of the cell[252]. Moreover, the hepatic metabolism of germ-free animals is different than that of colonized ones, and this difference is probably due to a higher presence of SCFAs in the liver: it has been shown that the liver uptakes acetate and propionate and uses them as substrates for lipo- and gluconeogenesis. Furthermore, SCFAs promote the differentiation and the proliferation of colon epithelial cells, probably through the modulation of genetic expression due to the butyrate-induced inhibition of the histone deacetylase (HDAC)[253]. Moreover, SCFAs are able to modulate gene expression by activating two different GPCR, GPR41 and GPR43, that partake in different pathways based on the cell type they are expressed in[254]. For example, the activation of GPR43, in neutrophiles, has an anti-inflammatory effect[255], whereas, in intestinal L cells, it induces the release of GLP-1[256]. By activating GPR41, the gut microbiome induces the release of PYY[257], and the genetic deletion of this receptor prevents the accumulation of fat. Although many studies demonstrate the interplay between GPR41/43, enteroendocrine cells activity, and metabolites produced by the gut microbiota, many lines of evidence suggest that other biologically active compounds, hence acting on different receptors, can induce the release of GLP-1/2 and PYY[258], for example bioactive lipids such as oleoylethanolamide (OEA) or 2-oleoyl-glycerol (2-OG), that bind the GPR119 receptor[259]. In fact, a very recent finding demonstrates the beneficial effects of OEA on the gut microbiota[260].

However, to date, little is known about the role that each bacterial species has in the production of bioactive metabolites. It has been observed that *Akkermansia muciniphila*, that breaks down mucine, produces propionate and butyrate, that bind the GPR43 receptor[261]. Moreover, a recent study reported that obese or insulin-resistant mice show a decrease of the abundance of this species, and that the daily administration of *A. muciniphila* for four weeks reverts this phenotype[262]. Moreover, a direct correlation between the abundance of this bacterial species and the secretory activity of L cells has been found[263].

The gut microbiome regulates lipid and glucidic metabolisms, acting both on the liver and on the production of bile acids. Colonized mice display higher levels of triglycerides stored in the liver, and an increase in the synthesis of very low-density lipoproteins (VLDL)[264], that are involved in the transport of triglycerides from the liver to other tissues. Furthermore, an increased production of triglycerides in colonized mice is associated to a reduction of the expression of the angiopoietin-like protein 4 (ANGPTL4) in the small intestine[251,265], that is a potent inhibitor of the lipoproteic lipase (LPL), a mediator of the cellular uptake of triglycerides. It has been demonstrated that germ-free mice knockout for the gene encoding for ANGPTL4 show an increase of fat mass and of the body weight, suggesting a role of both gut microbiota and ANGPTL4 in regulating adiposity[251,265].

Choline is an important constituent of plasma membranes, that can be either introduced with the diet (mainly through the consumption of eggs and meat) or endogenously synthesized[266]. It has a major role in lipid metabolism and in the synthesis of VLDL in the liver, and its inadequate supply is associated with an alteration of the gut microbiota and with hepatic steatosis, both in mice[267] and in humans[268]. In particular, low levels of Gamma-proteobacteria combined with high levels of *Erysipelotrichi* in human fecal microbiota are linked to hepatic steatosis[268]. The enzymatic activities of the gut microbiota and of the host interplay in the transformation of the choline into toxic metabolites, such as trimethylamine, that is further converted into trimethylamine-N-oxide in the liver[269,270], thus reducing choline availability, that may be the cause of the onset of the non-alcoholic fatty liver disease (NAFLD)

in mice[270]. Moreover, an increase in trimethylamine-N-oxide is linked to a higher cardiovascular disease risk[271].

Primary bile acids, such as cholic and chenodeoxycholic acids, are synthesized in liver from cholesterol, and partake in the solubilization of lipids, dietary fats and liposoluble vitamins, to aid their intestinal adsorption, and the gut microbiota is able to metabolize these compounds into secondary bile acids. In fact, higher levels of primary bile acids, and a lower variability in secondary bile acids, has been observed in germ-free rodents[272]. Moreover, bile acids bind nuclear receptors[273], as the farnesoid X receptor (FXR)[274], involved in the regulation and the synthesis of bile acids, and the G protein-coupled bile acid receptor 1 (or TGR5). Both these receptor are involved in the regulation of glucose metabolism in mice, negatively in the case of FXR, and positively in that of TGR5[275]. FXR is activated by primary bile acids, whereas TGR5 binds secondary bile acids, like deoxycholic (derived from cholic acid) and lithocholic (derived from chenodeoxycholic acid) acids. The signal induced by the activation of TGR5 in L enteroendocrine cells leads to an increase of GLP-1 secretion, improving glucose tolerance in obese mice[275]. Moreover, in the BAT and in the muscle, the activation of this receptor increases energy expenditure and protects from the onset of a DIO phenotype[276]. Commensal flora may contribute to the pathophysiological mechanisms underlying the onset of type 2 diabetes and obesity, by controlling lipid and glucidic metabolism through the regulation of the production of bile acids. Therefore, alterations in the capability of the commensal microflora of processing cholesterol, choline, and dietary lipids may contribute to the development of cardiovascular diseases[230,261].

1.4 Changes induced by the prolongated exposure to a high fat diet

1.4.1 Dysbiosis

The metabolites produced by the gut microbiota, apart from the intestinal function, directly influence the activity of the liver, of the CNS, of the adipose tissue and of the skeletal muscle, thus playing a crucial role in the regulation of energy homeostasis and in other physiological processes[230,249]. In fact, there is evidence supporting a strong link between a dysregulation of the gut microbiota and the onset of pathological conditions, such as obesity, type 2 diabetes, and cardiovascular diseases[254] (Fig. 1.4). A healthy microflora is characterized by a high diversity in bacterial species and by a high resistance to physiological stress[277], whereas, in pathological conditions, the gut microbiota loses these main features[278]. In fact, the dysbiosis, the imbalance of the gut microflora, has been associated with the development of several pathologies, such as colon cancer, irritable bowel syndrome, gastric ulcer, NAFLD, obesity, metabolic syndrome, asthma, allergies, hypertension, and behavioral alterations[230]. Although the gut microbiota profile is unique for each individual, and hence characterized by a high variability, it is known that the diet can alter its composition. For example, the gut microbiota of an African population, whose diet is mainly composed of polysaccharides obtained from plants, shows lower levels of *Firmicutes* and higher levels of *Bacteroidetes*, mainly *Prevotella* and *Xylanibacter*, compared to the Mediterranean population, whose gut microbiota is rich in *Shigella* and *Escherichia*[279]. *Prevotella* and *Xylanibacter* are known for their ability to degrade cellulose and xylans, and hence associated with a higher production of SCFA, maximizing the extraction of energy from foods rich in fibers. Changes in the daily consumption of carbohydrates, even for a short period of time, can influence specific bacteria. The consumption prebiotics, like inulin, has been linked to an increase of the levels of *F. prausnitzii* and *Bifidobacterium* sp. in humans[280]. Moreover, a reduction of adiposity and concentration of pro-inflammatory molecules produced by the gut microbiota (such as the lipopolysaccharide (LPS)) was observed in a mouse DIO model after prebiotic consumption[281]. Moreover, the diet supplementation with resistant starch

leads to an increase of *Ruminococcus bromii* and *Eubacterium rectale,* that are involved in the fermentation of dietary fibres[282]. Furthermore, the gut microbiota is influenced by the ingestion of fats. In fact, the exposure to HFD determines a decrease of *Bacteroidetes* abundance, and an increase of *Firmicutes* and *Proteobacteria*[283]. In particular, in obese mice, the most abundant class belonging to the phylum *Firmicutes* is the class *Mollicutes*[283], and the same profile has been observed in obese patients[278]. Similar alterations in the phyla have been observed after the exposure to both HFD and sucrose rich diet (SRD)[284]. In particular, it has been observed that the increase of fat is inversely correlated with the abundance of the genus *Akkermansia* (phylum *Verrucomicrobia),* while its directly correlated with the genus *Lactoococcus* (phylum *Firmicutes*) and *Allobaculum* (phylum *Bacteroidetes*)[284], and these changes can occur within 24 hours[285].

However, whether the changes of the gut microbiota are more due to the genetic background of the host or to the diet needed to be fully elucidated. To address this point, Carmody and colleagues conducted a study involving more than 200 species of mice, and exposing them to different diets, demonstrating that both the HFD and SRD lead to reproducible alterations of the gut microbiota, regardless of the genetic background of the host[286]. Moreover, another study demonstrated that obese prone (OP) rats have a different composition of the gut microflora compared to obese resistant (OR) rats, and that the fecal transplant from OP rats is able to induce the obese phenotype in germ-free animals[287]. Interestingly, the HFD-induced changes of the gut microbiome are associated to the type of dietary fats consumed[288].

Recently, many studies investigated the correlation between obesity and the alterations of specific bacterial taxa. For example, the levels of *Bilophila wadsworthia*[289], Clostridiales[190], Streptococcaceae[290], and *Oscillibacter*[291] are increased in obese (or exposed to HFD) rodents, whereas the phyla *Actinobacteria, Bifidobacteriaceae* (in particular the genus *Bifidobacterium*), *Verrucomicrobia* (in particular the species *Akkermansia muciniphila*), and *Prevotellaceae* (mainly the genus *Prevotella*) are decreased[292]. *B. wadsworthia* is known for its ability to produce hydrogen sulfide, a molecule with cytotoxic activity towards epithelial cells, that could be

linked to the disruption of intestinal permeability and inflammation observed in dysbiotic conditions[293]. Moreover, due to their LPS production, *Bilophila* sp. and *Oscillibacter* sp. may also partake in the development of these conditions[294]. On the other hand, in patients that underwent a Roux-en-Y gastric bypass (RYGB), a surgical procedure that induces weight loss and improves diabetes[295], an increase of *Faecalibacterium prausnitzii*, generally decreased by obesity and diabetes, was observed[296].

Overall, the HFD-induced dysbiosis is crucial for the onset of obesity and its metabolic disruptions. Moreover, it has been reported that the decrease of the richness of the gut microbiota exacerbates the dysmetabolic conditions[297]. The exact mechanisms underlying the contribution of the gut microbiota to the onset of obesity is still debated. However, it has been demonstrated that the microbiome of obese mice is able to extract more energy from food[298] by fermenting more indigestible fibers, that leads to the production of SCFA, that are an important source of energy[239]. In fact, it has been observed that lean and obese individuals show different concentrations of SCFA[299]. Moreover, the gut microbiome seems to be involved in the modulation of the expression of genes linked to fat storage[290]. It is believed that a reduction of fasting-induced adipose factor (FIAF) could take part in this process, since it has been shown that its expression is selectively silenced in germ-free mice after being colonized with the microbiota of obese animals[265].
Obesity, insulin-resistance and diabetes are all associated with systemic inflammation[300] and with altered intestinal permeability, probably caused by the reduction of the proteins of the tight junctions[301]. Moreover, the gut microflora of OP rats is characterized by increased levels of *Enterobacteriales*, an order particularly involved in inflammatory responses[302]. the gut microbiota in a source of molecules, such as LPS and peptidoglycan, that may contribute to the development of inflammation in peripheral tissues[303]. In fact, the administration of LPS in mice leads to inflammation of the adipose tissue and insulin-resistance[235]. Moreover, the increase of circulating LPS levels is associated with metabolic endotoxemia[304]. However, even though many studies reported an increased number of T lymphocytes[305] and mastocytes[306], and a decreased number of regulatory T lymphocytes[307] in

models of obesity, the interaction between the immune system and the gut microbiota is yet to be fully explained. Moreover, many studies showed a direct correlation between LPS circulating levels and consumption of dietary fats, both in mice[235] and humans[307], that may be due to either the inclusion of LPS in chylomicrons along with fats[308], or to the increased permeability of the intestinal epithelium of obese mice, through which LPS reaches directly the bloodstream[235]. The increase of the permeability is, moreover, associated with the increase of visceral fat deposition and hepatic steatosis[309], and patients with high abdominal adiposity and type 2 diabetes show elevated circulating levels of DNA from bacteria[310]. LPS binds the toll-like receptor 4 (TLR4), whereas peptidoglycans bind NOD-like receptors, and both activate a pro-inflammatory response[235,311]. It has been shown that the TLR4 is necessary for the development of insulin-resistance: in fact, in mice lacking the gene encoding for this receptor, the exposure to HFD failed to induce hyperinsulinemia and insulin-resistence, but did not fail to induce obesity[312]. LPS, peptidoglycan, and other pathogen-associated molecular patterns (PAMP) may bind to the family of NOD receptors containing the pyrin domain (NLRPs) and the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), thus forming a complex called inflammosome[313]. Obesity is associated with an increase of the NLRP3 in the adipose tissue, and the deletion of this receptor improves insulin sensitivity[314]. NLRP3, NLRP6 and ASC are also important regulators of the gut microflora, and the deletion of the genes encoding for these proteins leads to an increase of *Bacteroidetes* (*Prevotellaceae*) and *Candidatus Saccharibacteria*[267,315].

Moreover, recent studies suggest that gut microbiota may be responsible of the onset of inflammation in the CNS[316] through the leakage of inflammatory cytokines through the BBB[317], the stimulation of the microglia[318], and the activation of vagal afferents[319]. Apart from passing through the BBB, cytokines may induce inflammation in the CNS by engaging other pro-inflammatory mediators in the periphery and promoting their migration in the CNS[318,320], or by activating directly the vagus nerve[321].

In the past few years, many studies supported the role of the gut microbiota on the host's metabolism, that may contribute to the development of pathological conditions such as obesity, diabetes, and other associated pathologies[257,316]. Moreover, an increasing number of evidences point out the gut microbiota as a possible target for the treatment of metabolic disorders[322]. Therefore, the next step may be the development of novel experimental approaches in order to decipher the complex molecular mechanisms involved in the microbiota-induced signaling, to find novel markers and pharmacological targets for the treatment of metabolic disorders.

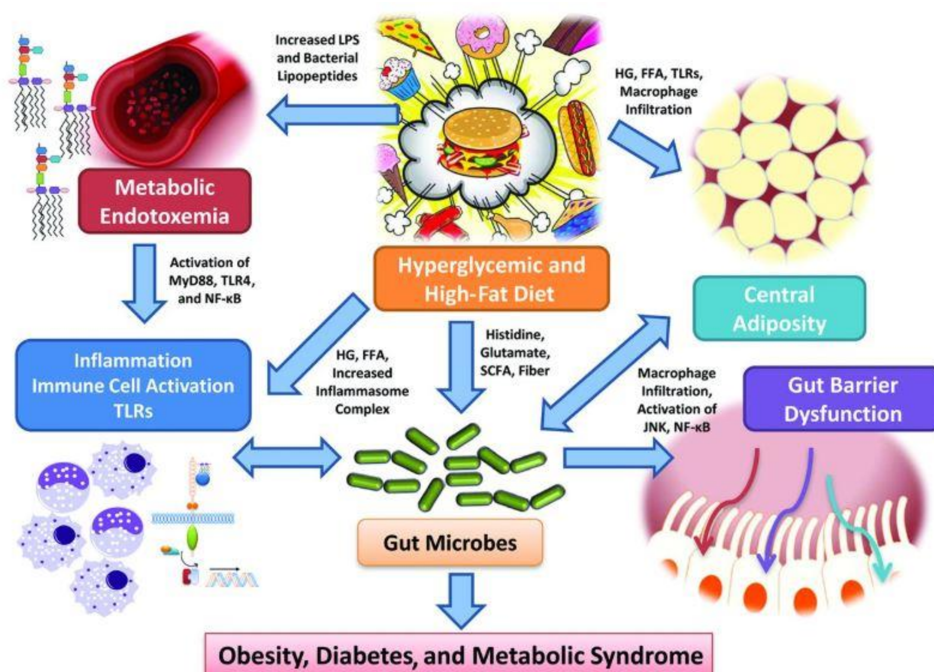

Fig. 1.4: Effects of the exposure to high-fat diet on the gut microbiota (dysbiosis).

1.4.2 Alterations of gene expression

Over the years, the effects of the prolonged exposure on both the transcriptomic and proteomic levels of regulation of gene expression have emerged[323,324], in particular in key homeostatic tissues, such as the hypothalamus, adipose tissue, liver and muscle[324,325]. In these tissues, the exposure to HFD induces the same gene expression profile, even when it is not paralleled by overweight[325].

In the hypothalamus, the NPY/AgRP and POMC/CART are under the control of circulating hormones, mainly leptin and insulin[326], and it has been demonstrated that the exposure to HFD leads to a reduction of the expression of leptin receptor, and to an increase of the expression of NPY and of the NPY/POMC ratio[327]. Moreover, these latter effects have been observed also in animals exposed to a cafeteria diet[328].

Regarding the adipose tissue, fat-rich diets increase the expression of genes related to FA uptake[327,329], that, in turn, alter the expression of genes regulating lipid metabolism, reducing the lipogenic capacity and activating lipolytic capacity[325,327]. In particular, the exposure to HFD regulates the expression of PPAR-γ, and this regulation depends on the period of exposure: short periods of exposure decrease PPAR-γ expression, and this effect is reverted upon long periods of HFD exposure[330]. In general, HFD exposure increases the expression of genes involved in FA catabolism, such as CPT 1[324,325]. However, the modulation of adipogenesis-related genes is unclear, since it seems to be influenced by the period of exposure and fat proportion[331]. Moreover, gender-specific considerations should be made in terms of modulation of gene expression in the adipose tissue. For example, in female rats, the cluster of differentiation (CD36) gene is more increased that in males upon HFD exposure, suggesting a higher accumulation of fats[327], and females show higher FAO in muscle while in males the expression of genes involved in thermogenesis increases[327,332].

During exposure to HFD, FA that are not uptaken or stored are metabolized by the liver into triglycerides, leading to NAFLD, that affects several cellular processes and leads to the alteration of the expression of genes involved in lipid metabolism[333]. In particular, the compensatory response to the higher fat consumption leads to an increase of expression of genes involved in FA catabolism in the liver, while decreases the expression of those related to lipogenesis[324,327,334]. Moreover, it has been showed that the increase of HFD consumption is paralleled by the increase of CPT-1 mRNA levels, that may be the cause of the insulin-mediated anti-lipolytic signaling[325,333,334]. Moreover, HFD consumption leads to an increase of mRNA expression of genes involved in the catabolism of FA, in particular PPAR-α[327],

while decreasing the gene expression of enzymes linked to FA anabolism[324,327,334]. However, these effects may vary depending on the fat content of the diet and on the duration of the exposure period: for instance, it has been demonstrated that the exposure to a diet containing more than 45% of calories from fat induces the upregulation of lipogenesis-related genes[335], whereas the exposure to diets containing less than 45% of calories from fat for a long period leads to uncertain results[333,336].

Skeletal muscle is the major site for FA and carbohydrate oxidation, and it is known to be able to adapt to physiological and pathophysiological changes in energy expenditure[337]. In fact, the exposure to HFD increases the number of muscle mitochondria, decreases the mRNA levels of genes involved in in FA synthesis and increases those of FA uptake, lipolysis and FAO[324,325,327], probably in reaction to the enhanced utilization of lipids[327,338].

The changes in gene expression observed upon the exposure to HFD alter also the response of the body during fasting, thus impairing restoration of a healthy genetic profile. As discussed before, the changes induced by HFD-exposure depend on the duration of the exposure and on the fat content of the diet[324]. In fact, it has been demonstrated that the exposure to a cafeteria diet in early life induces long-term changes in gene expression patterns[339,340], and the reversion to a control condition after a long-term exposure is still debated[341,342].

1.5 Oleoylethanolamide

The increasing incidence of obesity and other eating-related disorders highlighted the necessity of a discovery of a novel target for the pharmacological treatment of these diseases and their related comorbidities. Therefore, after the discovery of AEA as an EC neurotransmitter[343], the members of the FAE family gained a great deal of interest, that led to the discovery of these molules as modulators of feeding behavior[25]. This class of lipid mediators is found in animal and plant tissues[344], and is involved in a wide range of physio-pathological processes including feeding behavior, pain[345], innate immunity[346] and reward circuitry[347]. The relevance of these compounds in physiological functions emerged with the discovery of anti-allergic and anti-inflammatory properties of palmitoylethanolamide (PEA), the ethanolamide of palmitic acid[348] and, in particular, the ability of AEA to bind and activate the cannabinoid receptors[319]. Indeed, several studies demonstrated that AEA interacts with the same receptors activated by tetrahydrocannabinol (Δ^9-THC)[349]. Moreover, FAEs are synthesized on demand in response to many different stimuli, for example neural activation (in the rat brain)[350,351] or the exposure to metabolic stressors (in mouse epidermal cells)[352]. The FAEs that have been better characterized are palmitoylethanolamide (PEA) and OEA.

1.5.1 Synthesis, degradation, and distribution

OEA is synthesized in the enterocytes of the small intestine from the oleic acid released upon the ingestion of dietary fat[26], that is uptaken from the lumen by CD36, expressed on the apical surface of enterocytes in rodent duodenum and jejunum[353]. It has been demonstrated that the mobilization of OEA in the small intestine is induced by fat intake, not food intake alone. In fact, OEA levels in the small intestine increased after the intraduodenal infusion of a lipid emulsion, but not after di administration of carbohydrate, protein, or saline solutions[354,355].

OEA synthesis pathway is an enzymatic reaction in two steps: in the first step, oleic acid is transferred from the sn-1 position of a glycerophospholipid to the amine group of phosphatidylethanolamines, and

yields N-oleoylphosphatidylethanolamine (NOPE), that belongs to the NAPE family, and this reaction is catalyzed by a Ca^{2+}-dependent N-acyltransferase (NAT). Then, NOPE is cleaved to form OEA and phophatidic acid by a NAPE-specific phospholipase D (NAPE-PLD), that has been found to be expressed in the epithelium and in the lamina propria of the mouse duodenum[26]. However, it has been demonstrated that the genetic deletion of the NAPE-PLD does not impair OEA synthesis, suggesting that there are NAPE-PLD-indepented mechanisms for OEA synthesis[356]. However, the genetic deletion of NAPE-PLD induces the obese phenotype, with insuline resistance, adipose tissue inflammation and altered microbiota, suggesting that the synthesis of NAEs is crucial for the homeostatic regulation of energy balance[357].

It is known that feeding induces OEA formation in the proximal intestine[25,358], due to the increased levels of oleic acid-containing NAPEs, and to the activation of the NAPE-PLD[26]. Interestingly, food intake increases specifically the synthesis of OEA (and the analogue linoeoylethanolamide, LEA) without affecting the levels of other FAEs, such as PEA and stearoylethanolamide (SEA)[26]. Moreover, food intake does not change OEA level in the stomach, colon and submucosa of small intestine, but exclusively in the lumen[26]. Moreover, OEA synthesis is regulated by the feeding state: many lines of evidence demonstrate that OEA levels in the upper intestine are decreased by food deprivation and increased upon refeeding[25,26,355,358]. Interestingly, a novel study demonstrated that the feeding state does not affect OEA levels in the liver[359]. In genetically obese rats, duodenal levels of OEA are 12-fold higher than wildtype controls, prompting the idea that it might be an adaptive response to hyperphagia[360]. Conversely, in DIO animals jejunal OEA levels are blunted, suggesting that the prolonged exposure to HFD reduces the responsiveness fed or fasted states[355].

OEA is catabolized into oleic acid and ethanolamine by enzymatic hydrolysis, that can be catalyzed by three different enzymes: fatty acid amide hydrolase (FAAH or FAAH-1), FAAH-2 and N-acylethanilamine-hydrolyzing acid amidase (NAAA). FAAH is the main anzyme for NAE degradation[361], although it has higher affinity for AEA[362], and is expressed in the liver and in the small intestine[363], in particular in the epithelium and in the lamina propria

of the mouse duodenum[26]. However, the genetic deletion of the gene encoding for FAAH decreases NAEs in a tissue-specific manner, suggesting the presence of additional catabolic enzymes with different tissue distributions[364]. In fact, in FAAH knock out mice, OEA levels decrease more in the brain[364–366] than in the circulation and the heart[364,367].

FAAH-2, expressed in humans and primates, but not in rodents, has 20% sequence identity with FAAH-1, and has a higher affinity to OEA more than AEA[368]. NAAA is the third NAE-hydrolizing enzyme, and belongs to the lysosomal choloylglycine hydrolase family[369], and has a higher affinity for PEA than for OEA[370,371].

OEA is widely distributed throughout the body, even though plasma levels are lower than organ levels[372], suggesting that its signaling may be paracrine more than endocrine[26]. Moreover, in plasma, OEA is found in the lipoprotein-free fraction, likely carried by albumin[373]. In the brain, OEA has been detected in many brain structrures, such as cortex, hippocampus, thalamus, striatum, hypothalamus, cerebellum and brainstem[26]. In the liver, OEA levels increase after dietary oleic acid-intake, likely by providing the substrate for OEA synthesis[163,374], and, in the white adipose tissue, is triggered in rats after cold exposure[375] or after oxytocin administration[376].

1.5.2 Receptors

It is well known that OEA activates PPAR-α[340,377,378]. In fact, it has been demonstrated that OEA binds the ligand-binding domain of this receptor with a $K_D \sim$ 40nM and increases the transcription activity of this factor with a median effective concentration of ~120 nM[340]. When activated, PPAR-α dimerized with the retinoid receptor and, together, bind to specific sequences in the DNA called PPAR response elements (PPRE) to regulate gene transciption[379] of target genes, mainly involved in lipid transport and metabolism[380]. The OEA-induced activation of PPAR-α, and the resulting transcription of the PPAR-α-controlled genes, initiates the cascade of events leading to the induction of satiety and to the modulation of lipid metabolism exerted by this bioactive lipid. The PPAR-α-mediated OEA effects have been extensively studied in PPAR-α-KO mice, in which OEA fails to induce its

effects of feeding and on lipolysis[340,377]. Moreover, even though OEA activates PPAR-β/δ[340], it has been demonstrated that the effects of feeding are exclusively PPAR-α-dependent: in fact, PPAR-α agonists, like Wy-14643 and GW7647, are able to modulate feeding, whereas PPAR-β/δ and PPARγ failed to exert these effects[340]. Apart from the well-known transcriptional effects of OEA, PPAR-α exerts non trascriptional effects as well: it has been demonstrated that two PPAR-α agonists, GW-7647 and PEA, are able to induce a rapid decrease in the amplitude of evoked Ca^{2+} transient currents[381], and that PPAR-α can modulate the firing-rate of neurons by acting on nicotinic receptors[382].

OEA also binds the transient receptor potential vanilloid type-1 (TRPV1), highly expressed on sensitive vagal afferent neurons, after it has been phosphorylated by the protein-kinase C, leading to an increase of Ca^{2+} levels and thus to the depolarization of the membrane[383,384]. However, it has been demonstrated that the hypophagic effect of OEA is not TRPV1-dependent, since the peripheral administration of OEA still reduces short-term food intake in TRPV1-KO mice[384].

Finally, OEA is a medium potency agonist for GPR119, a G-protein coupled receptor expressed in rodent and human pancreatic and intestinal cells[385] that recognizes a broad panel of lipid molecules in addition to OEA[386]. However, also in the case of this receptor, it has been demonstrated that the genetic ablation of the gene encoding for GRP119 does not abolish the hypophagic effects of the periheral administration of OEA[387], thus further confirming the crucial role played by PPAR-α receptors in mediating the effects of OEA.

1.5.3 OEA's effects on feeding, gene expression, and lipid metabolism

The most known effect of OEA after peripheral administration is a long-lasting and dose-dependent reduction of food intake[378,388], that is not linked to fear or anxiety, does not change plasma corticosterone levels, and does not induce conditioned taste aversion in rats[25]. Moreover, the behavioral effects of OEA

are selective to feeding, since its peripheral administration does not alter water intake or sodium appetite[25].

The hypophagic action of OEA depends on the feeding state of the animal. In free-feeding rats, in fact, OEA increases the latency to eating onset, decreases the meal frequency, but does not affect meal size; conversely, in food-deprived rats, OEA is also able to decrease the size of the first meal, in addition to the effects on the other parameters[389]. Furthermore, OEA decreases gastric emptying in a dose-dependent manner, an effect that could contribute to the reduction of food intake[390].

Among NAEs, PEA and LEA also induce satiety[391]. However, LEA fails to induce satiety effects when administered at the same dose as OEA, even though it is found in higher concentrations in the upper small intestine[358]. On the other hand, PEA induces satiety to a lesser extent compared to OEA[25].

Peripheral OEA administration is known to increase the expression of both PPAR-α and PPAR-α-controlled genes, that encode for proteins involved in the control of lipid metabolism. In particular, it has been demonstrated that i.p. OEA administration increases mRNA levels of PPAR-α in the liver, duodenum and jejunum[340], as well as in adipose tissue and soleus muscle[377], and OEA-induced acrivation of PPAR-α induces the levels of expression of CD36[340,377,378] in all these tissues. Moreover, it has been shown that OEA is able to increase mRNA levels of the fatty acid binding protein (FABP), an intracellular protein with high affinity for fatty substrate[380], in the adipose tissue and soleus muscle[377], and, in particular, of the liver-specific isoform (L-FABP)[340]. Interestingly, CD36 is located, in enterocytes, on the apical membrane of brush border, suggesting its involvement in dietary fat uptake[392], further confirming its pivotal role in OEA's mechanism of action. Furthermore, in the liver, adipose tissue, and soleus muscle[377,378], OEA increases the expression of the uncoupling proteins (UCPs), whose expression levels are used to assess mitochondrial fatty acid β-oxidation. Moreover, OEA shows lypolitic effects and increases lipid mobilization from storage sites. In fact, it has been shown that OEA induces the release of non-esterified fatty acids (NEFA) and glycerol in rat dissociated adipocytes, without affecting glucose uptake and oxidation, in a concentration-dependent manner[377]. In the same

way, OEA concentration-dependently induced the release of NEFA and glycerol in a rat adipose cells colture, even though, interestingly, the incubation with the highest concentration inhibited these same effect[393]. Furthermore, OEA-induced NEFA and glycerol release has been demonstrated also in vivo, paralleled by an increase of 3-hydroxybutyrate[377], and another study demonstrated the decrease of lipid content in the liver of Zucker rats[378]. Overall, these findings show the lypolitic effects of OEA, that may contribute to the effects on body weight induced by this lipid mediator.

Many studies support the role of OEA in fatty acid β-oxidation, observed in cell coltures from rat skeletal muscle, heart, and liver cells incubated with OEA, with no effects on glucose metabolism[377]. Lastly, OEA lowers the levels of circulating lipids independently from its effects on body weight loss. In fact, rats that underwent a 1-week OEA treatment showed lower circulating levels of triglycerides compared to the pair-feeding group[25]. Moreover, peripheral OEA administration decreases circulating levels of cholesterol and triglycerides after one[393], two[378], and four[394] weeks, without affecting HDL cholesterol[393] or glucose[378] circulating levels.

1.5.4 OEA and the CNS

As described in the previous paragraphs, the CNS plays a pivotal role in the control of feeding and energy homeostasis thanks to the signals produced in the gut. Multiple lines of evidence demonstrate that OEA not only impacts lipid metabolism and oxidation[378,393], but also regulates neural circuits involved in the control of ingestive behavior. As already discussed, two of the main areas involved in the integration of the signals coming from the periphery are the NST and the AP, located in the brainstem. The NTS is the first relay station of vagal afferents and contains different populations of neurons that send projections to many different areas of the CNS, such as the hypothalamus[10]. In particular, the medial part of the caudal NST (SolM) is the site where the cell bodies of the noradrenergic A2 fibers are located[200]. It has been demonstrated by many studies that OEA increases Fos, both mRNA[25,395] and protein[205,396] levels, in these brain areas, where this activation is also paralleled by an increase of the noradrenergic tone[205,397]. In fact, it has been

demonstrated that peripheral OEA administration increases the levels of dopamine-β-hydroxylase (DBH, step-counter enzyme for noradrenergic synthesis) in both NST and AP, markedly in the SolM[205], that led to the investigation of the role played by the noradrenergic fibres in mediating the satiety effects of OEA. The lesion of A2 fibres with the injection of the retrograde toxin saporin (DSAP) conjugated to a monoclonal antibody against DBH into the PVN revealed that these neurons are crucial for the satiety effects[397]. Moreover, the ablation of the noradrenergic fibres led to the loss of the OEA-induced effects on the hypothalamus: in fact, it has been demonstrated that the peripheral administration of OEA induces the release of oxytocin from the PVN, the SON and the neurohypophysis, and the treatment with the antagonist of the oxytocin receptor in the 3rd ventricle abolishes OEA-induced satiety, demonstrating the pivotal role played by this neuropeptide in mediating OEA's effects[397,398]. Moreover, many studies showed that OEA activates oxytocin-expressing neurons in the PVN[397–399].

The histaminergic system controls many physiological processes, including feeding[186], and novel findings demonstrate that OEA requires an intact histaminergic system to exert its effcts on feeding and on the oxytocin secretion in the PVN[399]. All these findings demonstrate that OEA exerts multiple effects on the CNS by engaging several neural circuits, such as the noradrenergic[397], oxytocinergic[398] and histaminergic[399].

It is clear that OEA, to induce its effects on feeding and body weight, exerts many effects on both periphery and CNS. However, how the OEA-mediated signal could, from the periphery (where OEA is produced)[26], reach the CNS has been long debated. Many lines of evidence indicated that gut vagal afferents were responsible for NST activation and, in turn, for OEA's effects on the CNS. In fact, it had been observed that a total subdiaphragmatic vagotomy (TVX)[340] or the pretreatment with a neurotoxic dose of capsaicin[25] abrogated the hypophagic effects of OEA. However, a recent study conducted in rats that underwent a subdiaphragmatic vagal deafferentation (SDA), the most selective surgery targeting vagal afferents known to date, demonstrated that vagal afferent neurons are ot necessary for the hypophagic effect of OEA[388], thus weakening all previous hypotheses.

Therefore, attention was directed to the AP, a circumventrivular organ lacking a fully functional BBB, that not only receives direct inputs from vagal afferents[207] but is also able to detect bloodborne signals[203], and it has been demonstrated that the surgical lesion of this area abrogated the hypophagic and neurochemical effects of OEA[205]. Hence, as demonstrated in this work, OEA, after being produced in the small intestine, reaches the CNS through the bloodstream, and activates the SolM-projecting noradrenergic neurons in the AP. This leads to the activation of the A2 noradrenergic fibers project to the PVN (and possibly to the vTMN), increasing oxytocin neurosecretion, regulated also by the histaminergic activity of the vTMN (Fig. 1.5).

Moreover, OEA not only partakes in the homeostatic control of feeding behavior, but also on the non-homeostatic, by acting on the reward circuitry[400,401].

Overall, these findings demonstrate that, to induce satiety, OEA requires the involvement of multiple neural circuits, highlighting the multi-faceted effects this compound has on feeding behavior. However, OEA's central effects are not only restricted to feeding. In fact, it has been demonstrated that peripheral OEA administration improves memory consolidation[402]. attenuates depressive-like behavior[403] involving noradrenergic, serotonergic[404] and histaminergic[405] systems and ameliorates cognitive performances in a mouse model of ischemia[406].

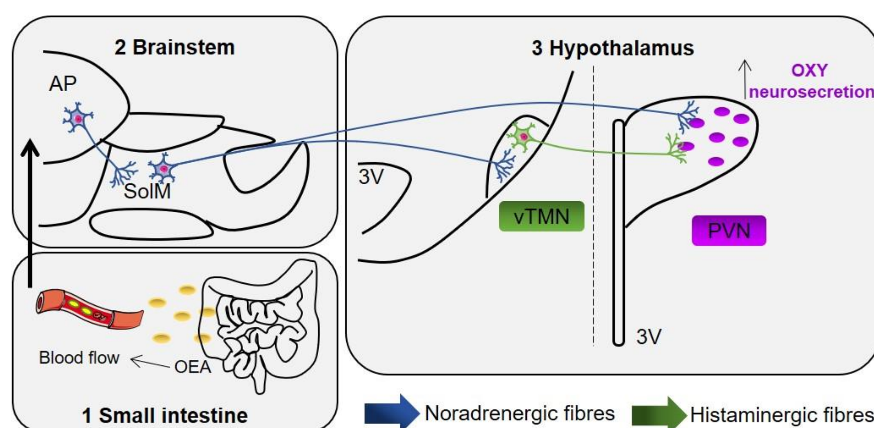

Fig 1.5: Mechanism of action of OEA. OEA is produced in the upper intestine upon the ingestion of dietary fat **[1]**, enters the bloodstream and is conveyed to the AP, where it activates the SolM-projecting noradrenergic neurons **[2]**. From the SolM, OEA activates A2 PVN-projecting (and possibly vTMN-projecting) noradrenergic fibers, increasing oxytocin neurosecretion, also with the aid of the histaminergic system **[3]**.

1.5 Aim of the study

According to the WHO, obesity is one of the most widespread chronic pathological conditions worldwide[1]. However, to date, there are no pharmacological treatments proven safe and effective. The obese phenotype is induced by the prolonged exposure to HFD, that has been linked to alterations in the mechanisms underlying the homeostatic control of energy balance. Moreover, it is known that the gut and the brain are constantly in communication through the so- called gut-brain axis, that plays a pivotal role in the regulation of feeding behavior and energy homeostasis[37]. In particular, the GI tract, after the ingestion of food, releases a variety of signals, like peptide hormons, such as CCK and PYY, and lipid mediators, that act both on the periphery and on the CNS to regulate feeding[118]. Among lipid mediators, OEA has gained a great deal of interest for its anti-obesity effects. OEA is a FAE synthesized in the small intestine upon the ingestion of dietary fat[26], and, in laboratory animals, the i.p. administration of OEA is able to induce a dose-dependent and long-lasting reduction of food intake and body weight[25,205,378], suggesting that it may be a potential pharmacological target for the treatment of obesity. The anorexiant effects of OEA require the PPAR-α, through which OEA is able to modulate lipid metabolism and FAO[377,393], and to induce gene expression, in particular in the liver and in the upper intestine[340]. PPAR-α is also required for the effects of OEA on the CNS: in fact, it is known to induce c-fos expression in areas involved in the control of feeding behavior, such as the NST and AP in the brainstem, and the PVN and SON in the hypothalamus[25,395]. Moreover, the central effects of OEA engage the oxytocinergic[398], noradrenergic[205,397] and histaminergic[205,399] neural circuits. However, how the OEA-mediated signal may be conveyed from the periphery to the CNS has long been debated. Since its hypophagic effects are abolished by total subdiaphragratic vagotomy[340] or the pretreatment with a neurotoxic dose of capsaicin[25], it has been suggested that this lipid compound may activate PPAR-α-expressing vagal afferents. However, novel findings demonstrate that OEA does not require intact vagal afferent system[388], but it rather requires an intact AP to exert its behavioral effects[205]. Moreover, the surgical lesion of the AP also prevents the neurochemical effects of OEA[205],

while the selective role of the vagal afferent system in mediating the OEA-induced activation of neural circuits involved in the control of feeding behavior still needs to be further elucidated.

Therefore, the aim of the 2nd chapter of this thesis is to address this point. In order to do so, we subjected male rats to the SDA surgery, the most selective surgery targeting vagal afferents. Sham animals were used as controls. Two hours after OEA administration, brains were removed from the skulls and cut at the cryostat in 20 µm-thick slices. Then, we performed Fos immunohystochemistry to assess whether vagal afferents are necessary for OEA to activate the AP, NST, PVN, Arc and vTMN; moreover, in the AP and NST, we investigated the expression of dopamine-β-hydroxilase (DBH) to evaluate the involvement of vagal afferents in the OEA-induced increase of the noradrenergic tone. Since it is known that oxytocin secretion is crucial for the satiety effect of OEA, we then performed a double staining, for Fos and oxytocin, in the PVN of both sham and SDA animals.

As aforementioned, OEA is able to modulate gene expression through PPAR-α activation: in particular, it has been demonstrated that i.p. OEA administration increases the expression of PPAR-α and CD36 in the liver and upper intestine[340], where it increases FAO and modulates lipid metabolism[377,393]. However, little is known about OEA effects on gene expression in the main areas involved in the homeostatic control of feeding behavior after peripheral administration. Therefore, the aim of the 3rd chapter of this thesis is to investigate the possible OEA-induced changes in gene epression, 2 and 4 hours after i.p. administration, in specific brain areas (AP, NST, Arc/ME and dHippo) and in peripheral organs (Liver, duodenum (Duo), and jejunum (JJ)). In order to do so, upon sacrifice, we microdissected, with the aid of brain matrix and surgical forceps, the brain areas, and collected samples of the peripheral organs, and analyzed gene expression by RT-qPCR.

The long-term exposure to HFD is responsible of the induction of the obese phenotype[407], and is known to disrupt the mechanisms underlying the homeostatic control of energy balance by acting on many different physiological processes[408]. In particular, the obese phenotype is characterized

by a significant reduction of OEA levels in the gut[355], and by changes in the gut microbiota, both in its composition and number of total bacteria[409]. Therefore, the aim of the last chapter of this thesis is to investigate the effects of the chronic peripheral administration of OEA in a rat model of DIO not only on its known effects on feeding and body weight, but also on the composition and number of bacteria of the gut microbiota, and on gene expression in the brain. In order to do so, we exposed male rats to HFD or LFD for 11 weeks to induce obesity, and then we subjected them to a chronic treatment with OEA (10 mg kg^{-1}, i.p.). During the chronic treatment, part of the rats that were gived HFD were shifted to LFD in order to mimic dieting. Upon sacrifice, we collected the brains and the fecal contents, and analyzed gene expression by RT-qPCR, measured the number of total bacteria and the relative abundance of the major phyla, classes and orders through PCR, targeting the gene encoding for the rRNA 16s.

Therefore, the main aim of this thesis is to investigate in depth the mechanism of action of this bioactive lipid compound, highlighting its complex role in modulating different aspects of the control of energy homeostasis.

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Chapter 2:
Role of vagal afferents on the neurochemical effects of oleoylethanolamide

Abstract
Oleoylethanolamide (OEA), a PPAR-α agonist, is a mediator of satiety. After peripheral administration, OEA induces Fos expression and activation in areas of the CNS involved in the control of feeding behavior and energy homeostasis, such as the nucleus of the solitary tract (NST) and in the area postrema (AP) in the brainstem, the hypothalamic paraventricular (PVN), supraoptic (SON) and ventral tuberomammillary (vTMN) nuclei. Moreover, it is known to increase the noradrenergic trasmission in the NST and AP, by increasing the expression of the dopamine-β-hydroxylase (DBH). Visceral ascending fibers were hypothesized to mediate such effects, but recent findings demonstrate that abdominal vagal afferents are not necessary for the anorectic effect of OEA. In fact, OEA is able to decrease food intake both in rats that underwent a subdiaphragmatic vagal deafferentation (SDA), a surgical procedure that eliminates all abdominal vagal afferents but spares about 50% of the vagal efferents, and in SHAM controls. Thus, the aim of the present work was to better elucidate the role of abdominal vagal afferents in mediating OEA's effects on the CNS. To meet this aim, we subjected rats to SDA surgery, using SHAM rats as control. By using immunohistochemistry, Fos and DBH expression patterns were investigated in the NST, in the AP, and in the hypothalamus after OEA administration (10 mg kg $^{-1}$).
Consistently with the behavioral results, OEA increases Fos expression in the NST and in the AP. Moreover, in these nuclei, SDA did not cause any alteration of DBH expression. In the hypothalamus, in line with the behavioral results, OEA is able to increase Fos expression in the PVN and the vTMN, even though in the latter does not reach statistical significance.

Overall, our findings indicate that vagal afferents are not strictly necessary for the satiety effect of OEA at both behavioral and neurochemical levels.

2.1 Introduction

To date, obesity represents one of the main health problems worldwide. This pathology is multifactorial in nature, and an increasing number of studies highlight how the exposure to an unhealthy diet can alter the mechanisms through which the central nervous system (CNS) regulates energy homeostasis and feeding behavior[1]. In fact, the CNS and the GI tract are constantly in reciprocal communication through the gut-brain axis, a complex network of neural and hormonal signals that links the periphery and the brain to control feeding behavior[2].

The vagus nerve is the tenth cranial nerve, and, since it reaches both the thoracic and the abdominal cavities, is one of the main connections of the gut-brain axis[3]. In fact, the signals produced by the variety of endogenous molecules acting in the periphery are conveyed, through the many receptors located on the vagal afferent system in the gut, to the brain areas involved in the regulation of feeding behavior[3]. The stimuli detected by the vagal afferents are mechanical, such as distention and contraction[4]; chemical, such as nutrients in the gut lumen[5] or hormonal, like CCK[6,7]and GLP-1[8]. Moreover, vagal afferents can detect neurotrasmitters and neuro-modulating agents (like serotonin[9] and somatostatin[8]), and cyotkines, as well as other inflammation mediators produced by the gut microbiome[10]. Therefore, vagal afferents play a pivotal role in conveying a large number of signals from the periphery to the nucleus of the solitary tract (NST)[11], a brain area located in the brainstem that is the main relay station of the vagal afferents in the CNS. In turn, the parts of the NST involved in the regulation of feeding behavior send projections to the hypothalamus[11], in particular towards the paraventricular and suraoptic nuclei (PVN and SON, respectively)[12], that are also linked to the endocrine system to regulate energy homeostasis[11].

In this scenario, in the last decade, the satiety factor oleoylethanolamide (OEA) has gained a great deal of interest as a possible novel therapeutic target to treat obesity and other aberrant eating-related disorders. In fact, OEA is known to reduce food intake in laboratory animals[13,14] without inducing stress or malaise[15]. OEA is a fatty acid

ethanolamide (FAE), synthesized in the small intestine upon the ingestion of dietary fat[13], that requires the intestinal peroxisome proliferator activated receptor-α (PPAR-α) to exert its prosatiety effects[16]. Moreover, the hypophagic effect of OEA (injected i.p. at the dose of 10 mg kg^{-1}) triggers many different effects in the CNS. In first place, peripheral OEA administration is paralleled by the activation of areas in the CNS involved in the control of feeding behavior, such as the NST and area postrema (AP) in the brainstem, and the PVN, the SON and the ventral tuberomammillary nucleus (vTMN) in the hypothalamus[17]. Furthermore, it has been demonstrated that the noradrenergic neurons that rise in the NST and project to the PVN are crucially involved in the activation of the hypothalamic areas induced by exogenous OEA, and that the ablation of these projections dampens OEA's satiety action[18].

In addition, in both PVN and SON, OEA increases *c-fos* mRNA in neurons expressing oxytocin (OXY), that leads to an increase of OXY mRNA levels and elevated circulating OXY levels[19]. All these effects on the oxytocinergic system are crucial in OEA's mechanism of action, as it has been demonstrated that the blockade of OXY receptors in the brain by intracerebroventricular infusion of the selective OXY antagonist, L-368,899, prevented the anorexic effects of OEA[19]. Furthermore, apart from the oxytocinergic system, OEA requires an intact histaminergic system to exert its pro-satiety effects[20]. Lastly, it has been long investigated whether OEA may exert its effects on feeding through the arcuate nucleus (Arc): in fact, previous experiments conducted in rats demonstrated that peripheral OEA administration failed to induce the expression of *c-fos* mRNA[15], whereas recent experiments conducted in mice show that OEA is able to increase Fos expression in this hypothalamic nucleus[21]. Moreover, the particular interest regarding this area rises from the fact that, like the AP, the Arc is a circumventricular organ, that receives bloodborne signals, such as leptin[22], to the CNS.

However, the mechanism through which the OEA-mediated signal is conveyed to the CNS is still uncertain. Many studies suggest the involvement

of visceral vagal afferents[13]: as a matter of fact, the OEA-induced satiety effect is abolished in animals that were treated with capsaicin[15] or that underwent a truncal subdiaphragmatic vagotomy (TVX)[16].

On one hand, both vagal afferents and efferents are lesioned in the TVX, that impairs the normal crosstalk from and to the CNS, leading to disruptions in sensory mechanisms, gastrointestinal secretions and motor functions of the GI tract[23].

On the other hand, the lesion induced by systemic capsaicin treatment is not specific for vagal afferents[24], as it removes unmyelinated visceral sensory neurons of both vagal and spinal afferents. Moreover, up to 20% of vagal afferents are left intact because are myelinated[25,26]. Furthermore, 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[27,28].

Therefore, a novel approach to selectively investigate the role of vagal afferents in the behavioral effects of OEA was used, the subdiaphragmatic vagal deafferentation (SDA)[29]. This surgery completely and selectively eliminates all abdominal vagal afferents, while sparing approximately half of the efferents. It is the most selective surgery targeting only vagal afferents, unlike TVX, and, unlike capsaicin treatment, it eliminates both myelinated and unmyelinated vagal fibers. In striking contrast with the previous findings[15,16], this study demonstrated that vagal afferents are not necessary for the hypophagic effects of OEA. Moreover, a recent study demontrated that, conversely, the AP is crucial for both the hypophagic and neurochemical effects of OEA[17]. The AP is a circumventricular organ, devoid of a functional bloo-brain barrier (BBB), where the receptors for many mediators of feeding behavior (such as amylin, GLP-1 and ghrelin) may be found[30,31].

Therefore, the aim of this work is to selectively investigate the role of abdominal vagal afferents in mediating the OEA-induced activation of the areas in the CNS involved in the control of feeding behavior. Moreover, we aim to investigate whether SDA surgery may impact the effects of OEA on the noradrenergic and oxytocinergic system.

2.2 Materials and methods

2.2.1 Animals

Male Sprague Dawley rats, weighing 180-200 gr upon arrival (Charles Rivers, Sulzeld, Germany) were used in this experiment. After arrival, the animals were individually housed in acrylic infusion cages in a temperature- and humidity-controlled room (22 ± 2 °C and 60% relative humidity), with a 12:12 h light/dark cycle with light on at 6.30 p.m. All the animals had free access to water and standard chow pellets (N 3433 diet, caloric density: 3.11 kcal g^{-1}, Provimi Kliba SA, Switzeland). All rats were implanted with an intraperitoneal catheter to minimize animal handling during treatments. All procedures were approved by the Veterinary Office of The Canton of Zurich.

2.2.2 Drugs and treatments

Prior to surgery, rats received a s.c. injection of antibiotics (4 mg kg^{-1} of trimethoprim and 20 mg kg^{-1} of sulfadoxine, Borgal 24%; Intervet/Shering-Plough Animal Health, Kenilworth, NJ) for infection prophylaxis. Fifteen minutes before surgery, animals received an i.p. injection of atropine (0.05 mg kg^{-1}; Sintetica, Mendrisio, Switzerland), followed by an injection of a mixture of 80 mg kg^{-1} ketamine (Ketasol-100; Dr. E. Gräub AG, Bern, Switzerland) and 5 mg kg^{-1} xylazine (Rompun; Bayer, Leverkusen, Germany), the final volume being 1.2 mg kg^{-1}.

After surgery, 5 mg kg^{-1} of carprofen (Rimadyl; E. Gräub, Bern, Switzerland) and 4 mg kg^{-1} Borgal 24% were injected for 2 days for analgesia and infection prophylaxis, respectively. All rats were allowed to recover from surgery for at least 2 wk before the experiments started. OEA (Sigma-Aldrich) was dissolved in sterile saline/PEG/Tween 80 (90/5/5, v/v/v; 2 ml kg^{-1}) and administered by i.p. injection 10 min before dark onset at the dose of 10 mg kg^{-1}. Control animals received an i.p. injection of vehicle, saline/PEG/Tween 80 (90/5/5, v/v/v; 2 ml/Kg). Each solution was freshly prepared on the experimental day.

2.2.3 Intraperitoneal catheter implantation

Catheter implantation was performed under aseptic conditions. Instruments were autoclaved, and the catheters were sterilized (Kodan Forte Farblos, Schulke & Mayr, Switzerland) prior to use. The proximal end of the catheter was led subcutaneously from the neck to a 4 cm midline incision in the abdomen and inserted in the abdominal cavity through a puncture hole. Intraperitoneal catheters ended in the peritoneal cavity and were anchored on the left side of the abdominal wall with Histoacryl glue. The abdominal muscle wall and skin were closed with absorbable sutures (3–0 and 5–0 Vicryl, respectively; Ethicon, Norderstedt, Germany). All rats were allowed to recover from surgery for at least 2 weeks before starting the experiments.

2.2.4 Subdiaphragmatic vagal deafferentation (SDA)

Twenty-seven rats were subjected to either SDA (n=15) or sham (n=12) surgery. In the SDA, the left dorsal (afferent) vagal rootlet at the level of the brainstem and the dorsal (left) esophageal trunk of the vagus in the abdomen were visualized and sectioned as previously described[32,33]. This procedure results in a complete elimination of all vagal afferents from below the diaphragm, while leaving approximately half of the abdominal vagal efferents intact (Fig 2.1). The sham procedure consisted of similarly exposing the vagal rootlets and abdominal vagus without manipulating them. Five milliliters of warm of Ringer lactate solution (Ri-Lac; B. Braun Medical AG, Sempach, Switzerland) were injected intraperitoneally after closing the abdomen.

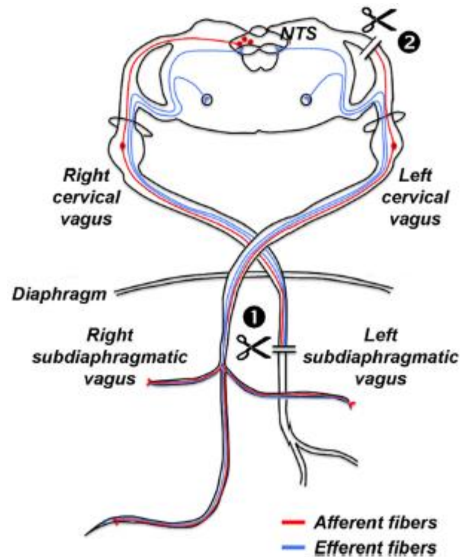

Fig 2.1: Schematic illustration of vagal fibers targeted by SDA. Afferent and efferent fibers are represented in red and blue, respectively. In the SDA procedure, the left (dorsal) subdiaphragmatic trunk of the vagus nerve is fully transected (indicated by the first scissor symbol), leading to a disconnection of both afferent and efferent fibers in the left (dorsal) trunk of the vagus nerve. The right (ventral) subdiaphragmatic trunk of the vagus nerve is left intact. In addition, a left-sided intracranial vagal rhizotomy is performed (indicated by the second scissor symbol) to selectively disconnect the remaining vagal afferents. This SDA procedure induces a complete (100%) disconnection of vagal afferents while leaving 50% of the vagal efferents functionally intact.

2.2.5 Immunohistochemistry

On the day of the terminal experiment, food was removed from the cages 1 h prior to dark onset, and rats were administered or OEA (10 mg kg-1, i.p.) or vehicle (saline/PEG/Tween80, 90/5/5, v/v/v, i.p.) 30 minutes prior to dark onset. Two hours after drug administration, animals were deeply anesthetized with pentobarbital sodium (80 mg kg-1; Kantonsapotheke, Zurich, Switzerland) and transcardially perfused with ice-cold sodium phosphate buffer (0.1 M PBS, pH 7.4), followed by fixative solution containing 4% paraformaldehyde. Fixed brains were removed from the skull, 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.

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 and DBH chromogenic immunostaining

OEA is known to reduce food intake[15–19,34], and this effect is paralleled by the activation of brain areas linked to the control of feeding behavior[17,18,34]. Since it has been previously described that SDA does not abrogate the satiety effect of OEA[29] (Fig 2.2), we aimed to investigate whether SDA is able to impair OEA's central effects.

One series containing the NST (n= 4-5 per group), the AP (n= 3-4 per group), and the dorsal motor nucleus of the vagus (DMV) (n= 4-5 per group) (from -13.68 to -14.30 mm from Bregma[35]), the PVN (n= 5-7 per group) (from -1.5 to -2.12 mm from Bregma[35]), the Arc (n= 5-7 per group) (from -2.12 to -4.52 mm from Bregma[35]) and the vTMN (n= 5-7 per group) (from -3.80 to -4.52 mm from Bregma[35]) was used for Fos immunostaining, and one more series containing the NST and AP (n= 5-7 per group for both areas) was used for dopamine-β-hydroxylase (DBH) immunostaining. Both analyses were conducted using the 3,3'-diaminobenzidine (DAB)-H_2O_2-horseradish peroxidase detection method. Briefly, sections were rehydrated in PBS (pH 7.4) and then incubated for 1.5 h in a solution containing 2% normal donkey serum (Jackson Immunoresearch) in 0.1% Triton X-100, followed by incubation with the primary antibody (rabbit polyclonal anti-c-fos primary antibody 1:5000, Santa Cruz Biotechnologies, Santa Cruz, California or mouse monoclonal anti-DBH antibody, MAB308, Millipore) at 4°C. Sections were then incubated with the secondary antibody (donkey anti-rabbit biotinylated secondary antibody, Jackson Immunoresearch, 1:500 or donkey anti-mouse biotinylated secondary antibody, Jackson Immunoresearch, 1:400) in 0.1% PBST for 2h at room temperature. After incubation for 1h 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).

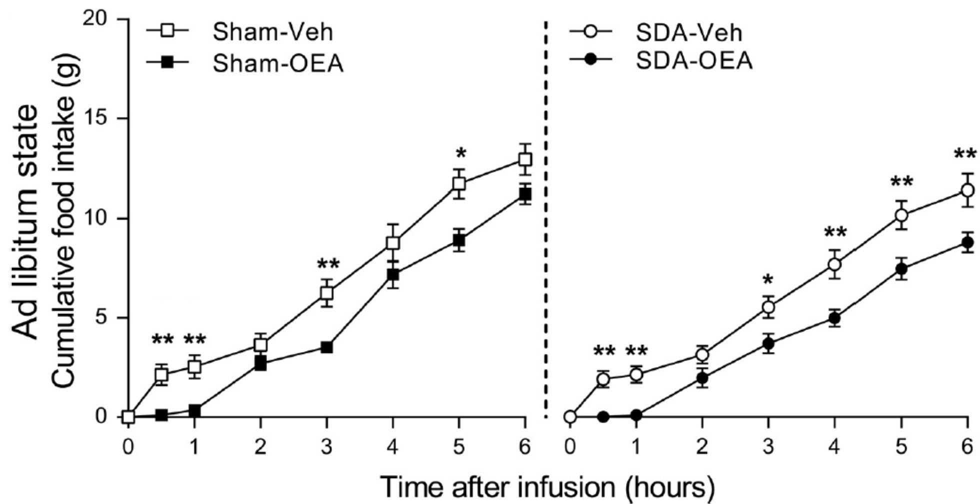

Fig 2.2: Effects of peripheral OEA administration on feeding of sham and SDA rats. Intraperitoneal OEA injection reduced food intake in sham (n=12; left) and SDA (n=15; right) rats during the first hour following injection. The reduction in cumulative food intake was still present to some degree between 3 and 6 h. Data were analyzed for individual time points by the Wilcoxon signed rank test. No significant differences in the food intake reduction by OEA were observed between SDA and sham rats (Mann-Whitney U-test). *P < 0.05, **P < 0.005 vs. vehicle in the same surgery group. Data are presented as means ± SE.

Fos and DBH double fluorescent immunostaining

A second series of sections containing AP and NST (n=1 per group for both areas) (from -13.68 to -14.30 mm from Bregma[35]) was double-stained for Fos and DBH to qualitatively assess their colocalization within these brainstem structures. Sections were rinsed with PBS and incubated for 1 h in a solution containing 0.3% Triton X-100 (Sigma-Aldrich) and 2% of normal goat serum (Jackson Immunoresearch) in PBS. Sections were then incubated with the primary antibodies (rabbit polyclonal anti-c-fos primary antibody 1:500 dilution, Santa Cruz Biotechnologies, Santa Cruz, California; mouse anti-DBH monoclonal primary antibody, 1:1000 dilution, MAB308, Millipore) for 2 overnights at 4 °C. Sections were then incubated with the secondary antibodies (anti-rabbit Alexa Fluor 594 secondary antibody 1:300 dilution; Invitrogen; anti-mouse Alexa Fluor 488 secondary antibody 1:250 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 (Sigma-Aldrich).

Fos and OXY double fluorescent immunostaining

A second series of sections containing the PVN (n=5-7 per group) (from -1.5 to -2.12 mm from Bregma[35]) was double-stained for Fos and OXY to assess their colocalization within this hypothalamic structure. After rehydration in PBS, sections were incubated in 2% normal goat serum (Jackson ImmunoResearch) in 0.3% PBST for 1 h. Sections were then incubated for one overnight at room temperature with rabbit polyclonal anti-c-fos antibody (1:5000 dilution, Ab-5, Calbiochem) and a mouse monoclonal anti-OXY antibody (1:1000 dilution; MAB 5296, Millipore). Sections were then rinsed in PBST and incubated with a goat anti-mouse Alexa Fluor 488 (1:400 dilution; Invitrogen) and a goat anti-rabbit Alexa Fluor 594 (1:300 dilution; Invitrogen) for 2 h. After additional washes, the sections were cover-slipped with Fluoromount (Sigma-Aldrich).

2.2.6 Image analyses

All the brain sections obtained from sham and SDA 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 in bright field or epifluorescent conditions. The rat brain atlas by Paxinos and Watson[35] was used as reference for the localization of the brain areas of interest.

The DAB- immunostaining was 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. For the double immunofluorescence analyses, Fos- or OXY-positive cells were manually counted and the colocalization was assessed as the percentage of OXY-positive cells within Fos-positive neurons. The investigator was blinded to animal treatment, and measurements were obtained in at least three consecutive tissue sections per animal containing the desired structure.

2.2.7 Statistical analyses

Immunohistochemical and immunofluorescence 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 $P<0.05$.

2.3 Results

2.3.1 OEA induces the expression of Fos and DBH in the AP and in the NST of both sham and SDA rats

Neurons of the AP and NST receive direct inputs from vagal afferents, and, in turn, project to brain areas that partake in the central control of food intake[36–40]. Moreover, previous studies demonstrated that the behavioral effects of OEA are paralleled by a selective induction of c-fos in these brain areas[34,41]. Since we already demonstrated that the behavioral effects of OEA are not abolished after SDA[29], we wanted to further demonstrate whether the SDA surgery may prevent the effects of OEA on Fos and DBH expression in the AP and in the different subnuclei of the NST (Fig 2.3A).

We found that SDA did not prevent the OEA-induced Fos expression of the AP (Fig 2.3B). Moreover, Fos optical density was increased in most of the NST subnuclei of both SHAM and SDA rats treated with OEA, as compared to respective controls treated with VEH (Fig 2.3 panels C-F). The Fos immunostaining of the SolVL of both SHAM and SDA rats was unaffected by OEA treatment (Fig 2.3F). Surprisingly, in the SolC of SDA rats the OEA-induced increase in Fos expression is attenuated compared to SHAM rats treated with OEA (Fig 2.3C), suggesting an involvement of vagal afferents in the activation of this subnucleus after OEA administration.

In addition, we aimed to investigate the effects of peripheral OEA administration on vagal efferents. To do so, we measured Fos optical density in the DMV, and we found that OEA increases Fos expression in both sham and SDA rats (Fig 2.3G). As for the SolC, the increase of Fos expression in the SDA animals treated with OEA is attenuated compared to sham OEA-treated controls, suggesting that a vago-vagal reflex may play a role in OEA's mechanism of action.

We demonstrated that the noradrenergic fibers that from the NST project to the PVN are responsible of the activation of this hypothalamic nucleus[18], and that the peripheral administration of OEA is able to induce DBH expression in the AP and NST[17]. Hence, the second aim of this study was to evaluate the role of abdominal vagal afferents in mediating the OEA-

induced increase in DBH expression in the brainstem. The results were similar to those obtained from measuring Fos optical density (Fig 2.4 panels A-E). In fact, OEA treatment led to an increase of DBH expression in the AP and in most of the NST subnuclei analyzed of both SHAM and SDA rats, compared to respective controls treated with VEH. Again, the DBH immunostaining of the SolVL of both SHAM and SDA rats was unaffected by OEA treatment (Fig 2.4E). 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 2.3 and 2.4, respectively.

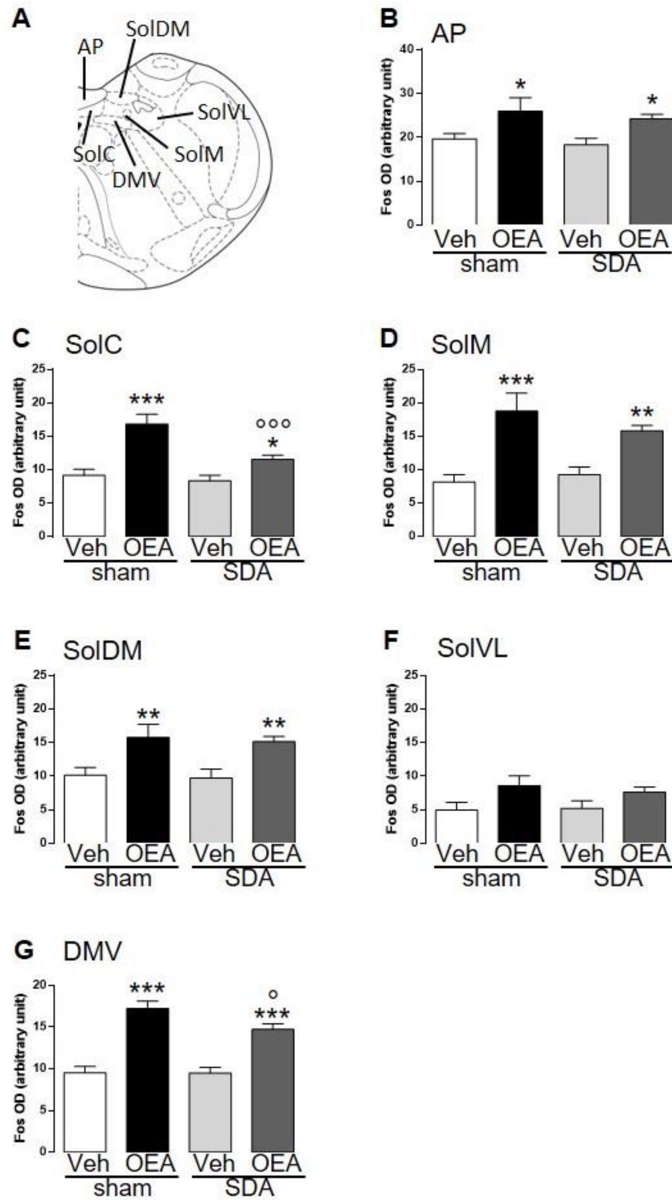

Fig 2.3: Effects of peripheral OEA administration on the activation within the DVC. Rat brain diagram taken from Paxinos brain atlas showing NST subnuclei, AP and DMV, in which Fos signal was quantified **(A)**. Semiquantitative analysis of Fos immunostaining within the AP **(B)**, commissural (SolC) **(C)**, medial (SolM) **(D)**, dorsomedial (SolDM) **(E),** ventrolateral (SolVL) **(F)** parts of the NST, and DMV **(G)** of both sham and SDA rats, treated with either vehicle (saline solution, PEG, Tween 80, 90/5/5 v/v/v; 2ml kg^{-1}) or OEA (10 mg kg^{-1}, i.p.). Data are expressed as mean ±SEM. *p<0.05; **p<0.01; ***p<0.001 vs vehicle in the same surgery group; °p<0.05; °°°p<0,001 vs sham in the same treatment group. AP: n= 3-4 per group; NST and DMV: n=4-5 per group.

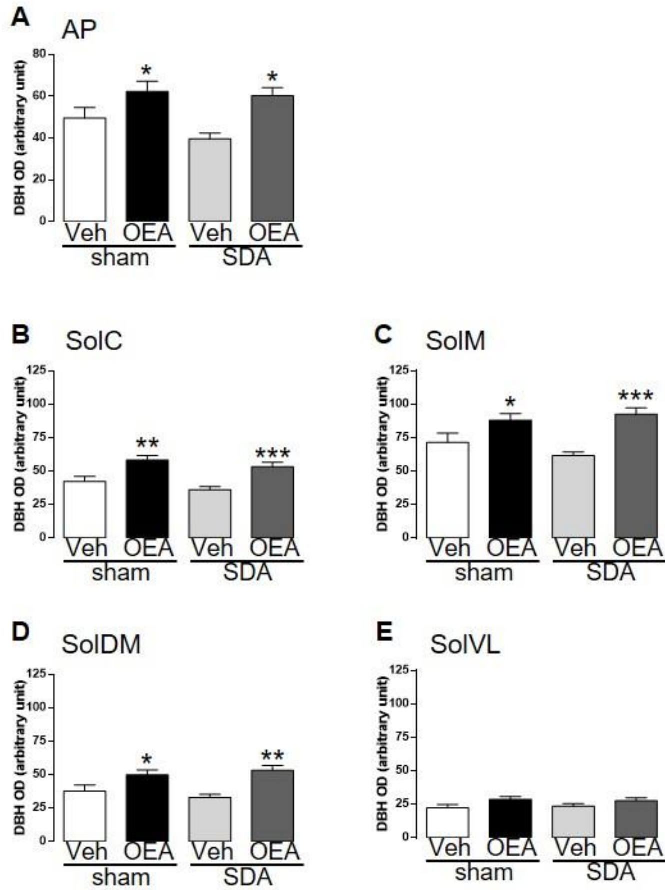

Fig 2.4: Effects of peripheral OEA administration on the noradrenergic transmission within the DVC. Semiquantitative analysis of DBH immunostaining within the AP **(A)**, commissural (SolC) **(B)**, medial (SolM) **(C)**, dorsomedial (SolDM) **(D)** and ventrolateral (SolVL) **(E)** parts of the NST of both sham and SDA rats, treated with either vehicle (saline solution, PEG, Tween 80, 90/5/5 v/v/v; 2ml kg^{-1}) or OEA (10 mg kg^{-1}, i.p.) (n=5-7 per group). Data are expressed as mean ±SEM. *p<0.05; **p<0.01; ***p<0.001 vs vehicle in the same surgery group. AP and NST: n=5-7 per group.

		$F_{treatment}$	$F_{surgery}$	$F_{interaction}$	df
Fos	AP	10.148 (P<0.01)	0.636 (P=0.438)	0.012 (P=0.914)	1/15
	SolC	28.594 (P<0.001)	8.929 (P<0.01)	4.884 (P<0.05)	1/18
	SolM	22.995 (P<0.001)	0.643 (P=0.433)	0.852 (P=0.368)	1/18
	SolDM	16.687 (P<0.001)	0.069 (P=0.795)	0.04 (P=0.843)	1/18
	SolVL	5.692 (P<0.05)	0.232 (P=0.636)	0.121 (P=0.732)	1/18
	DMV	50.974 (P<0.001)	1,28 (P=0.273)	0,439 (P=0.516)	1/18
DBH	AP	10.19 (P<0.01)	2.132 (P=0.158)	0.151 (P=0.701)	1/23
	SolC	27.025 (P<0.001)	3.541 (P=0.073)	0.011 (P=0.917)	1/23
	SolM	16.911 (P<0.001)	0.339 (P=0.566)	1.071 (P=0.311)	1/23
	SolDM	15.492 (P<0.001)	0.056 (P=0.815)	0.744 (P=0.397)	1/23
	SolVL	2.671 (P=0.116)	0.255 (P=0.618)	0.117 (P=0.735)	1/23

Table 2.1: Results of the two-way ANOVA analyses of Fos and DBH expression observed in the NST subnuclei of both SHAM and SDA rats after intraperitoneal injection of OEA or vehicle. Area postrema (AP), commissural (SolC), medial (SolM), dorsomedial (SolDM) and ventrolateral (SolVL) parts of the NST, dorsal motor nucleus of the vagus (DMV).

2.3.2 SDA may not prevent OEA-induced increase of Fos expression in DBH+ neurons in the NST and AP

We qualitatively investigated, performing double immunofluorescence analyses, the colocalization of Fos and DBH in the AP (Fig 2.5A) and in the SolM (Fig 2.5B) of SHAM and SDA rats. The images analyzed seemed to follow, for both Fos and DBH, the densities observed in the immunohistochemistries carried out singularly (see previous chapter) in all groups. Moreover, the images likely supported that OEA induces Fos in DBH-expressing cells in both SHAM and SDA animals. These analyses, therefore, would be in line with the behavioral results[29], and previous findings[17], highlighting the role of the noradrenergic projections that rise from the NST in mediating OEA's central effects.

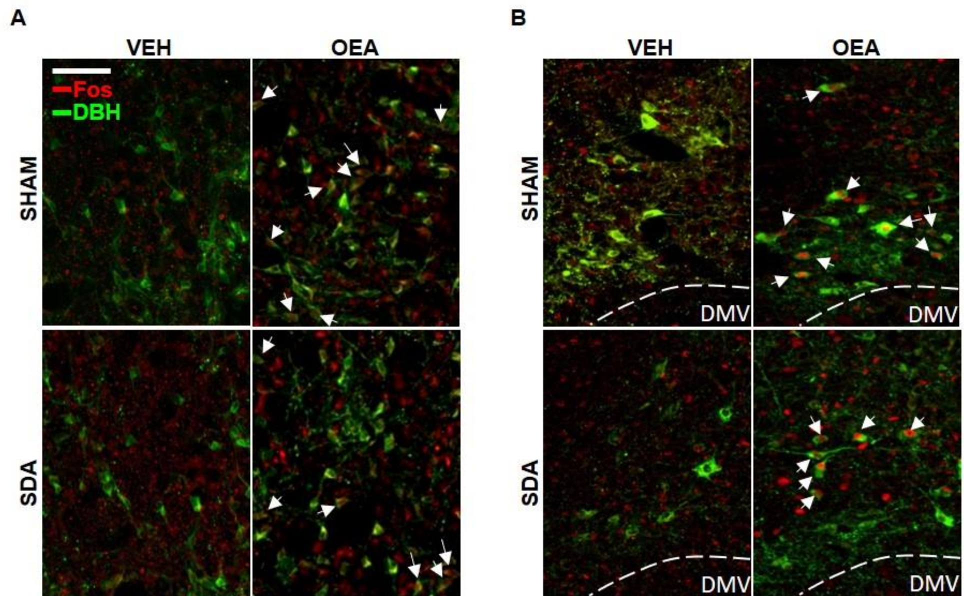

Fig 2.5: Effects of peripheral OEA administration on the activation of noradrenergic neurons within the DVC. Representative photomicrographs (x20 magnification, scale bar = 100 μm) of double fluorescent Fos/DBH immunostaining (red/green) within the AP **(A)** and the SolM **(B)** of both sham and SDA rats, treated with either OEA (10 mg kg^{-1}, i.p.) or vehicle (saline, PEG, Tween 80: 90/5/5 v/v/v; 2 ml kg^{-1}).

2.3.3 SDA does not prevent OEA-induced increase of Fos expression in OXY+ neurons in the PVN

Our previous work demonstrated that the PVN is activated after OEA administration[17]. Therefore, in the present work we evaluated whether this effect could be prevented by SDA surgery. We measured the optical density of Fos in the PVN (Fig 2.6B), and the results by two-way ANOVA showed a significant effect of treatment ($F_{treatment}$= 16.883, df= 1/21, P<0.001), but not of surgery ($F_{surgery}$= 3.139, df= 1/21, P= 0.090) nor of the interaction between the two factors ($F_{interaction}$= 0.865, df= 1/21, P= 0.362). The results from the test for multiple comparisons showed that, in keeping with our previous observations[17], OEA treatment significantly induced Fos expression in the PVN of SHAM-operated rats (P<0.01 vs VEH-treated controls), and, in line with the behavioral results[29], in SDA rats (P<0.05 vs VEH-treated controls). In our previous work we demonstrated that OEA induces Fos in OXY-expressing

cells of the PVN[17–19]. Hence, we wanted to assess whether vagal afferents are necessary for this effect. Our results show that the percentage of Fos^+/OXY^+ cells is increased in the PVN after OEA treatment in both SHAM and SDA rats. In particular, the results from the two-way ANOVA revealed significant effects of the treatment ($F_{treatment}$= 8.896; df= 1/23, P<0.01), but no significant effect of the surgery ($F_{surgery}$= 2.198, df= 1/23, P= 0.152) nor of the interaction between the treatment and the surgery ($F_{interaction}$= 0.000, df= 1/23, P= 0.992). Moreover, the results from the test for multiple comparisons showed that OEA treatment significantly induced Fos expression in OXY-positive cells the PVN of SHAM-operated rats (P<0.05 vs VEH-treated controls), and, in line with the behavioral results[29], of SDA-operated rats (P<0.05 vs VEH-treated controls) (Fig 2.6C).

Also, we analyzed separately the activation of the parvocellular (PaP) and magnocellular (PaM) portions of the PVN. Again, following the results obtained from the analysis of the PVN in toto, we found that OEA increases Fos expression in both sham and SDA rats. In particular, in both portions, data obtained from the two-way ANOVA analysis revealed significant effects of the treatment (PaM: $F_{treatment}$= 11.313, df= 1/18, P<0.01; PaP: $F_{treatment}$= 13.690; df= 1/18, P<0.01), but no significant effect of the surgery (PaM: $F_{surgery}$= 0.648, df= 1/18, P= 0.431; PaP: $F_{surgery}$= 0.011, df= 1/18, P= 0.918) nor of the interaction between the treatment and the surgery (PaM: $F_{interaction}$= 0.040; df= 1/18; P= 0.844; PaP: $F_{interaction}$= 0.138, df= 1/18, P= 0.715). Moreover, the results from the test for multiple comparisons showed that OEA treatment significantly induced Fos expression in the PaM and PaP of both sham- (P<0.01 vs veh-treated controls) and SDA-operated rats (P<0.05 vs veh-treated controls) (Fig 2.6D-E).

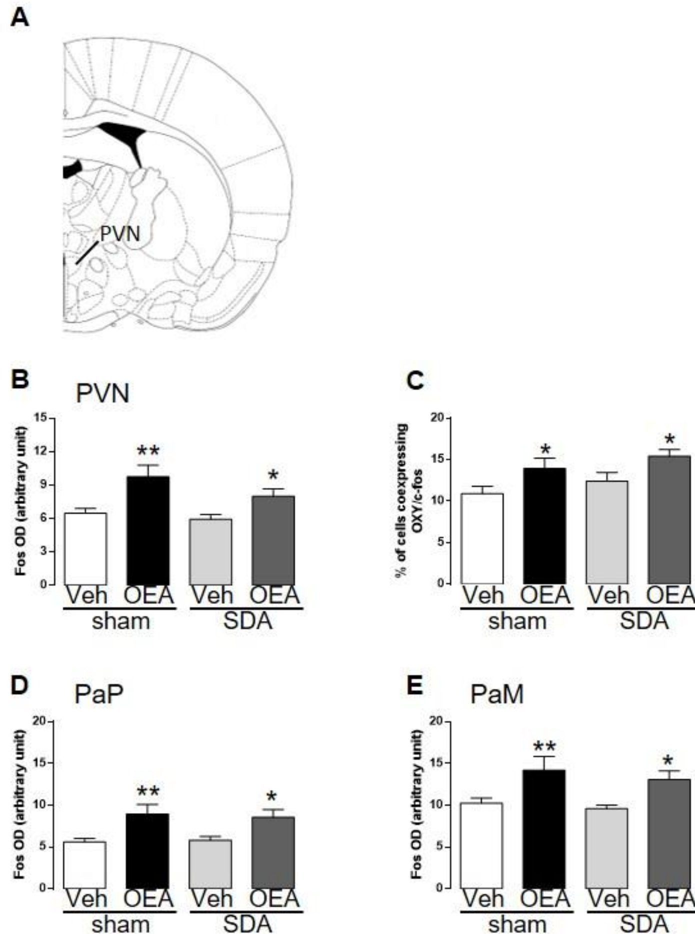

Fig 2.6: Effects of peripheral OEA administration on the activation of the PVN. Rat brain diagram showing the PVN **(A)**. Semiquantitative analysis of Fos immunostaining **(B)** and percentage of cells coexpressing Fos/OXY **(C)** within the PVN; semiquantitative analysis of Fos immunostaining within the parvocellular (PaP) **(D)** and magnocellular (PaM) **(E)** portions of the PVN, of both sham and SDA rats, treated with either vehicle (saline solution, PEG, Tween 80, 90/5/5 v/v/v; 2ml kg^{-1}) or OEA (10 mg kg^{-1}, i.p.) (n=5-7 per group). Data are expressed as mean ±SEM. *p<0.05; **p<0.01 vs vehicle in the same surgery group.

2.3.4 Effects of SDA on OEA-induced Fos expression in the hypothalamus

Our previous work demonstrated that the vTMN participates in OEA's effect on eating[17,20]. Therefore, we aimed to investigate whether the lesion of vagal afferents may prevent OEA's effects on this hypothalamic nucleus. We measured the optical density (Fig. 5B), and data obtained from the two-way ANOVA analysis showed a statistical significance of the treatment ($F_{treatment}$= 9.438, df= 1/15, P<0.01), but not of the surgery ($F_{surgery}$= 0.194, df= 1/15, P=

0.665) or the interaction between the two factors ($F_{interaction}$= 0.508, df= 1/15, P= 0.486). The results from the test for multiple comparisons showed that OEA increases Fos expression in SHAM animals (P<0.01 vs VEH-treated controls) but not in SDA animals, were the same trend is observed, although it does not reach the statistical significance (Fig 2.7B).

Previous studies showed that the peripheral administration of OEA (10 mg kg^{-1}) does not induce changes in the expression of c-fos in the Arc[15]. Conversely, recent experiments conducted in mice demonstrated that OEA increases Fos expression in the Arc[21]. Our results show that OEA has no effect on the activation of this hypothalamic nucleus (Fig 2.7C). In fact, the results from the two-way ANOVA did not show a statistical significance of the treatment ($F_{treatment}$= 0.013, df= 1/19, P= 0.910), nor of the surgery ($F_{surgery}$= 0.574, df= 1/19, P= 0.458) or the interaction between the two factors ($F_{interaction}$= 1.535, df= 1/19, P= 0.230).

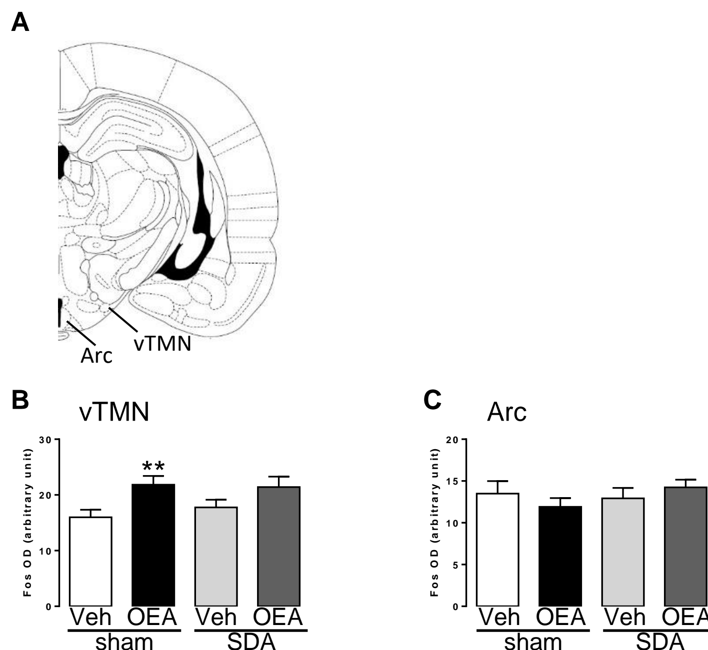

Fig 2.7: Effects of peripheral OEA administration on other areas of the hypothalamus. Rat brain diagram taken from Paxinos brain atlas showing the PVN **(A)**. Semiquantitative analysis of Fos immunostaining within the ventral part of the tuberomammilary (vTMN) **(B)** and arcuate (Arc) **(C)** nuclei, of both sham and SDA rats, treated with either vehicle (saline solution, PEG, Tween 80, 90/5/5 v/v/v; 2ml kg^{-1}) or OEA (10 mg kg^{-1}, i.p.) (n=5-7 per group). Data are expressed as mean ±SEM. **p<0.01 vs vehicle in the same surgery group.

2.4 Discussion

The study of the gut-brain axis, and, in particular, of all the molecules that partake in the control of energy balance, is of crucial importance in order to find novel therapeutic targets to treat obesity. Among the molecules that play a role in the control of feeding, OEA has gained a great deal of attention due to its anorexiant effects[15–19,34], however, to date, the machanism through which it reaches the CNS still needs to be fully elucidated.

It has been reported that both the TVX[16] and the systemic treatment with a neurotoxic dose of capsaicin[15] are able to abolish the anorexiant effect of OEA, strongly suggesting an involvement of the vagal afferent system in its mechanism of action. However, there are limits to both these approaches: with the TVX surgery, both afferent and efferent fibers are lesioned, leading to changes in the sensory mechnisms and motor functions of the GI tract[23]; on the other hand, is toxic to unmyelinated fibres, so both vagal and spinal neurons are lesioned[25,26]. Moreover, capsaicin is not able to lesion since the myelinated vagal afferent fibres, are up to 20% of the total[25]. Therefore, because of the limits presented by these techniques, the SDA surgery was used in this study. SDA is the most selective surgery targeting vagal afferents, and eliminates all of the afferent fibers while sparing around 50% of the efferent ones[32,33]. Using this approach, it is possible to avoid the side effects induced by the TVX[23], and to lesion selectively the afferent fibers of the vagus nerve, both myelinated and unmyelinated[24].

A recent study tested the hypophagic effect of OEA in rats subjected to SDA surgery and, surprisingly, demonstrated that vagal afferents are not necessary for the hypophagic effects of peripherally administered OEA[29]. Moreover, a novel study conducted by our research team demonstrated that the lesion of the AP, a cirumventricular organ that lacks a functional BBB, abolishes both the behavioral and the neurochemical effects of OEA[17].

Hence, to broaden our knowledge about the mechanism of action of this molecule, this study we investigated the role of vagal afferents in the neurochemical effects of OEA.

First, we focused our attention on the activation of the DVC. In particular, we carried out the densitometric analysis of the immunostaining directed towards Fos in the AP, the DMV, the NST and its subnuclei, namely the commissural (SolC), medial (SolM), dorsomedial (SolDM) and ventrolateral (SolVL) subnuclei.

It is known that the AP, together with the NST, receives direct inputs from the vagus nerve[27,28]. Moreover, as already demonstrated by our laboratory, the peripheral administration of OEA is able to induce the expression of Fos in the AP[17].

In the present study, we demonstrate for the first time that the OEA-induced activation of the AP does not require intact vagal afferents: in fact, in line with the behavioral results[29], OEA induces the expression of Fos in both sham and SDA rats. This supports the hypothesis that OEA does not act mainly through the vagus nerve, as supported by prevoius evidence[15,16], but through this circumventrivular organ[17], even though these results may seem in contrast. Regarding the lesion of vagal afferents with capsaicin, a possible explanation may be found in the fact that the peripheral administration of this neurotoxic compound may damage neurons of the AP that receive direct inputs from the vagal umyelinated fibres[27,28]. Thus, one interpretation accommodating the data we obtained with the previous capsaicin data is that unmyelinated spinal visceral afferents mediate the eating-inhibitory effect of OEA. On the other hand, the reason behind the loss of OEA's pro-satiety effect after TVX surgery, that leaves the spinal visceral afferents intact, is less clear. One possible explanation, other than the side effects that this surgery can induce[42,43], lies in a potential role of vagal efferents in mediating OEA's effects: in fact, in this study we report for the first time that OEA induces the expression of Fos in the DMV, and this effect does not require an intact vagal afferent system. Moreover, preliminary data obtained in our laboratory show that the OEA-induced increase of Fos expression in the DMV is blunted in animals that underwent a surgical lesion of the AP, further supporting the hypothesis that this circumventricolar organ plays a pivotal role in mediating OEA's effects.

Previous studies conducted by our laboratory demonstrated that the hypophagic effect of OEA is paralleled by the increase of Fos expression in the subnuclei of the NST[17,34].

In the present study, the results obtained by the densitometric analyses of the subnuclei of the NST show that, as already demonstrated[17,34], OEA induces the expression of Fos in all subnuclei of the NST, except for the SolVL, in SHAM rats. Moreover, in line with the behavioral results[29], the peripheral administration of OEA is able to induce Fos expression in all subnuclei of the NST, except for the SolVL, in SDA animals. Surprisingly, the increase of Fos expression in the SolC of OEA-treated SDA animals is attenuated compared with sham OEA-treated animals, suggesting a role played by vagal afferents in activating this portion of the NST after OEA administration.

Moreover, OEA induces the major increase in Fos expression at the level of SolM, a subnucleus particularly involved in the regulation of food intake, since it responds to the peripheral signals involved in the control of feeding behavior, such as leptin[44]. As already showed by our previous work[17], this portion of the NST plays a pivotal role in OEA's mechanism of action, since it receives projections from the AP [12], and is where the cell bodies of A2 noradrenergic neurons that send projections to the PVN[12] are located.

The same results obtained in the subnuclei of the NST for Fos expression were obtained after the densitometric analysis of the expression of DBH. In particular, along with Fos expression, peripheral OEA administration increases the DBH expression in both sham and SDA rats. Again, as expected[17],and in keeping with our previous observation this increase is mainly observed at the level of the SolM.

Based on our previous observations, that show that OEA activates the noradrenergic neurons of both the AP and SolM[17], we further qualitatively investigated whether this effect is still observed in SDA animals. In line with the behavioral results[29], peripheral administration of OEA is able to increase Fos expression in DBH-positive cells in both sham and SDA rats, further

confirming that vagal afferents are not necessary for the activation of this pathway, and, hence, for OEA's satiety effect.

We have already demonstrated that the NST-PVN noradrenergic pathway is crucial for OEA's mechanism of action[18]. Moreover, the noradrenergic fibers projecting to the PVN have their cell bodies in the SolM[12]. In particular, evidence demonstrate that the saporin-induced lesion of these fibres injected in the PVN[18] induces the loss of the cell bodies in the SolM, specially where the A2 cells are located, that directly project to the oxytocinergic neurons of the PVN[45]. Hence, we evaluated the activation of this hypothalamic area and the effects on the oxytocinergic system induced by the peripheral administration of OEA. As it has been already demonstrated, OEA is able to induce Fos expression in the PVN of sham rats[17]. In this study, for the first time, we demonstrate that the activation of this hypothalamic nucleus does not require intact vagal afferent system. We then analyzed the two major oxytocinergic sub-population of neurons of the PVN separately, the PaP (that project to several other brain areas) and the PaM (that send projections to the neurohypophysis)[46]. As for the PVN, SDA surgery does not impair the OEA-mediated activation of these two distinct portions of oxytocinergic neurons. Moreover, since it is known that OEA induces Fos in OXY^+ cells[17–19], we finally investigated if this effect is mediated by vagal afferents. The results we obtained by counting Fos^+/OXY^+ double-labeled cells show that the activation of the oxytocinergic cells exerted by OEA does not require an intact vagal afferent system.

Recents studies involving mice with a genetic deletion of the gene coding for histidine dexarboxylase (HDC-KO mice) showed that OEA's hypophagic action requires an intact histaminergic system[20]. Based on these findings, we analyzed the activation of the vTMN, the only source of histminergic neurons in the CNS[47]. The data obtained show that the peripheral administration of OEA induces Fos expression in the vTMN of sham rats, and the same trend is observed in SDA animals, even though it does not reach statistical significance. However, since the activation of the vTMN is necessary for the satiety effect of OEA[17,20], and SDA does not

prevent OEA's effects on feeding, we can presume that the increase in Fos expression in the vTMN of SDA rats, although not significant, is still sufficient for OEA to exert its anorexic effects.

Finally, since the results regarding the activation of the Arc after OEA administration are unclear, we analyzed the activation of this nucleus and the hypothetical role of vagal afferents in this effect. Recent experiments conducted in mice demonstrated that OEA increases Fos expression in the Arc[21], whereas, in the present work, OEA does not activate this hypothalamic nucleus. The difference between the two results could be due to the feeding state of the animals: in fact, the animals in the former work were starved for 12 h before OEA administration, and food deprivation has been demonstrated to influence the activation of the Arc nucleus[48]. Therefore, our results are in line with previous results[15], where peripheral administration of OEA has no effect on the activation of this circumventricular organ.

In conclusion, the data obtained with the present study show that the OEA-induced effects on the CNS do not require an intact vagal afferent system. Conversely, the present work further supports the pivotal role played by the AP, but not other circumventricular organs such as Arc, in mediating such effects.

Due to the lack of efficient pharmacological therapies for treating obesity, discovering new aspects about the role of the mediators of the gut-brain axis in the regulation of feeding behavior is of crucial importance. Almost 20 years of preclinical and clinical research clearly support the hypothesis that OEA may represent a successful canditate to treat obesity and aberrant eating-related disorders.

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Chapter 3:
Role of oleoylethanolamide in regulating gene expression in both brain and peripheral organs

Abstract

Oleoylethanolamide (OEA), is a fatty acid ethanolamide (FAE), known to induce satiety by modulating the meal pattern in laboratory animals. It has been observed that these behavioral effects require the activation of the peroxisome proliferator-activated receptor-α (PPAR-α), of which OEA is a high affinity ligand. PPAR-α is a transcription factor and, among the genes under its control, an important role is played by the cluster of differentiation 36 (CD36) and interleukin 6 (IL-6), for their effects on the modulation of uptake of FA and inflammatory responses, respectively. After its peripheral administration (10 mg kg^{-1}, i.p.), OEA is able to modulate lipid metabolism and increase FA oxidation in the liver in a PPAR-α dependent fashion; at the level of the central nervous system (CNS), OEA engages neural circuits involved in the control of feeding behavior. In particular, in our previous work, we demonstrated that the area postrema (AP) is crucial not only for the behavioral effects of OEA, but also is responsible of the activation of the pathways that underlie OEA central effects. Therefore, the aim of the present work is to evaluate if the effects induced by OEA peripheral administration (10 mg kg^{-1}, i.p.) are paralleled by changes in gene expression. In particular, we collected brain areas (AP, nucleus of the solitary tract (NST), arcuate nucleus, median eminence (Arc/ME), dorsal hippocampus (dHippo) and peripheral organs, in particular Liver, duodenum (Duo) and jejunum (JJ), from rats that were treated with vehicle or OEA (10 mg kg^{-1}, i.p.) and sacrificed at different time points, and analyzed the expression of PPAR-α, CD36 and IL-6 through RT-qPCR.

Overall, our findings confirm the pivotal role played by AP in the hypophagic effect of OEA and deepen our knowledge about its mechanism of action in peripheral organs, especially in the liver.

3.1 Introduction

Oleoylethanolamide (OEA), is a gut-derived fatty acid ethanolamide (FAE), produced in the small intestine upon the ingestion of dietary fat[1]. OEA is known to reduce food intake through the modulation of the meal pattern[2], by activating the peroxisome proliferator-activated receptor-alpha (PPAR-α)[3]. In fact, it has been demonstrated that OEA is able to activate this receptor at nanomolar concentrations, and that its genetic deletion abrogates the hypophagic effects of OEA[3]. PPAR-α, moreover, is a transcriptional regulator of genes involved in peroxisomal and mitochondrial β-oxidation, FA transport[4] and hepatic glucose production[5], and is particularly abundant in peripheral districts with high FA oxidation (FAO) rates, such as liver and brown adipose tissue, although it is also expressed in the intestine and the vascular endothelium[6]. Hence, by activating this receptor, OEA acts in the periphery by increasing FAO[7]. These effects are due to OEA's ability to regulate gene expression in the liver, jejunum and duodenum of wild-type mice, and these effects are absent in PPAR-α KO mice[3].

On the other hand, OEA acts in the central nervous system (CNS) by activating key brain areas involved in the control of feeding[8–11]: in fact, it has been shown that OEA's behavioral effects rely on the activation of the noradrenergic[10,11], oxytocinergic[11,12], and the histaminergic[11,13] neural circuits. In our previous work, we demonstrated the crucial role played by the area postrema (AP), a circumventricular organ that lacks a functional blood-brain barrier (BBB) located in the brainstem, in mediating OEA's both behavioral and neurochemical effects[11]. In the same work, we further demonstrated that PPAR-α is expressed in this brain region, and thus we hypothesized that OEA may enter the CNS through the fenestrated capillaries of the AP, activate PPAR-α receptors and then, in turn, exert all the downstream events involving the brain structures that partake in OEA's mechanism of action[11]. AP is in close contact with the nucleus of the solitary tract (NST), the primary relay station of vagal afferents[14], that is also strongly activated by OEA administration[8,9,11]. The NST is known to send projections towards many brain areas, in particular the hypothalamic areas that partake in OEA's mechanism

of action[10]. Moreover, in our previous work[11], we hypothesized that OEA may reach the AP through the bloodstream, and, to date, it seems to be the only circumventricular organ that partakes in OEA's mechanism of action.

Obesity alters fat uptake[15] and induces inflammation, that may lead to cognitive impairment[16]. In fact, recent findings demonstrate that a prolonged exposure to a high fat diet increases the risk of hippocampal alterations, leading to deficits in learning and memory[17], suggesting that a disruption of FA homeostasis in the dorsal hippocampus (dHippo) plays a role in cognition. Moreover, it has been observed that both the administration of PPAR-α agonists[18] and OEA[19] improve memory by activating this brain area.

It is known that OEA, by activating PPAR-α, triggers gene expression in peripheral organs, such as liver, duodenum, and jejunum[3]. However, little is known about the effects that OEA may exert at the gene expression level in the CNS. Therefore, the aim of the present work is to assess the effects of acute peripheral administration of OEA on gene expression in the CNS, in particular in the AP; the NST; the caudal part of the hypothalamus containing the arcuate nucleus (Arc) and the median eminence (ME) (Arc/ME), to investigate whether these circumventricular organs partake in OEA's mechanism of action; and in the dorsal part of the hippocampus (dHippo). Moreover, we carried out these analyses in peripheral organs such as liver, where OEA increases PPAR-α mRNA levels, and modulates lipid metabolism and FAO[7,20], duodenum (Duo) and jejunum (JJ), where OEA is known to be produced[1]. In order to do so, we treated rats with an acute i.p. administration of vehicle (veh) or OEA (10 mg kg^{-1}) and sacrificed them at different time points. We then collected samples of the peripheral organs and brain areas and analyzed gene expression levels through RT-qPCR. In order to assess the effects of OEA on fat uptake and inflammation, the genes we targeted are PPAR-α and two genes under its control, the cluster of differentiation 36 (CD36), also known as FA translocase (FAT), and interleukin 6 (IL-6). CD36, whose gene expression is positively regulated by PPAR-α agonists[21] coordinates the uptake and processing of free FA[22] and plays a role in the regulation of energy balance. Therefore, it is involved in the onset of

metabolic disorders such as insulin resistance, type 2 diabetes mellitus, obesity, and non-alcoholic hepatic steatosis[23]. On the other hand, IL-6, whose gene expression is repressed by PPAR-α[24], is a known pro-inflammatory cytokine and myokine.

3.2 Materials and methods

3.2.1 Animals

A total of 112 male Wistar-Han rats (Janvier Labs, Le Genest-Saint-Isle, France) were used in this study. All animals, weighing 275–325 g upon arrival, were individually housed in wire mesh cages under a 12:12 h 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 Kliba, Gossau, Switzerland) *ad libitum*. 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[25], dissolved in the vehicle (veh; saline/PEG/Tween80, 90/5/5 v/v/v, 2 ml kg^{-1}), and administered 10 mg kg^{-1} via i.p. injections. Both veh and OEA solutions were freshly prepared on each test day and administered about 30 min before dark onset.

3.2.3 Behavioral experiment and organ harvesting

On test day, food was weighed and temporarily removed from the cages 1 h prior to dark onset. Starting from 30 minutes before the dark phase, all the animals received an i.p. injection of either veh or OEA and were again given free access to food. After 2.5, 5, 15, 30, 60, 120 or 240 minutes, animals were deeply anesthesized with isoflurane, and blood, peripheral organs (Liver, Duo and JJ), and brain areas (AP, NST, Arc/ME and dHippo) were harvested. Briefly, samples of the peripheral organs taken in consideration were dissected, collected in an eppendorf tube and immediately snap frozen in liquid nitrogen. Regarding the brain areas, Arc/ME and dHippo were dissected with the aid of a rat brain matrix, whereas AP and NTS were extracted with the aid of curved forceps and a scalpel, and immediately snap frozen in liquid nitrogen. Food was measured again after sacrifice to assess the amount of food consumed by the animals after OEA or veh administration (n= 8 per group).

3.2.4 RT-qPCR analyses

All samples from 120 and 240 minutes were analyzed by RT-qPCR to assess the changes in gene expression after OEA administration (n= 4-8 per group). Total RNA was extracted using, according to the manufacturer's instructions, the ReliaPrep™ RNA Tissue Miniprep System (Promega, Fitchburg, WI, USA) from all the tissues. cDNA was then synthesized using the SensiFAST™ cDNA Synthesis Kit (BIOLINE, London, UK) from 1 µg of total RNA. qPCR was performed with an ABI 7500 instrument and software (Applied Biosystems, Foster City, CA, USA). The following conditions were used for amplification: an initial holding stage of 10 min at 95 °C, then 40 cycles consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60. Each sample was measured in duplicate during the same run. Data are normalized to the GAPDH mRNA expression. The sequences of the primers used are listed in Table 3.1.

GAPDH	Fwd	AAGAAGGTGGTGAAGCAGGC
	Rev	TCCACCACCCAGTTGCTGTA
CD36	Fwd	TGAGCCTACATTATGCACTAGC
	Rev	CACACCACCGTTTCTTCAAC
IL-6	Fwd	CACTTCACAAGTCGGAGGCT
	Rev	TCTGACAGTGCATCATCGCT
PPAR-α	Fwd	CGGGATGTCACACAATGCAATC
	Rev	CAGATCGTGTTCACAGGTAAGG

Table 3.1: List of the primers used in the study.

3.2.5 Statistical analyses

Feeding data were statistically analyzed by Student's *t*-test for mean comparison between veh- and OEA-treated animals at the same time-point (IBM SPSS, version 22, IBM Analytics). PCR data were statistically analyzed, using the $2^{-\Delta Ct}$ method, by two-way ANOVA, with "time" and "treatment" as the two factors. Tukey's test was used as a post hoc test to perform multiple comparisons.

In all instances, the threshold for statistical significance was set at $P<0.05$.

3.3 Results

3.3.1 Behavioral results

OEA is known to reduce food intake when administered i.p. to free feeding rats few minutes before dark onset, at a dosage (10 mg kg^{-1}) that does not readily allow penetration into the brain[9,10,12]. In this experiment, cumulative food intake was monitored after the sacrifice at each time point considered (2.5, 5, 15, 30, 60, 120 and 240 min). In particular, the results of the *t*-test analyses performed for each time point showed that OEA is able to significantly decrease food intake at 5 ($P<0.05$), 15 ($P<0.001$), 30 ($P<0.001$), 60 ($P<0.01$) and 120 ($P<0.05$) min, whereas no significant reduction was observed at 2.5 and 240 min time point (Fig 3.1).

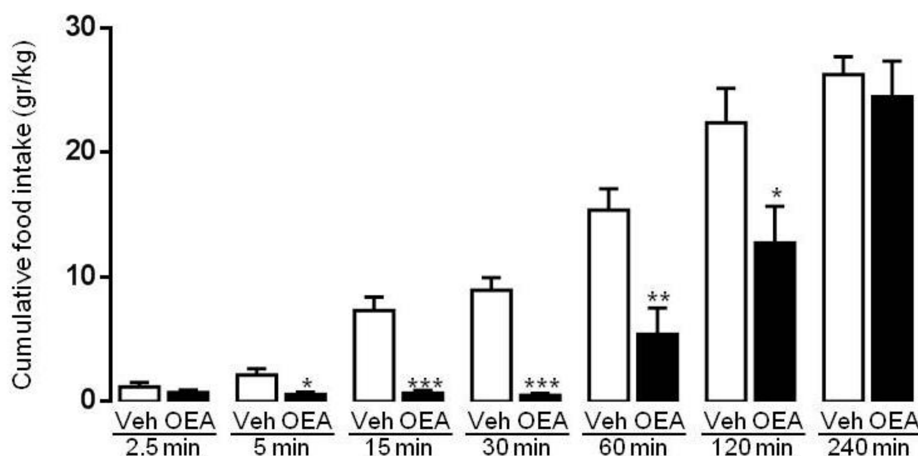

Fig 3.1: Effects of peripheral OEA administration on food intake at different time points. Time course of the cumulative food intake of animals sacrificed at different time points, treated with either vehicle (saline solution, PEG, Tween 80, 90/5/5 v/v/v; 2ml kg^{-1}) or OEA (10 mg kg^{-1}, i.p.) (n=8 per group). Data are expressed as mean ±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs vehicle at the same time point.

3.3.2 Effects on gene expression in different brain areas

Regarding the gene expression analyses, we started analyzing OEA's effects on gene expression in the AP, a circumventricular organ located in the brainstem. The AP in known to be the site of action of OEA[11], where it could possibly activate the PPAR-α receptor, its main pharmacological target[3]. In fact, our results show that the treatment with OEA (10 mg kg^{-1}, i.p.) is able to

induce PPAR-α expression 120 min after administration. The results of the Two-way ANOVA did not show a significant effect of the time (F_{time}= 3.441; df= 1/31; P= 0.074), nor of the treatment ($F_{treatment}$= 2.936; df= 1/31; P= 0.098) or of the interaction between these two factors ($F_{interaction}$= 3.441; df= 1/31; P= 0.074). Moreover, the results of Tukey's test for multiple comparisons showed that OEA is able to significantly increase the levels of expression of PPAR-α 120 min after administration in the AP (*P<0.05 vs 120 min veh). Interestingly, PPAR-α mRNA levels significantly decrease 4h after administration (°P<0.05 vs 120 min in the same treatment group) (Fig. 3.2). No differences were observed in the levels of expression of the other genes analyzed (Table 3.2).

Since it is well known that peripheral administration of OEA (10 mg kg^{-1}, i.p.) induces the activation of the NST[8,9,11], we aimed to investigate if this activation is paralleled by changes in gene expression at this level. Surprisingly, no changes in the expression of PPAR-α or its downstream target genes were observed in the NST (Table 3.2), suggesting that other mechanisms underlie the activation of the NST (Fig. 3.2).

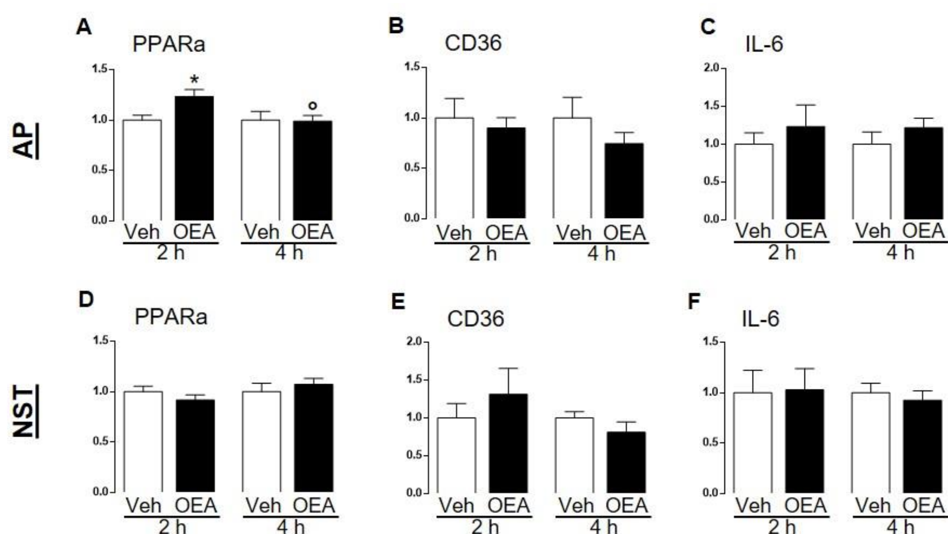

Fig. 3.2: Effects of peripheral OEA administration on gene expression in different brain areas 2 and 4 hours after administration. mRNA expression levels of PPAR-α, CD36 and IL-6 in the AP **(A-C)** and in the NST **(D-F)** of animals treated with veh or OEA (10 mg kg^{-1}, i.p.) and sacrificed 120 or 240 min after treatment (AP: n= 8 per group; NST: n= 5-6 per group). Data are expressed as mean ±SEM. *p<0.05 vs vehicle at the same time point; °p<0.05 vs 120 min in the same treatment group.

Moreover, we aimed to investigate if OEA, through the bloodstream, could reach other circumventricular organs. Therefore, we evaluated the effects of OEA on gene expression in the caudal part of the hypothalamus, containing the ME and the Arc nuclei, already known to receive blood-borne signals such as leptin[26]. We found that the peripheral administration of OEA has no effects on the expression on PPAR-α or on the IL-6 mRNA levels in these hypothalamic nuclei (Table 3.2). Surprisingly, i.p. OEA administration (10 mg kg^{-1}) increased mRNA levels of CD36: the results of the Two-Way ANOVA showed a significant effect of the time and of the interaction between time and treatment (F_{time}= 5.451; df= 1/30; P= 0.027; $F_{interaction}$= 5.451; df= 1/30; P= 0.027), but not of the treatment alone ($F_{treatment}$= 2.548; df= 1/30; P= 0.122). Moreover, the results of the Tukey's post hoc test for multiple comparisons showed that CD36 mRNA levels are significantly increased 120 min after administration (*P<0.05 vs veh-treated controls at the same time point), and they decrease over time (°°P<0.01 vs 120 min in the same treatment group) (Fig.3.3).

No changes were observed in the mRNA levels of PPAR-α and IL-6 (Table 3.2) within the hippocampus, whereas the peripheral administration of OEA (10 mg kg^{-1}, i.p.) is able to increase CD36 mRNA levels in this brain area, although, as we observed in the Arc/ME, this effect is not paralleled by an increase of PPAR-α mRNA levels as we might have expected. In particular, the results of the Two-way ANOVA did not show a significant effect of time, nor of treatment or of interaction between these two factors (F_{time}= 1.130; df= 1/19; P= 0.304; $F_{treatment}$= 2.760; df= 1/19; P= 0.116; $F_{interaction}$= 1.130; df= 1/19; P= 0.304). Moreover, the Tukey's post-hoc test for multiple comparisons showed that OEA is able to significantly increase CD36 mRNA levels 120 min after administration (*P<0.05 vs veh treated controls at the same time point) (Fig. 3.3).

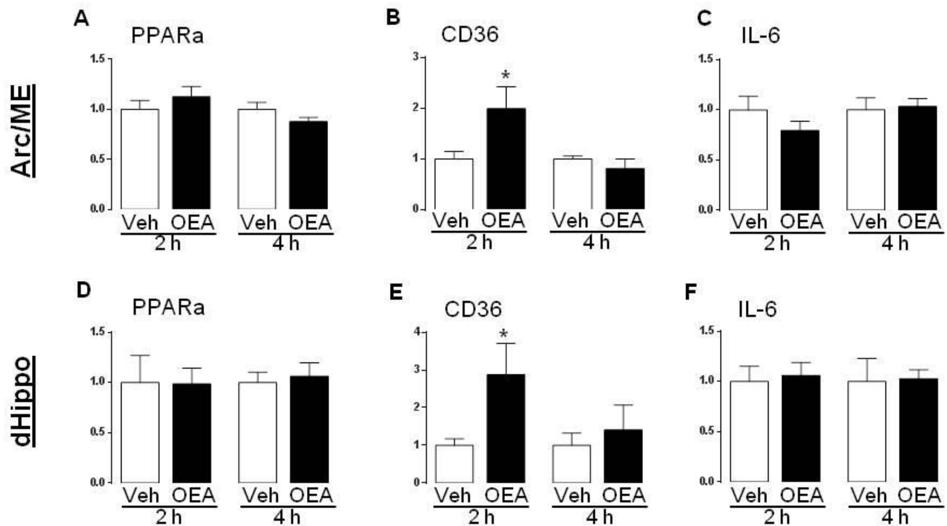

Fig. 3.3: Effects of peripheral OEA administration on gene expression in different brain areas 2 and 4 hours after administration. mRNA expression levels of PPAR-αlpha, CD36 and IL-6 in the Arc/ME **(A-C)** and in the dHippo **(D-F)** of animals treated with veh or OEA (10 mg kg^{-1}, i.p.) and sacrificed 120 or 240 min after treatment (Arc/ME: n= 7-8 per group; dHippo: n= 5-6 per group). Data are expressed as mean ±SEM. *p<0.05 vs vehicle at the same time point.

3.3.2 Effects on gene expression in different peripheral organs

It is known that OEA acts in the periphery, where it regulates lipid metabolism and FAO[7,20]. In particular, it has already been shown that it increases mRNA levels of PPAR-α and its target genes (such as CD36 and FABP-1) in the liver[3]. In the present work we found that, as expected, peripheral OEA administration (10 mg kg^{-1}, i.p.) increases the expression of PPAR-α: the results of the Two-way ANOVA showed a significant effect of time (F_{time}= 26.147; df= 1/28; P<0.001) and of interaction between time and treatment ($F_{interaction}$= 26.147; df= 1/28; P<0.001), but no significant effect of treatment ($F_{treatment}$= 3.682; df= 1/28; P= 0.066). Moreover, the Tukey's post hoc test for multiple comparisons revealed that PPAR-α mRNA levels significantly increased 120 min after OEA administration (***P<0.001 vs veh-treated controls at the same time point) and decreased 240 min after treatment (°°°P<0.001 vs 120 min in the same treatment group). Interestingly, at this time point, PPAR-α mRNA levels are lower compared to veh-treated controls (*P<0.05 vs veh at the same time point) (Fig. 3.4). Moreover, OEA treatment

(10 mg kg^{-1}, i.p.) seems to reduce CD36 mRNA levels, even though it does not reach statistical significance (P=0.050). No differences were observed in the expression of IL-6 (Table 3.2).

As next step of our experiments, we analyzed gene expression in the upper intestine, were OEA is produced[1]. In both the Duo and JJ, as expected[3], peripheral OEA administration (10 mg kg^{-1}, i.p.) increases PPAR-α mRNA levels. In particular, the results of the Two-way ANOVA did not show, in the Duo, a significant effect of time, nor of treatment or of interaction between these two factors (F_{time}= 2.851; df= 1/28; P= 0.104; $F_{treatment}$= 4.224; df= 1/28; P= 0.050; $F_{interaction}$= 2.851; df= 1/28; P= 0.104). Moreover, the results of the Tukey's post hoc test for multiple comparisons show that PPAR-α mRNA levels increase 120 min after OEA administration (*P<0.05 vs veh at the same time point) and decrease 240 min after treatment (°P<0.05 vs 120 min in the same treatment group). Regarding the JJ, peripheral OEA administration (10 mg kg^{-1}, i.p.) increases PPAR-αlpha mRNA levels 120 min after its administration. In particular, the results of the Two-way ANOVA showed a significant effect of the treatment ($F_{treatment}$= 4.940; df= 1/26; P<0.05), whereas no significant effect was observed for either the time or for the interaction between the two factors (F_{time}, $F_{interaction}$= 3.242; df= 1/26; P= 0.085). Moreover, the results of the Tukey's post hoc test for multiple comparisons showed that PPAR-α mRNA levels significantly increase 120 min after administration (*P<0.05 vs veh-treated controls at the same time point) and decrease 240 min after administration (°P<0.05 vs 120 min in the same treatment group).

We further analyzed the levels of expression of CD36: surprisingly, in the Duo, i.p. administration of OEA (10 mg kg^{-1}) did not induce changes in mRNA levels of this transporter (Table 3.2), whereas, in the JJ, the results of the Two-way ANOVA showed a significant effect of the treatment ($F_{treatment}$= 5.510; df= 1/29; P<0.05), while no significant effect of the time or of the interaction between the two factors was observed (F_{time}, $F_{interaction}$= 0.666; df= 1/29; P= 0.422). Moreover, in line with previous findings[3] the results of the Tukey's test for multiple comparisons show that the peripheral administration

of OEA is able to significantly increase CD36 mRNA levels 240 min after administration (*P<0.05 vs veh at the same time point). Lastly, in both the Duo and the JJ, peripheral OEA administration increases IL-6 mRNA levels 120 min after administration. In particular, in the Duo, the results of the Two-way ANOVA showed a significant effect of the time, the treatment and of the interaction between the two factors (F_{time}= 8.611; df= 1/21; P<0.01; $F_{treatment}$= 15.127; df= 1/21; P<0.01; $F_{interaction}$= 8.611; df= 1/21; P<0.01). Moreover, the Tukey's post hoc test for multiple comparisons showed that IL-6 mRNA levels significantly increase 120 min after OEA i.p. administration (***P<0.001 vs veh-treated controls in the same time point) and decrease 240 min after treatment (°°P<0.01 vs 120 min in the same treatment group). In the JJ, the results of the Two-way ANOVA showed a significant effect of the time, the treatment and of the interaction between the two factors (F_{time}= 12.899; df= 1/26; P<0.01; $F_{treatment}$= 5.455; df= 1/26; P<0.01; $F_{interaction}$= 12.899; df= 1/26; P<0.01). Moreover, the Tukey's post hoc test for multiple comparisons showed that IL-6 mRNA levels significantly increase 120 min after OEA i.p. administration (***P<0.001 vs veh-treated controls in the same time point) and decrease 240 min after treatment (°°°P<0.001 vs 120 min in the same treatment group).

		F_{time}	$F_{treatment}$	$F_{interaction}$	df
AP	CD36	0.232 (P=0.634)	1.207 (P=0.281)	0.232 (P=0.634)	1/29
	IL-6	0.002 (P=0.969)	1.304 (P=0.264)	0.002 (P=0.969)	1/29
NST	PPAR-α	1.019 (P=0.326)	0.004 (P=0.952)	1.019 (P=0.326)	1/22
	CD36	2.036 (P=0.170)	0.127 (P=0.726)	2.036 (P=0.170)	1/22
	IL-6	0.136 (P=0.716)	0.220 (P=0.884)	0.136 (P=0.716)	1/22
Arc/ME	PPAR-α	2.456 (P=0.128)	0.001 (P=0.972)	2.456 (P=0.128)	1/30
	IL-6	1.188 (P=0.285)	0.627 (P=0.435)	1.188 (P=0.285)	1/30
dHippo	PPAR-α	0.340 (P=0.856)	0.015 (P=0.904)	0.340 (P=0.856)	1/21
	IL-6	0.010 (P=0.920)	0.078 (P=0.783)	0.010 (P=0.920)	1/21
Liver	CD36	0.725 (P=0.402)	4.205 (P=0.051)	0.725 (P=0.402)	1/29
	IL-6	0.188 (P=0.669)	2.869 (P=0.103)	0.188 (P=0.669)	1/29
Duo	CD36	0.576 (P=0.454)	0.001 (P=0.978)	0.576 (P=0.454)	1/30

Table 3.2: Results of the two-way ANOVA analyses of mRNA expression levels observed in brain areas and peripheral organs after intraperitoneal injection of OEA or vehicle. Area postrema (AP), nucleus of the solitary tract (NST), arcuate nucleus/median eminence (Arc/ME), dorsal hippocampus (dHippo), liver and duodenum (Duo).

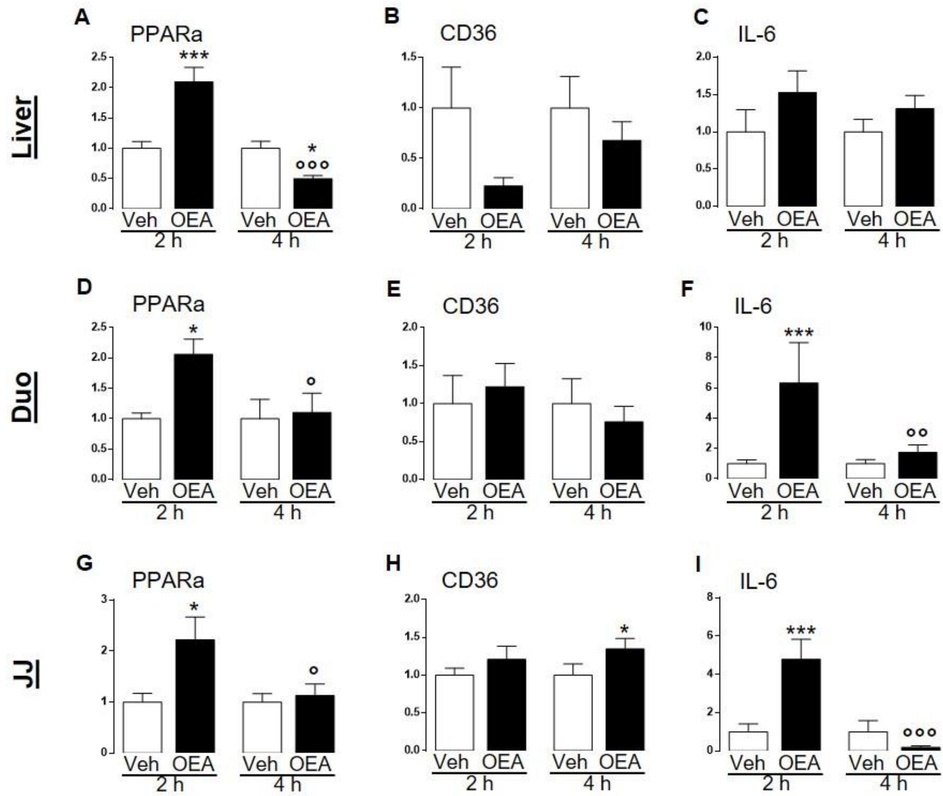

Fig. 3.4: Effects of peripheral OEA administration on gene expression in different peripheral organs 2 and 4 hours after administration. mRNA expression levels of PPAR-α, CD36 and IL-6 in the Liver **(A-C)**, in the Duo **(D-F)** and the JJ **(G-I)** of animals treated with veh or OEA (10 mg kg^{-1}, i.p.) and sacrificed 120 or 240 min after treatment (Liver and Duo: n= 7-8 per group; JJ: n= 6-7 per group). Data are expressed as mean ±SEM. *p<0.05; ***p<0.001 vs vehicle at the same time point; °p<0.05; °°°p<0.001 vs 120 min in the same treatment group.

3.4 Discussion

OEA induces a long-lasting and dose-dependent reduction of food intake in laboratory animals in a PPAR-α-dependent fashion[3]. The behavioral results of the present work, are in line with numerous previous findings[3,8–12,27], and highlight the ability of OEA to reduce food intake over time. Moreover, by activating PPAR-α, OEA is able, in the CNS, to trigger a complex neuronal cascade underlying its behavioral effects[8–11]. In our previous work, we demonstrated that the AP, a circumventricular organ located in the brainstem, is crucial for OEA's behavioral and neurochemical effects[11]. In the present work, we demonstrate, for the first time, that the acute peripheral administration of OEA (10 mg kg^{-1}, i.p.) induces the transcription of PPAR-α in the AP selectively, although this effect is not followed by the modulation of the expression of the genes known to be under its control. This may seem in contrast with the role of PPAR-α as a transcription factor, however, many lines of evidence suggest that PPAR-α participates also to non-transcriptional activities. In particular, it has been demonstrated that PPAR-α agonists (GW-7647 and palmitoylethanolamide), in mouse sensory neurons co-cultured with tumor cells, decrease the amplitude of Ca^{2+}-evoked transient currents[28]. Similarly, it has been reported that PPAR-α activity modulates, on one hand, the firing rate of neurons acting through nicotinic receptors[29], and, on the other, the calcium-activated K^+ channels, crucial for peripheral nociception[30]. It has been hypothesized that the short-term hypophagic effect of OEA, is due to PPAR-α's effect on ion channels[31], that could explain the reduction of food intake observed even 5 min after administration. Moreover, it may be hypothesized that the OEA-induced increase in PPAR-α expression observed at 120 min time point could enhance this effect, that could be the mechanism underlying the long-lasting effect of peripheral administered OEA observed up to 24 h after its administration[11]. In the present work, we further demonstrate that the increase of PPAR-α mRNA expression selectively occurs in the AP: in fact, no changes in mRNA expression of PPAR-α or its target genes is observed in the NST, even though this area is in proximity with the AP. We previously suggested that the activation of neurons in the AP might be

synaptically transmitted to noradrenergic neurons of the NST, particularly to the noradrenergic neurons present in the medial part of the NST. The data we obtained in the present work suggest that 1) peripherally administered OEA activates PPAR-α in the AP; 2) this event leads to a depolarization of AP noradrenergic neurons, that, in turn, 3) increase Fos and DBH levels in the NST[11], hence supporting our hypothesis.

The caudal hypothalamus plays a major role in regulating feeding behavior[32], and contains neurons that are sensitive to the presence of free FA thanks to the expression of several transporters, including CD36[33,34]. Since our findings support that peripherally administered OEA reaches the AP, we then investigated if it can reach and induce changes in gene expression in the Arc/ME through the same route. Our results demonstrate for the first time that peripheral OEA administration induced the transcription of CD36 in this brain area, whereas, to date, this effect of OEA has only been investigated in the upper intestine[3,20]. Unexpectedly, OEA fails to increase PPAR-α mRNA levels, even though this effect is observed in peripheral organs, thus leading us to hypothesize either that 1) the increase of PPAR-α mRNA levels occurs before the time points considered, or 2) the mechanism underlying this increase is OEA-related but might not be PPAR-α-dependent, as has been suggested[3]. In fact, it has been observed that PPAR-α mRNA levels significantly increase, in hepatoma cell cultures, not only in presence of the PPAR-α agonist Wy 14,643, but also of the fatty acid analog perfluorodecanoic acid, and, with the highest efficacy, dexamethasone[35,36]. Hence, it could be hypothesized that the OEA-induced activation of PPAR-α (with the consequent transcription of its target genes) and its increase in mRNA levels might follow two distinct, although parallel, pathways, that surely need further investigation.

As mentioned before, OEA improves memory and cognition by acting on the hippocampus[19]; interestingly, both cognition and memory might be impaired by the exposure to a high fat diet-inducing FA imbalance[17]. Therefore, it may be hypothesized that the beneficial effects of OEA on memory and cognition may be due to the upregulation of CD36, that, in turn, may ameliorate the FA sensing and uptake. Similar results were obtained in

microglial cells from a mouse model of Alzheimer Disease, were an increased expression of CD36 improved the clearance of Aβ plaques[37].

Regarding peripheral organs, we confirmed that OEA is able to increase PPAR-α mRNA levels in the Liver, Duo and JJ, as previously described[3]. However, in contrast with previous observations, we found that OEA decreased CD36 mRNA levels in the liver. Novel findings demonstrate that the pharmacological inhibition of CD36 improves obesity by reducing visceral fat accumulation and insulin resistance in obese mice[38]. Moreover, a recent clinical study showed that an increased expression of PPAR-α and a decreased expression of CD36 in the liver is observed in patients that underwent a Roux-en-Y gastric bypass (RYGB) surgery[39], and increased OEA signaling has been observed in a rat model of RYGB[40]. Hence, peripheral OEA administration may mimic the beneficial adjustments that occur after RYGB, that is one of the most effective and used surgical approaches to treat obesity[41,42].

Surprisingly, OEA treatment (10 mg kg^{-1}, i.p.) increased IL-6 mRNA levels 120 min after administration in the Duo and the JJ, and this effect is no longer observed after 240 min. In the small intestine, we demonstrated that OEA induces an increase of PPAR-α mRNA levels, that represses the transcription of the *IL6* gene via protein-protein interaction[24]. Hence, the PPAR-α products deriving from the OEA-induced increase in PPAR-α mRNA levels 120 min after administration could possibly be responsible of the decrease in IL-6 mRNA levels observed 240 min after administration. Moreover, this is in line with the well-known anti-inflammatory and anti-obesity effects of OEA, since a reduction of inflammatory cytokines[43] and of food intake[11,27] are observed long after administration.

Overall, the present work better elucidates the mechanism of action of OEA: in first place, it confirms the primary role that AP has in the OEA-induced neurochemical cascade, it elucidates the role played by OEA in modulating lipid sensing in the caudal hypothalamus and in the hippocampus; lastly, it gives new insights on the effects of OEA in peripheral organs, in particular in the liver associated with its anorexigenic effect.

3.5 References

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Chapter 4: Role of oleoylethanolamide in the metabolic changes induced by a prolonged exposure to a high fat diet

OEA is an endogenous lipid signal produced in the small intestine upon the ingestion of dietary fat. Although it is a structural analog of anandamide, it does not activate endocannabinoid receptors, but it binds the peroxisome-proliferator activated receptor-α, through which it stimulates lipolysis and reduces feeding and body weight. As a drug, OEA is able to reduce food intake and body weight gain (BWG) in both lean and obese rodents and has recently emerged as a potential novel pharmacological target for the treatment of obesity.

Based on these premises, in this study we investigated the anti-obesity effects of OEA in a rat model of diet-induced obesity (DIO), not only focusing on the regulation of feeding behavior and BWG, but also on the composition of the gut-microbiota, and on the expression of genes involved in the control of feeding behavior in key regions of the central nervous system, such as the brainstem and the hypothalamus.

The rat model of DIO was obtained by exposing male rats to high fat diet (HFD) for 11 weeks. Then, all the animals were chronically treated (14 days) with either vehicle or OEA (10 mg kg^{-1}, i.p.) during which they were exposed to HFD, low fat diet (LFD) or underwent a shift of the diet (SHIFT animals, that were given HFD for the first part of the experiment (11 weeks), then shifted to LFD during the chronic treatment. Moreover, an additional experimental group of pair-feeding rats (that received vehicle injection and was given the average amount of food consumed by OEA-treated rats in the matching diet group) was monitored to investigate a possible indirect effect of OEA on body weight.

We found that OEA reduces food intake and body weight gain independently from the diet regimen adopted and contributes to the reduction of body weight gain regardless of its anorexic action. In addition, two weeks of OEA treatment affected the general composition of the gut microbiota increasing the number of total bacteria and its diversity in all the groups (LFD, HFD, SLFD).

Analysis of gene expression in the brainstem reveals that the exposure to HFD and OEA treatment together increase the expression of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), PPAR-α, and c-fos genes. In the hypothalamus, OEA is able to increase the expression of the melanocortin 4 receptor (MC4R) in both HFD-fed and SFLD animals compared to the LFD-fed animals.

Overall, our study provides important new information on the therapeutic potential of OEA for the treatment of obesity.

4.1 Introduction

The communication between the gut and the brain, the so- called gut-brain axis, plays a pivotal role in the regulation of feeding behavior and energy homeostasis[1]. In particular, the gastrointestinal (GI) tract, after the ingestion of food, releases a variety of signals, like peptide hormones, such as CCK and PYY, and lipid mediators to regulate feeding[2]. Among the latter ones, N-acylethanolamines (NAEs) and N-acylphosphatidilethanolamine (NAPEs) are known to mediate satiety[3], and, due to its effects on food intake, the most studied NAE in this context is oleoylethanolamide (OEA), an unsatured analog of anandamide that does not bind CB receptors, but exerts its action through the peroxisome proliferator-activated receptor-alpha (PPAR-α)[4].

It has been demonstrated that OEA, mainly produced in the upper intestine upon the ingestion of dietary fats[5], acts as a satiety factor in rodents, and, when peripherally administered, alters the meal pattern by prolonging the latency to eat and the meal frequency, without affecting the quantity of food consumed[4,6,7]. Moreover, peripheral OEA administration exerts several effects in the central nervous system (CNS): in fact, it activates key areas involved in the control of feeding behavior, both in the brainstem and in the hypothalamus[8], and engages noradrenergic[9], oxytocinergic[8–10], and histaminergic[11] systems to modulate feeding.

It has been reported that the excessive ingestion of a high fat diet (HFD) is responsible of the induction of the obese phenotype (diet-induced obesity DIO)[12], characterized by a significant reduction of OEA levels in the gut[13], and by a change in the microbiome, both in its composition and number of total bacteria[14]. Moreover, it has been shown that a prolonged exposition (7-14 days) to HFD significantly reduces, in a DIO animal model, the concentration of anorexiant NAEs, including OEA, that could lead, in turn, to the hyperphagia observed in this experimental model[15,16]. This hypothesis was recently supported by a study that showed how the feeding-induced mobilization of OEA is disrupted in the same animal model[13].

The incidence of dysbiosis, an imbalance in the composition of the gut micorbiome correlated to other pathologies associated with incorrect eating habits, is increasing[17]. In fact, the prevalent consumption of fat-enriched

foods, along with the reduction of the consumption of fruits and vegetables, has noxious effects on the gut microbiome, that lead to alterations in its quality that contribute to the development of obesity and diabetes[18,19]. In fact, many studies have demonstrated that the exposure to HFD, obesity and metabolic syndrome induce changes in the composition and total number of gut bacteria[20,21].

On the other hand, whether long-term exposure to a HFD induces changes in gene expression in the CNS is still unclear. Exogenous OEA is reportedly a PPAR-α agonist that, in turn, induces the transcription of its target genes[4]. Hence, the aim of this work is to evaluate, in a rat model of DIO, the impact of peripheral OEA administration on i) food intake and body weight; ii) on the diet-induced changes of the gut microbiome; iii) on the changes of gene expression in key areas of the CNS involved in the control of feeding behavior, such as the brainstem and the hypothalamus. In particular, we focused our attention, in the brainstem, on *ppara*, *napepld* and *cfos*, to investigate the effects of prolonged exposure to HFD on OEA's main pharmacological target (*ppara*), on its synthesis (*napepld*) and on one of its main effects in this area (*cfos* induction). On the other hand, in the hypothalamus, we focused our attention on the melanocortin system by analyzing the effects of HFD on *mc4r*, due to its involvement in the reduction of food intake[22].

To this aim we exposed male Wistar-Han rats to either HFD or low-fat diet (LFD). After 11 weeks of exposure to HFD in order to induce obesity, all rats underwent a chronic treatment (2 weeks) of either vehicle (veh, saline/PEG/Tween80, 90/5/5/, v/v/v) or OEA (10 mg kg^{-1}). Moreover, during the chronic treatment, the animals were divided in different diet groups: i) animals that were fed with a LFD *ad libitum* throughout the whole experiment; ii) animals that were given a HFD *ad libitum* throughout the entire experiment; iii) animals that were exposed to HFD *ad libitum* during the first 11 weeks of induction of obesity and then were shifted to LFD *ad libitum* during the 2 weeks of treatment; iv) pair-feeding animals, that were given the average amount of food consumed by the OEA-treated animals in the matching diet groups.

4.2 Materials and methods

4.2.1 Animals and diets

A total of 96 male Wistar-Han (Charles River, Calco, Italy) were used in this study, weighing 305-315 gr upon arrival. All the animals were single housed in plexiglass cages in a temperature- and humidity-controlled room (T=22±2 °C; 60% of relative humidity), with a 12:12 h dark/light cycle. All the animals had free access to water and food, unless otherwise stated. After one week of housing, during which were exposed to standard chow diet, all the animals were exposed either to a purified LFD, with a total of 10% of the calories coming from fats (Open Source DIETS, D12450B), or to a purified HFD, with a total of 60% of the calories coming from fats (Open Source DIETS, D12492). The caloric density of the diets was 3.85 kcal/gr and 5.24 kcal/gr, respectively.

Housing, animal maintenance, and all experiments were conducted in accordance with the Council Directive of the European Community (86/609/EEC) of the Italian Decreto Legislativo n.26 (2014) and National Institutes of Health guidelines on animal care and were approved and supervised by a veterinarian.

Induction of obesity

In order to induce the obese phenotype, 60 out of the 96 animals were exposed *ad libitum* to the HFD for 11 weeks, while the remaining were fed with LFD *ad libitum*. The induction of the obese phenotype was assessed monitoring the weight of the animals daily and was considered established when the body weight (BW) of HFD-fed animals was significantly higher than that of LFD-fed animals (Fig 4.1). At the end of the induction of obesity, the HFD animals whose BW gain (expressed as percentage of the weight of day 1) were below the average of BWG% of LFD animals were considered obese-resistant, and therefore excluded from the study.

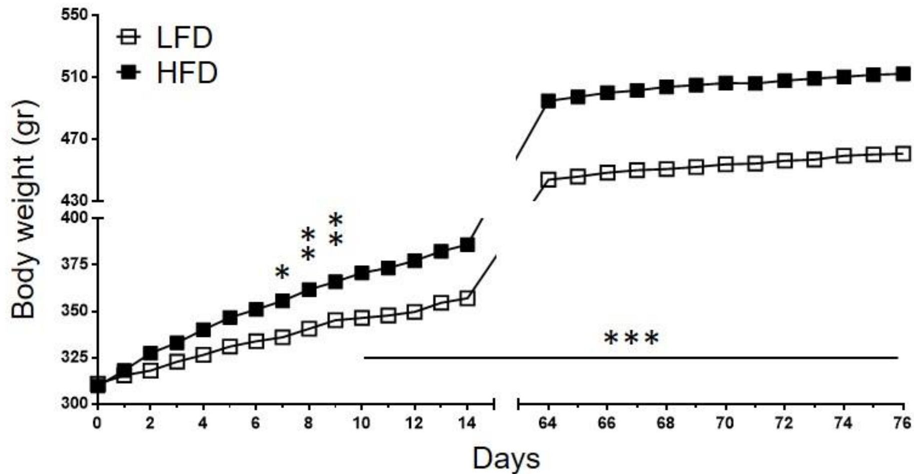


Fig. 4.1: Induction of obese phenotype. Time course of the body weight gain in rats exposed to LFD or HFD for 11 weeks. Data are expressed as means ±SEM. *P<0.05; **P<0.01; ***P<0.001 vs LFD.

4.2.2 Drugs and treatments

OEA (Sigma-Aldrich) was dissolved in the vehicle (veh; saline/PEG/Tween80, 90/5/5 v/v/v, 2 ml kg^{-1}), and administered 10 mg kg^{-1} via i.p. injections for 14 consecutive days. Both veh and OEA solutions were freshly prepared on each test day and administered about 30 min before dark onset.

4.2.3 Chronic treatment

Starting from the 12th week, all the animals were divided into two main experimental groups, named group A and B. All the rats belonging to the A group, 40 exposed to the HFD and 24 to the LFD, had free access to food throughout the whole experiment, whereas the animals belonging to the B group did not have free access to food during the 2 weeks of chronic treatment. Moreover, the HFD animals from the A group were divided in two subgroups: half of them were given HFD until the end of the experiment, and the other half was given LFD in the last two weeks of the experiment. This last group was named SHIFT and was introduced in order to mimic the fat and calories restriction observed in dieting individuals. In the same way, half of the HFD rats of the B group were maintained on HFD until the end of the experiment, while the other half became part of the SHIFT group. No changes were introduced in the LFD-fed group.

During the chronic treatment, all the animals of the A group were treated with either veh or OEA (10 mg kg $^{-1}$, i.p.) and had free access to food, while all the animals of the B group (pair-feeding group), were treated only with the veh, and received the average of the amount of food consumed by the OEA-treated animals of the A group in the matching diet group (pHFD, pLFD and pSHIFT). This group was introduced to investigate whether the OEA-induced weight loss is only due to its effect on the feeding or also to effects on the metabolism.

BW and food and caloric intakes of all animals were monitored throughout the whole experiment, both during the induction of obesity and the chronic treatment.

At the end of the experiment, in order to evaluate the effects of chronic administration of OEA (10 mg kg $^{-1}$, i.p.), we evaluated: the energy intake (EI) expressed in Kcal of each day of treatment, normalized by the weight of the animal; the BW gain expressed as percentage of weight of the last day normalized for the weight on day1 of chronic treatment; the BW gain expressed as percentage of weight of each day of chronic treatment normalized for the weight on day1.

The EI, expressed in Kcals, was calculated multiplying the grams of food consumed by the caloric density of each diet type, and was then normalized by the BW of the animal (Kcal/kg). For the behavioral experiment, the n= 10-12 per group.

The detailed timeline of the experiment is depicted in Fig 4.2.

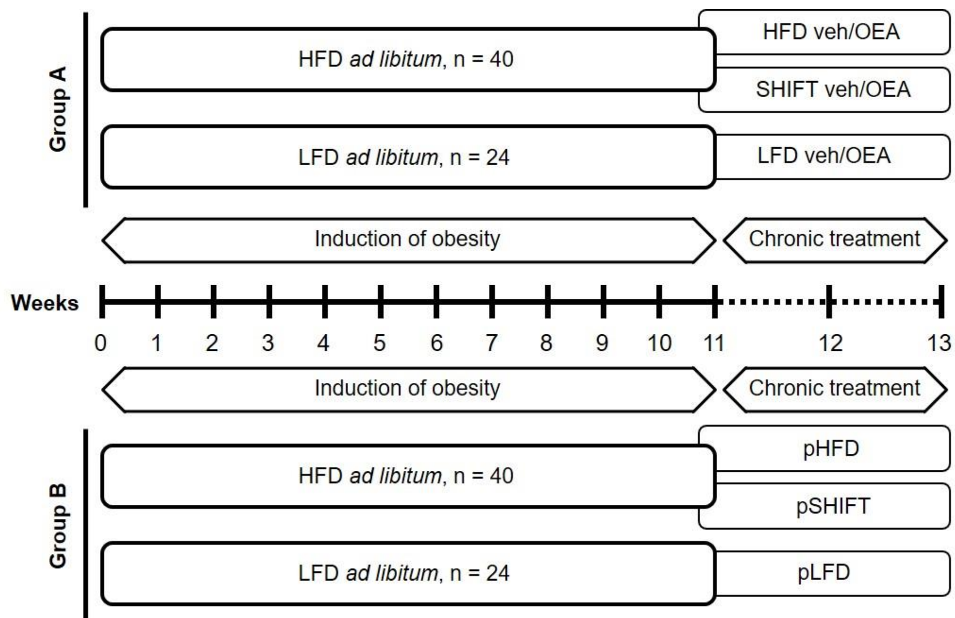


Fig. 4.2: Experimental paradigm. The diagram depicts the time course of the experiment, the diets and the treatments the animals were exposed to.

4.2.4 Terminal experiment

On the day of the terminal experiment, all food was removed from the cages 1 h prior to dark onset. All the animals were administered with either veh or OEA (mg kg $^{-1}$, i.p.) 10 minutes before the dark phase, and then had again free access to food. One hour after the administration, animals were deeply anesthetized with chloral hydrate (Sigma-Aldrich), and sacrificed through decapitation. Brains were extracted and immediately snap-frozen in ice-cold isopentane (Sigma-Aldrich), and cecal content was collected in sterile conditions to avoid any contamination from the experimental environment.

4.2.5 Quantification of total bacteria: qPCR

The quantification of the total bacteria through quantitative polymerase chain reaction (qPCR) was performed in collaboration with Prof. Patrice D. Cani, from the Metabolism and Nutrition research group at the Louvain Drug Research Institute (LDRI), Université catholique de Louvain (Brussels, Belgium). Briefly, the metagenomic DNA from each sample (85-120 mg; n= 3-6 per group) was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following manufacturer’s instruction. Then, the

concentration of the DNA was quantified using a Thermo Scientific NanoDrop™ 2000/2000c, and each sample was diluted to a concentration of 10 ng DNA/µl using a Tris-EDTA (TE) buffer.

The qPCR was performed using the STEPonePLUS Real-Time PCR system instrument and software (Applied Biosystems, Foster City, CA, USA), using Sybr Green for signal detection. A standard curve was set up with seven 5-fold serial dilutions of known amounts of DNA (colony forming units, CFU, were used), in order to quantify the number of total bacteria in each sample. Data were then expressed as Log_{10} (CFU/gr of sample).

The following conditions were used for amplification: an initial holding stage of 3 min at 95 °C, then 30 cycles consisting of denaturation at 95 °C for 15 s, annealing for 20 s, and extension at 72 °C for 10 s. Each sample was analyzed in duplicate in the same run.

A highly preserved sequence of the gene coding for the rRNA 16S was used to quantify the total amount of bacteria, using the following primers:

Rev: ATTACCGCGGCTGCTGG; -Fwd: ACTCCTACGGGAGGCAGCAG.

4.2.6 Pyrosequencing of the barcode rRNA 16s gene

Pyrosequencing is a method used to analyze a DNA sequence during its synthesis, thanks to a charge coupled device able to detect the light signal coming from the inorganic pyrophosphate released during DNA elongation.

Briefly, metagenomic DNA was extracted as described in the previous paragraph (n= 4-6 per group), and the highly variable part (V1-V3 region) of the rRNA 16S gene was amplified. This region, unlike the one used for the quantification of total bacteria, is less conserved, hence it allows to distinguish between phyla, classes, and orders. The high-throughput results of the sequencing of the amplicons were then analyzed using the Roche FLX Genome Sequencer, based on titanium dioxide nanoparticles technology (Titanium Chemistry, DNA Vision, Gosselies, Belgium). Data obtained were analyzed, in turn, with Quantitative Insights into Microbial Ecology v1.7.0 (QIIME) pipeline. The abundance of both identified and unidentified taxa, after the exclusion of those taxa representing less than 0.01% of the total abundance, were converted using the Hellinger method. The principal

coordinate analysis (PCoA) was measured using the unweighted UniFrac distance, that measures the beta-diversity relying on phylogenetic information comparing different microbial communities. Moreover, the operational taxonomic units (OTUs) were classified using the uclust consensous taxonomic classifier, with the threshold set at 0.97, and consulting the Greengenes database. Finally, phylogenetic trees were generated and developed using QIIME software v1.7.0 and displayed using iTOL v2.2.2.

4.2.6 RT-qPCR: analysis of gene expression in the brainstem and hypothalamus

The analysis of the alterations of gene expression due to a chronic treatment with OEA (10 mg kg^{-1}, i.p.) was carried out through retrotranscription qPCR (RT-qPCR), in both the brainstem and hypothalamus isolated from the brains previously collected and snap frozen during the terminal experiment (n= 4-5 per group). Briefly, total RNA was extracted with Trizol (Sigma-Aldrich) and chloroform. After centrifugation, isopropanol was added to allow total RNA precipitation, followed by washes in EtOH 75%. The pellet obtained was then vacuum dried and diluted in nuclease-free water. The concentration of RNA was obtained measuring the absorbance at 260 nm wavelength with a spectrophotometer, and the purity of the RNA was assessed measuring the 260 nm/ 280 nm wavelength ratio.

Then, total RNA was converted into cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). The qPCR was performed using the STEPonePLUS Real-Time PCR system instrument and software (Applied Biosystems, Foster City, CA, USA), using Sybr Green for signal detection.

The following conditions were used for amplification: an initial holding stage of 10 min at 95 °C, then 50 cycles consisting of denaturation at 95 °C for 10 s, annealing for 30 s at +57°C, and extension at 72 °C for 30 s. Each sample was analyzed in duplicate in the same run. The relative expression of the genes of interest (*napepld*, *cfos*, *PPAR-α* in the brainstem, and *MC4R* in the hypothalamus) was normalized to the expression of the GAPDH and expressed as $2^{-\Delta\Delta Ct}$ for statistical analysis.

4.2.7 Statistical analyses

Regarding the behavioral experiment, the data obtained from the daily monitoring of the BW gain and EI were analyzed with a Two-way ANOVA for repeated measures (within the same diet group), setting "treatment" (veh, OEA, pair-feeding) and "time" as fixed variables, and the Bonferroni's test was used as *post-hoc* analysis for multiple comparisons. On the other hand, the BW gain between the first and the last day of chronic treatment was analyzed by Two-way ANOVA, with "treatment" (veh, OEA, pair-feeding) and "diet" (HFD, LFD, SHIFT) as fixed variables, and Tukey's test was used as *post-hoc* for multiple comparisons.

Regarding the analyses of the microbiome and gene expression, the differences in the number of total bacteria and in the expression of *napepld*, *cfos*, *PPAR-α* and *MC4R* were both analyzed by Two-way ANOVA, with "treatment" (veh, OEA, pair-feeding) and "diet" (HFD, LFD, SHIFT) as fixed variables, and Tukey's test was used as *post-hoc* for multiple comparisons. Finally, for the analyses of the pyrosequencing, Student's *t*-test was used to compare the differences between two groups within the same *phylum*, order or class.

All the Two-way ANOVA analyses were carried out using SPSS Statistics (IBM Corporation, Armonk, NY, USA), while the *t*-tests were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

In all instances, the significance threshold was set at $P<0.05$.

4.3 Results

4.3.1 OEA decreases energy intake in all diet groups

In order to evaluate the hypophagic effect due to OEA administration (10 mg kg^{-1}, i.p.), the EI (Kcal/kg) was monitored throughout the two weeks of chronic treatment. In particular, in LFD animals, the Two-way ANOVA for repeated measures analysis showed a significant effect of time (F_{time}= 2275.787; df= 1/35; °°°P<0.001), of treatment ($F_{treatment}$= 26.165; df= 1/35; °°°P<0.001), and of interaction between these two factors ($F_{interaction}$= 9.222; df= 1/35; °°°P<0.001). Moreover, the Bonferroni post-hoc analysis for multiple comparisons showed that OEA induces a statistically significant reduction of EI from the third day of treatment (LFD V vs LFD O = °P<0.05), and until the end of the experiment (°°P<0.01 on the fourth day and °°°P<0.001 from the eleventh to the last day) (Fig 4.3A).

We further analyzed the EI intake of HFD animals. The results obtained with the Two-way ANOVA for repeated measures analysis showed a significant effect of time (F_{time}= 1938.974; df= 1/28; °°°P<0.001), treatment ($F_{treatment}$= 8.585; df= 1/28; °°P<0.01) and interaction between these two factors ($F_{interaction}$= 15.899; df= 1/28; °°°P<0.001). Furthermore, the post hoc analysis showed that OEA significantly decreases the EI in HFD animals from the eighth day of treatment (HFD V vs HFD O = °P<0.05), and until the end of the treatment (°°P<0.01 at days 9 and 10; °°°P<0.001 from the eleventh day until the end of the experiment) (Fig. 4.3B).

Lastly, we analyzed the EI of the SHIFT animals, where the Two-way ANOVA for repeated measures analysis showed a significant effect of time (F_{time}= 869.836; df= 1/26; °°°P<0.001), treatment ($F_{treatment}$= 14.760; df= 1/26; °°°P<0.001), and interaction between these two factors ($F_{interaction}$= 19.443; df= 1/26; °°°P<0.001). Moreover, the Bonferroni post hoc analysis for multiple comparisons showed that OEA is able to significantly decrease the EI of SHIFT animals from day 6 (°P<0.05), and until the end of the treatment (°°P<0.01 at the seventh day; °°°P<0.001 from the eighth day until the end of the experiment) (Fig. 4.3C).

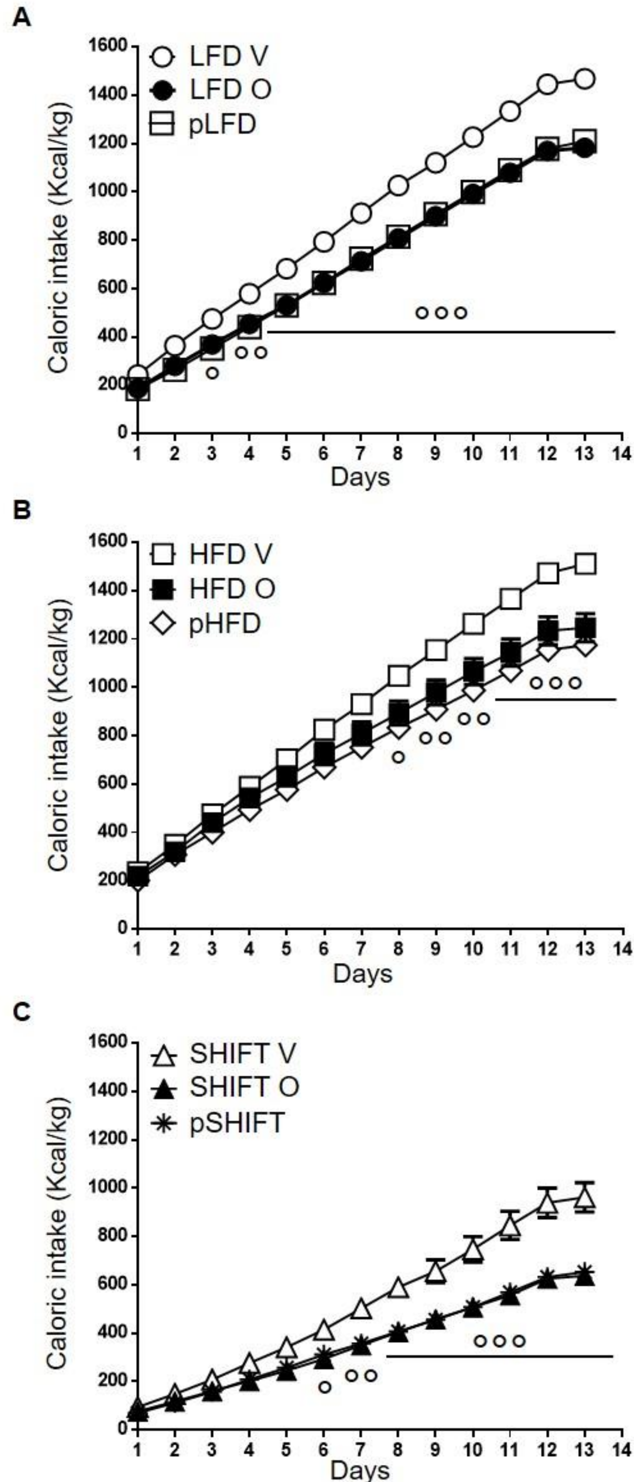


Fig. 4.3: Peripheral OEA administration decreases caloric intake regardless the diet regimen. Time course of the caloric intake of animals exposed to LFD **(A)**, HFD **(B)** or SHIFT **(C)** diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data are expressed as mean±SEM. °P<0.05, °°P<0.01; °°°P<0.001 vs veh-treated controls. N= 10-12 per group.

4.3.2 The OEA-induced weight loss is observed in all diet groups, and is linked to changes in the metabolism

In order to evaluate the anti-obesity effects of OEA, we evaluated both the cumulative, expressed as the difference between the last and the first day of the experiment, and the day-to-day decrease in BW gain.

Regarding the cumulative BW gain (Fig. 4.4), the results obtained with the Two-way ANOVA showed a significant effect of diet and treatment (F_{diet}= 138.960; df= 1/91; ***P<0.001; $F_{treatment}$= 42.696; df= 1/91; ***P<0.001), but not of the interaction between these two factors ($F_{interaction}$= 0.817; df= 1/91; P= 0.518). Moreover, the Tukey post hoc analysis for multiple comparisons showed that the chronic exposure to HFD induces *per se* a significant increase of BW compared to the chronic exposure to LFD (LFD V vs HFD V = *P<0.05). Furthermore, the shift from HFD to LFD induces *per se* a decrease of BW compared to the chronic exposure to HFD or LFD (SHIFT V vs LFD V = ***P<0.001; SHIFT V vs HFD V = ***P<0.001). In addition, chronic OEA administration (10 mg kg^{-1}, i.p.) induced a significant BW loss in all diet groups compared to veh-treated controls (veh-treated vs OEA-treated = °°°P<0.001 in all diet groups).

Lastly, we investigated whether the OEA-induced weight loss is only due to the reduction of the amount of food consumed. By comparing the weight loss of OEA-treated and pair-feeding animals in each diet group, we observed that OEA-treated animals showed a higher decrease of BW compared to pair-feeding animals independently of the diet group (^^^P<0.001 HFD O vs pHFD; ^^P<0.01 LFD O vs pLFD; SHIFT O vs pSHIFT), suggesting that the anti-obesity effects of OEA are due to effects on the metabolism rather than only the reduction of the amount of food consumed.

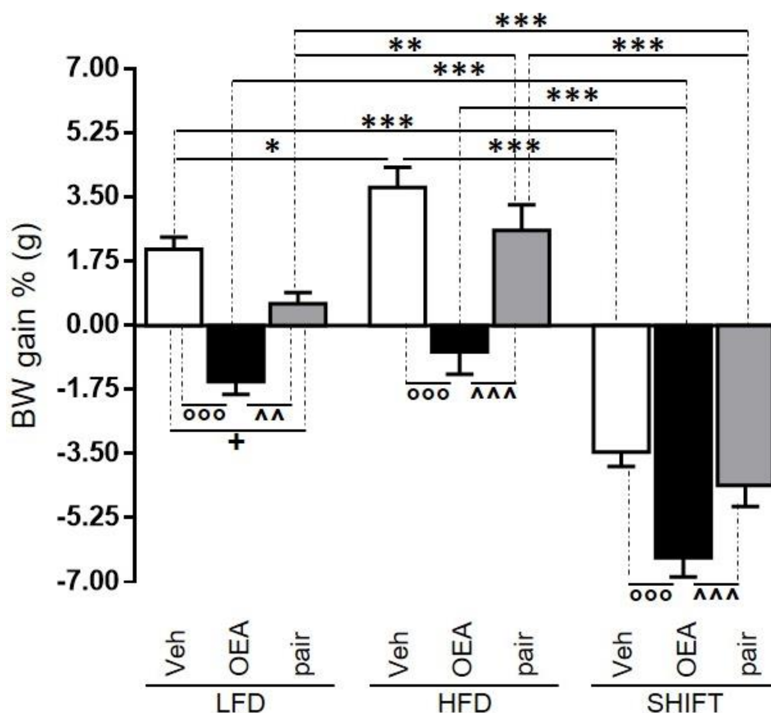


Fig. 4.4: OEA decreases the BW gain regardless of diet regimen. BW gain expressed as the difference between the first and the last day of treatment, in rats exposed to LFD, HFD o SHIFT diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for 2 weeks. Data are expressed as mean±SEM. $^{+}$P<0.05 LFD veh vs pLFD; °°°P<0.001 veh vs OEA in the same diet group; ^^P<0.01, ^^^P<0.001 OEA vs pair-feeding in the same diet group; *P<0.05, **P<0.01, ***P<0.001 vs other diet regimen in the same treatment group. N= 10-12 per group.

Regarding the day-to-day BW gain monitoring, the results of the Two-way ANOVA for repeated measures analysis, showed, in LFD animals, a significant effect of time (F_{time}= 2.479; df= 1/35; *P<0.05), of treatment ($F_{treatment}$= 23.393; df= 1/35; ***P<0.001), and of interaction between the two factors ($F_{interaction}$= 8.173; df= 1/35; ***P<0.001). Moreover, the Bonferroni post hoc test for multiple comparisons showed that, from the fifth day of treatment, chronic OEA administration is able to significantly decrease the BW of LFD animals (LFD V vs LFD O= °°P<0.01 on the fifth day; °°°P<0.001 from the sixth day to the end of the treatment (Fig. 4.5A).

In the same way, the results obtained with the Two-way ANOVA for repeated measures showed, in HFD animals, a significant effect of time (F_{time}= 12.719; df= 1/28; ***P<0.001), of treatment ($F_{treatment}$= 8.397; df= 1/28; **P<0.01) and of interaction between the two factors ($F_{interaction}$= 10.913; df=

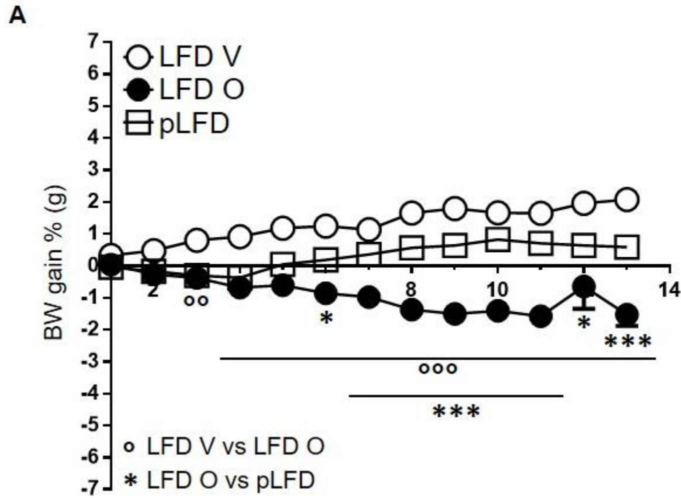
1/28; ***P<0.001). Furthermore, the Bonferroni post hoc analysis for multiple comparisons showed that OEA is able to significantly decrease the BW of HFD-fed animals from the sixth day of chronic administration to the end of the treatment (HFD V vs HFD O= °°P<0.01 on the sixth day; °°°P<0.001 for the remaining days) (Fig. 4.5B).

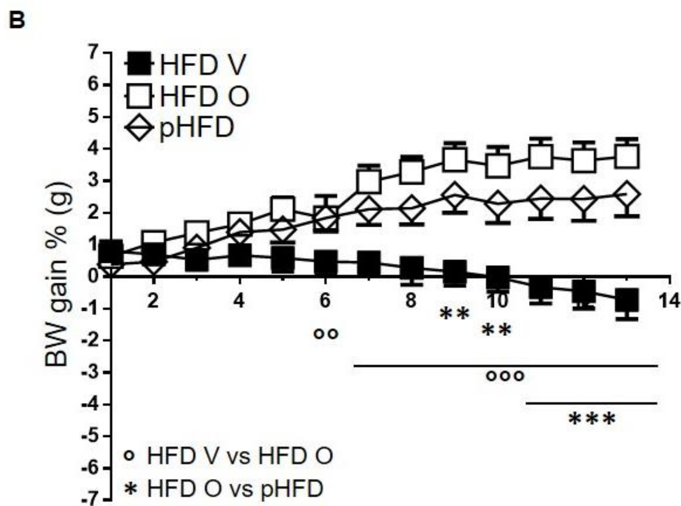
The chronic treatment with OEA (10 mg kg^{-1}, i.p.), in addition, is able to decrease the BW gain in SHIFT animals. In fact, the results of the Two-way ANOVA for repeated measures showed a significant effect of time (F_{time}= 125.040; df= 1/26; ***P<0.001) and of interaction between time and treatment ($F_{interaction}$= 6.550; df= 1/26; ***P<0.001), but not of treatment alone ($F_{treatment}$= 2.126; df= 1/26; P=0.141). Moreover, the Bonferroni post hoc test for multiple comparisons showed that OEA is able to decrease BW in SHIFT animals from the day 11 to the end of the treatment (SHIFT V vs SHIFT O= °°P<0.01 on day 11-14) (Fig. 4.5C).

Interestingly, for all diet groups, the decrease of BW gain in OEA-treated animals is higher than in pair-feeding animals. In particular, the Bonferroni post hoc test for multiple comparisons showed that OEA treatment (10 mg kg^{-1}, i.p.) induces, in the LFD group, a significant decrease of BW compared to pLFD animals from the seventh day of treatment (LFD O vs pLFD= *P<0.05 on day 7; ***P<0.001 from day 8 to the end of the treatment) (Fig. 4.5A).

Regarding HFD animals, the results obtained with the Bonferroni post hoc test for multiple comparisons showed that a significant, OEA-induced decrease of BW gain, compared to the pHFD group, is observed from the ninth day of treatment until the end of the experiment (HFD O vs pHFD= **P<0.01 on days 9 and 10; ***P<0.001 from the eleventh day) (Fig. 4.5B).

Finally, the Bonferroni post hoc test for multiple comparisons showed that, in SHIFT animals, OEA induces a significant decrease of BW gain on days 11-13 compared to veh-treated animals (SHIFT O vs SHIFT V= °°P<0.01 on day 11; °°°P<0.01 on day 12 and 13), and on days 12 and 13 compared to pSHIFT animals (SHIFT O vs pSHIFT= *P<0.05) (Fig. 4.5C).





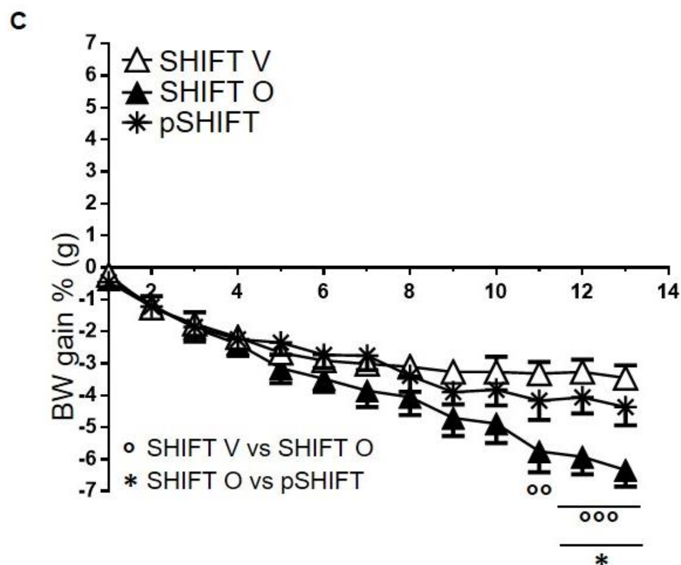


Fig. 4.5: Peripheral OEA administration decreases the body weight gain regardless of diet regimen. Time course of the body weight gain of animals exposed to LFD **(A)**, HFD **(B)** or SHIFT **(C)** diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data are expressed as mean± SEM. °°P<0.01; °°°P<0.001 vs veh-treated controls; *P< 0.05, **P<0.01, ***P<0.001 vs pair-feeding animals in the same diet group. N= 10-12 per group.

4.3.3 Microbiota analysis: total number of bacteria and β-diversity

Apart from the behavioral analyses, we aimed to investigate whether a chronic treatment with OEA (10 mg kg^{-1}, i.p.) could modulate the amount, the composition or the beta-diversity of the gut microbiota. Moreover, through the sequencing of the barcode genes, we investigated any possible OEA-induced changes in the *phyla*, orders or classes.

It is already known that the obese phenotype is characterized by lower amount and variability of the gut microbiota[23]. The data obtained show that OEA treated animals tend to have a higher number of bacteria in the LFD and HFD groups, whereas no differences were observed between the veh-treated and the pair feeding animals. Therefore, it could be suggested that this trend is induced by the treatment and not by the reduction of food intake (Fig. 4.8). In particular, the results of the Two-way ANOVA revealed a significant effect of treatment ($F_{treatment}$= 4.138; df= 1/44; *P<0.05), but no significant effect of diet or interaction between the two factors (F_{diet}= 0.824; df= 1/44; P= 0.44; $F_{interaction}$= 0.718; df= 1/44; P= 0.585). Moreover, the Tukey post hoc analysis for multiple comparisons showed that, in LFD animals, OEA significantly increases the number of total bacteria compared to pLFD rats (LFD O vs pLFD= °P < 0,05). Interestingly, the same trend is observed in HFD rats, even though it does not reach statistical significance (HFD O vs pHFD= P=0.054). Lastly, the total amount of bacteria in higher in pSHIFT compared to pHFD animals (*P<0,05) (Fig. 4.6).

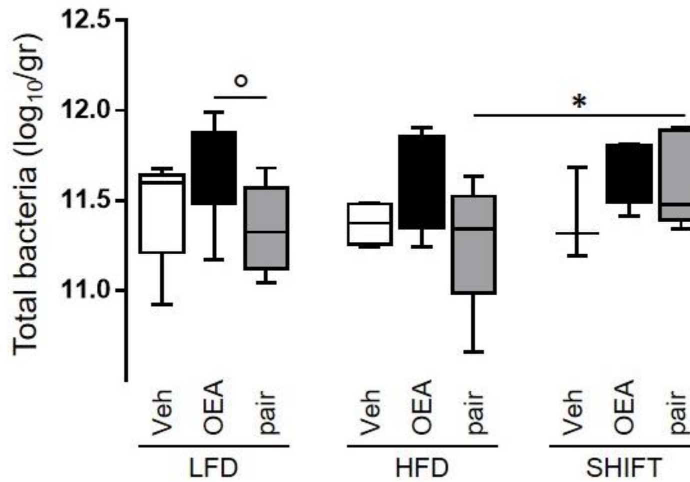


Fig. 4.6: Peripheral OEA administration increases the number of total bacteria compared to caloric restriction alone. Number of total bacteria of animals exposed to LFD, HFD or SHIFT diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data are expressed as mean±SEM. °P<0.05 OEA-treated vs pair-feeding rats; *P<0.05 vs other diet regimen in pair-feeding rats. N= 3-6 per group.

As for the number of total bacteria, HFD reduces the beta-diversity of the gut microbiota. Moreover, from the data obtained from the PCoA, using the unweighted UniFrac distance to measure the distance between different taxa, show that the chronic treatment with OEA (10 mg kg^{-1}, i.p.), in line with the results already showed, increases the beta-diversity of the gut microbiota, in all diet groups and, as for the previous results, OEA's action on beta-diversity is not linked only to its hypophagic effects. In particular, taking in consideration only the LFD V, HFD V, HFD O, and pHFD groups, a difference is observed between the OTUs of veh- and OEA-treated animals in the HFD group. In fact, our data show that the exposure to HFD leads to a decrease in OTUs, while the OEA treatment reverts this effect (Fig. 4.7).

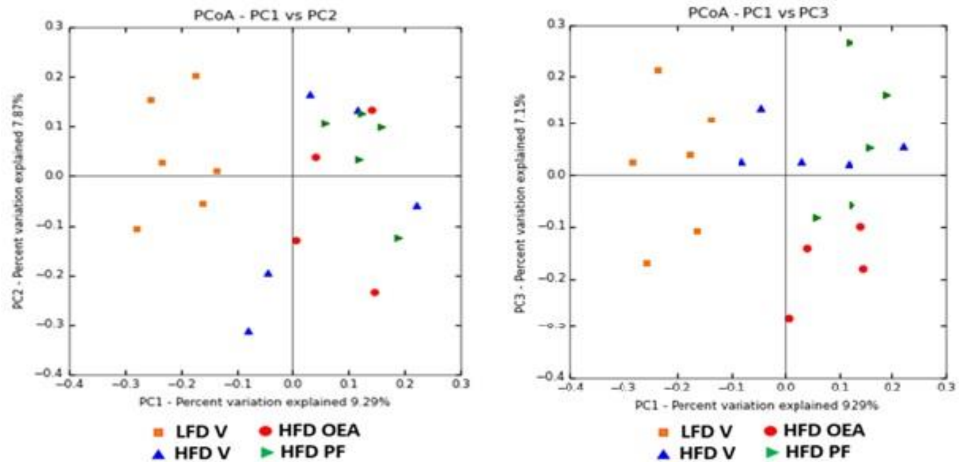


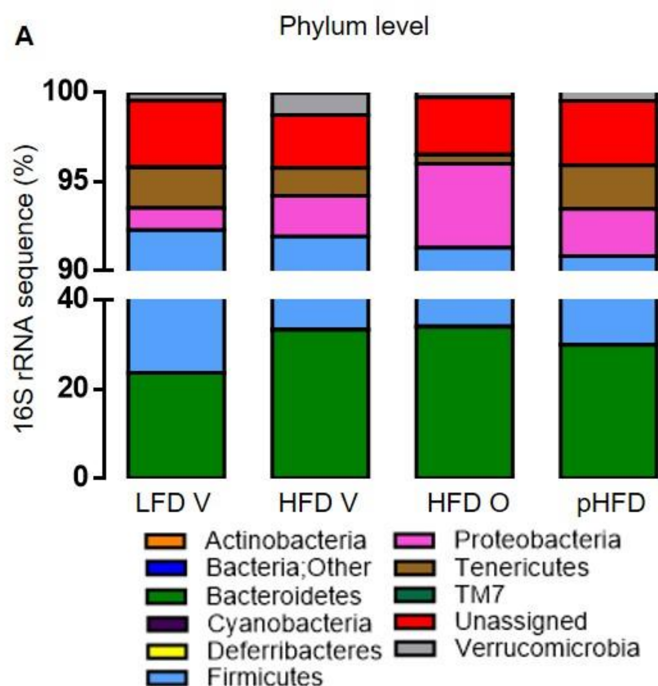
Fig. 4.7: Peripheral OEA administration increases the β-diversity of the gut microbiota. Plot of the β-diversity of the gut microbiota of animals exposed to LFD, HFD or SHIFT diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data were analyzed using the Principal Coordinates Analysis (PCoA), measuring the unweighted UniFrac distance. Each dot represents a sample, and the distance between dots represents the difference of the gut microbiota. In particular, in the left panel, all the animals exposed to LFD cluster on the left, whereas the HFD groups cluster on the right. Moreover, in the right panel, the majority of veh-treated animals cluster on the upper part of the graph, while the OEA-treated animals cluster on the bottom right, and, interestingly, do not overlap with pHFD animals. N= 4-6 per group.

4.3.4 Microbiota analysis: relative abundance and the main phyla, classes and orders

After the analysis on the number of total bacteria and the beta-diversity, we focused on the variations of the composition of the gut microbiota that could be induced by the chronic treatment with OEA (10 mg kg^{-1}, i.p.).

In particular, the sequencing of the rRNA 16S gene allowed us to evaluate the differences in phyla, classes and orders thanks to the QIIME software, that separates the microbial communities using the variability that characterizes this gene. We analyzed the relative abundance of the major phyla (*Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, *Tenericutes*, *TM7*, and *Verrucomicrobia*), and the major orders and classes belonging to them. However, no statistically significant results were obtained by these analyses, exception made for the phylum *Tenericutes*. In fact, the treatment with OEA induced a reduction of the abundance of the phylum *Tenericutes* in HFD animals (HFD V: mean= 1.552; SEM= 0.361; HFD O: mean= 0.506; SEM= 0.160: *P<0.05) (Fig. 4.9A). Moreover, among the classes in this phylum, and OEA-induced reduction of

the class *Mollicutes* has been observed in the HFD group (HFD V: mean= 1.550; SEM= 0.362; HFD O: mean= 0.483; SEM= 0.158; *P<0.05) (Fig. 4.9B). In the orders belonging to this class, the *t*-test analysis showed an OEA-induced reduction in the *RF9* order in the HFD group (HFD V: mean= 1.523; SEM= 0.363; HFD O: mean= 0.370; SEM= 0.164; *P<0.05) (Fig. 4.9C).



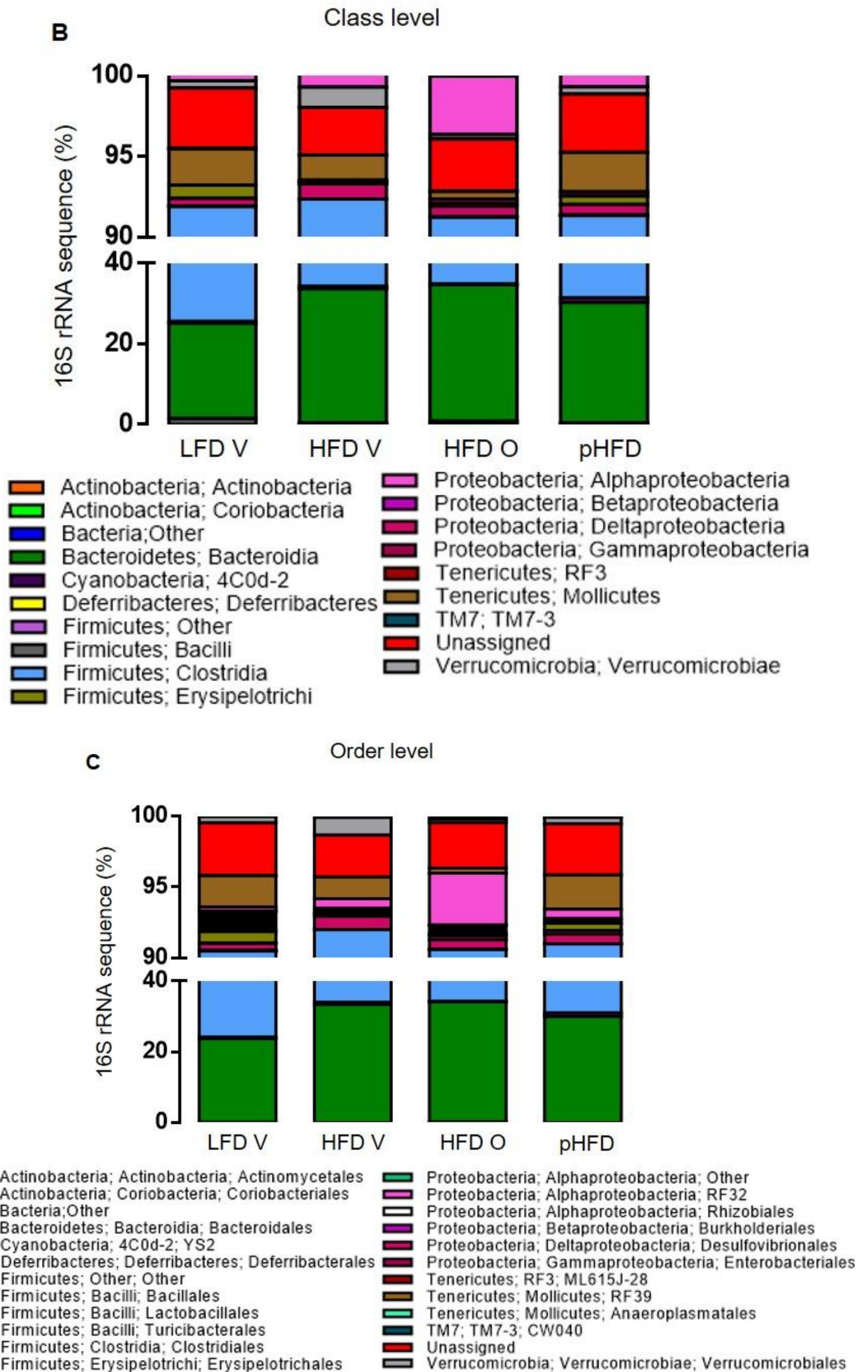


Fig. 4.9: Peripheral OEAinduces changes in the composition of the gut microbiota. Chronic peripheral OEA administration decreases the abundance of the phylum *Tenericutes* **(A)**, and, in particular of the class *Mollicutes* **(B)** and the order *RF39* **(C).** Data were obtained with the pyrosequencing of the gene encoding for the rRNA 16S. N= 4-6 per group.

4.3.5 Effects of OEA on gene expression in the brainstem and hypothalamus

We then investigated the effects of the chronic treatment with OEA (10 mg kg^{-1}, i.p.) on gene expression in the brainstem and in the hypothalamus, two key brain areas in the control of feeding[2]. In particular, we analyzed the expression of PPAR-α, NAPE-PLD and c-fos in the brainstem, and of MC4R in the hypothalamus.

For the brainstem, the Two-way ANOVA analysis revealed a significant effect of the diet (F_{diet}= 6.233; df= 1/26; **P<0.01), but no significant effect of the treatment or interaction between the two factors ($F_{treatment}$= 0.80; df= 1/26; P= 0.780; $F_{interaction}$= 2.889; df= 1/26; P= 0.078). Moreover, the Tukey's post hoc test for multiple comparisons showed a significant increase of PPAR-α expression in OEA-treated HFD animals, compared to both LFD and SHIFT animals (HFD O vs LFD O= °°P<0.01; HFD O vs SHIFT O= ^^P<0.01) (Fig. 4.10A).

Regarding the expression of the NAPE-PLD, the results of the Two-way ANOVA showed a significant effect of the diet (F_{diet}= 9.306; df= 1/24; **P<0.01), but no significant effect of the treatment nor of the interaction between the two factors ($F_{treatment}$= 3.055; df= 1/24; P= 0.780; $F_{interaction}$= 0.584; df= 1/24; P= 0.587). Moreover, the Tukey's post hoc for multiple comparisons revealed that the HFD *per se* significantly increases the expression of this enzyme (HFD V vs LFD V= °P<0.05), and the same difference is maintained in OEA-treated animals (HFD O vs LFD O= °P<0.05). In addition, the shift of the diet reduces NAPE-PLD mRNA expression in OEA-treated animals (HFD O vs SHIFT O= ^^P<0.01) (Fig. 4.10B).

We then proceeded analyzing c-fos mRNA levels. The results of the Two-way ANOVA showed a significant effect of the diet and of the treatment (F_{diet}= 13.980; df= 1/26; ***P<0.001; $F_{treatment}$= 0.874; df= 1/26; P= 0.360), but no significant effect of the interaction between the two factors ($F_{interaction}$= 6.680; df= 1/26; **P<0.01). Furthermore, the Tukey's post hoc test for multiple comparisons revealed that the exposure to HFD significantly increases c-fos mRNA expression in OEA-treated rats compared to both LFD and SHIFT animals (HFD O vs LFD O= °°°P<0.001; HFD O vs SHIFT O= ^^^P<0.001).

Moreover, in HFD animals, OEA increases c-fos mRNA levels compared to veh-treated animals (**P<0.01 vs veh in the same diet group) (Fig. 4.10C).

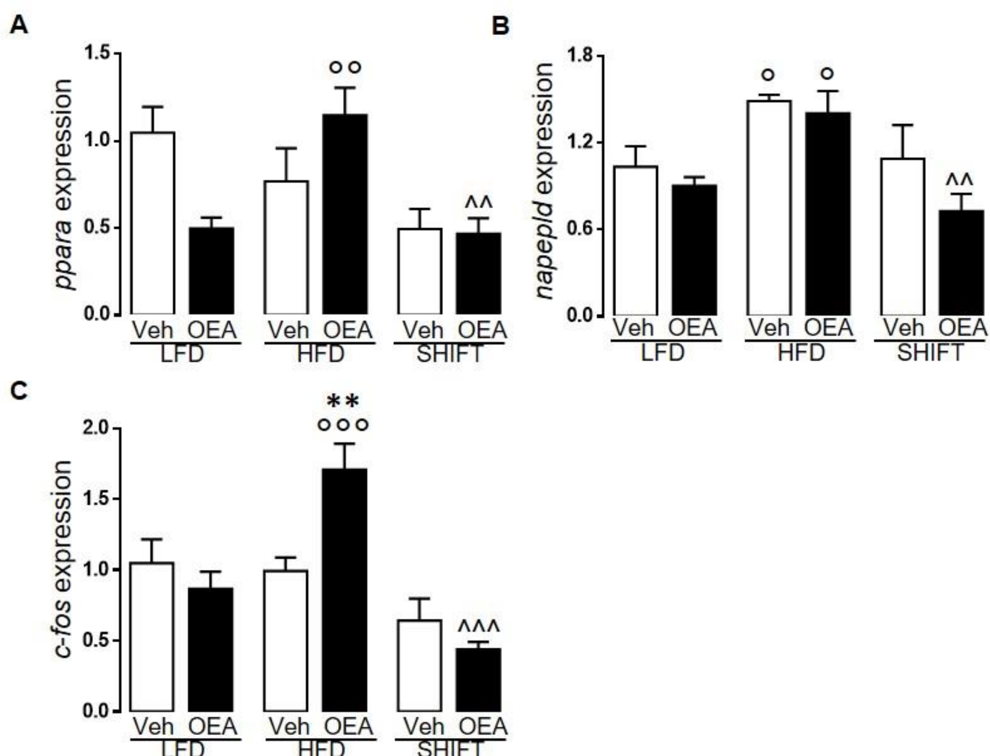


Fig. 4.10: Modulation of gene expression in the brainstem after chronic OEA administration. Gene expression of PPAR-α **(A)**, NAPE-PLD **(B)** and c-fos **(C)** in the brainstem of animals exposed to LFD, HFD or SHIFT diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data are expressed as mean±SEM. °P<0.05, °°P<0.01; °°°P<0.001 vs LFD in the same treatment group; ^^P<0.01; ^^^P<0.001 vs SHIFT in the same treatment group; **P<0.01 vs veh-treated controls in the same diet group. N= 4-5 per group.

In the hypothalamus, we investigated the levels of expression of MC4R. The results of the Two-way ANOVA showed a significant effect of the diet and of the interaction between the diet and the treatment (F_{diet}= 4.317; df= 1/27; *P<0.05; $F_{interaction}$= 5.362; df= 1/27; *P<0.05), but no significant effect of the treatment alone ($F_{treatment}$= 1.691; df= 1/27; P=0.207). Moreover, the Tukey's post hoc test for multiple comparison revealed that the HFD leads to a significant increase of this receptor in the OEA-treated group compared to LFD animals (HFD O vs LFD O= °°P<0.01). Furthermore, In the HFD animals,

OEA induces the expression of MC4R compared to veh-treated rats (**P<0.01) (Fig. 4.11).

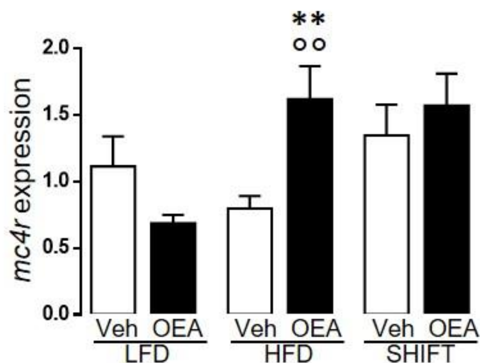


Fig. 4.10: Modulation of gene expression in the hypothalamus after chronic OEA administration. Gene expression of MC4R in the hypothalamus of animals exposed to LFD, HFD or SHIFT diet and treated with either vehicle or OEA (10 mg kg^{-1}, i.p.) for fourteen days. Data are expressed as mean±SEM. °°P < 0.01 vs LFD/SHIFT in the same treatment group; **P < 0.01 vs veh-treated controls in the same diet group. N= 4-5 per group.

In conclusion, these analyses revealed that both the exposure to HFD and OEA treatment (10 mg kg^{-1}, i.p.) play a role in the expression of the genes analyzed. These data, together with the ones obtained from the analyses of the behavior and of the gut microbiota, support the thesis that OEA does not only affect FAO, lipid metabolism[24,25] and neurotransmission[8,9,11,26,27], but also gene expression.

4.4 Discussion

The fight against obesity, considered by many as the epidemic of the 21st century, and the lack of a pharmacological treatment proven safe and effective[28], is one of the main challenges for clinical research. In this scenario, a great deal of interest has been gained by OEA, due to its anorexiant effect[4,8–10,26,29,30]. It is known that the excessive consumption of high fat foods is responsible of the development of the obese phenotype in both laboratory animals and humans, and that a prolonged exposure to HFD (7-14 days) decreases the endogenous levels of anorexiant NAEs in the small intestine[31,32]. This effect may, at least in part, promote hyperphagia, responsible of the development of obesity. Furthermore, it has been demonstrated that the exposure to HFD decreases the synthesis and the mobilization of OEA[13].

Based on these premises, the aim of the present work was to analyze the effects of the chronic administration of OEA in a rat model of DIO, in which the obese phenotype was induced by exposing animals to HFD *ad libitum* for 11 weeks. Moreover, the effects of OEA were analyzed in animals that underwent a shift of the diet (SHIFT animals) from HFD to LFD *ad libitum* from the first day of treatment, in order to mimic dieting. The data obtained from the behavioral analyses confirmed the already known hypophagic effect of OEA[4,8–10,26,29,30], in all diet groups. Interestingly, in HFD animals, the anorexiant effect of OEA appears to be delayed compared to LFD and SHIFT groups: the delay of the onset of the hypophagic effect of OEA may be due to the disruption of the OEA signaling pathway that occurs upon the exposure to HFD, where the synthesis and mobilization of OEA is dampened[13]. Moreover, the data obtained in the present work showed that the hypophagic effect of OEA is observable also in SHIFT animals, although, as expected[14,33], the change of the diet induces *per se* a reduction in the caloric intake during the first days of treatment. Interestingly, the hypophagic effect of OEA, in SHIFT animals, is observed earlier compared to HFD animals. This observation led us to hypothesize that the shift of the diet may, in part, restore the sensitivity to the biological action of OEA, and the imbalance between orexiant and anorexiant mediators that is disrupted upon the exposure to HFD[34–36].

Therefore, novel studies are required to investigate the possible involvement of OEA in the secretion and action of other mediators that regulate feeding. Recently, it has been reported that NAEs, and OEA in particular modulate the secretion of GLP-1, GLP-2 and PYY[30] by binding the GPR119 expressed in the enteroendocrine L cells[37,38].

We further analyzed the BW gain of the animals in all diet groups, both as difference between the first and the last day and as time course. Our data demonstrate that OEA is able to decrease BW gain in all diet groups compared to veh-treated animals, as expected. In this regard, pair feeding groups were introduced in order to investigate whether the effects of OEA on BW are only due to the reduction of the food consumed or to effects on the metabolism. We found that the BW of OEA-treated animals was significantly lower than that of pair-feeding animals in all diet groups. Regarding the day-to-day monitoring of the BW, we found that the OEA-induced weight loss, as for the EI, has a different onset depending on the diet group. In fact, in HFD animals, the reduction of the BW gain is delayed compared to the other diet groups. This observation further confirms the hypothesis that OEA signaling is disrupted by the prolonged exposure to HFD[16], that leads to a reduced responsivity to its anorexigenic effect[39]. Regarding the SHIFT animals, the OEA-induced reduction of BW, seems to be delayed compared to the onset of the effect on the EI. It could be hypothesized that OEA's effect is dampened by the effect that the shift of the diet already has on weight loss.

These observations highlight how the anti-obesity effects of OEA are not only due to the reduction of the food consumed, but also to effects on the metabolism. In fact, many lines of evidence report that OEA modulates lipid metabolism[24,25], mainly through the activation of PPAR-α[4]. OEA, like other PPAR-α agonists, is able to increase fatty acid oxidation (FAO)[24], and to increase circulating levels of β-hydroxy-butyrate (BHB), a marker of FAO and ketogenesis[30]. Moreover, it has been demonstrated that chronic treatment with OEA reduces the levels of circulating lipids and hepatic fat deposition[40].

It is well known that the exposure to HFD decreases the diversity of the gut microbiota and the number of total bacteria, increasing the risk of the development of obesity[41,42]. Hence, modulation of the gut microbiota could be

a novel approach for the treatment of obesity[43]. Therefore, in the present work, we aimed to evaluate the possible effects of chronic treatment with OEA on the gut microbiota, in particular on the number of total bacteria and the β-diversity. The data obtained in this study show that OEA increases the abundance of total bacteria in HFD animals compared to LFD animals. Notably, this effect is not only due to the hypophagic effect of OEA, since the microbiota abundance in pair-feeding animals is comparable to veh-treated rats. As for the number of total bacteria, OEA influences the β-diversity of the gut microbiota. In fact, the data obtained from the PCoA analysis show that the chronic treatment with OEA modulates the β-diversity in all diet groups. In particular, OEA treatment reverts the decrease of OTUs observed by the exposure to HFD. Interestingly, this effect is not only due to the reduction of food consumption, since the trends of OEA-treated and pair-feeding animals are not comparable. These effects are confirmed by very recent finding showing the beneficial effects of the subchronic treatment with OEA on gut microbiota[44].

Overall, the data obtained with the analysis of the number of total bacteria and the β-diversity highlight the impact that the chronic exposure to HFD has on the gut microbiota, and how OEA is able to revert this trend, with effects that go beyond the sole reduction of food intake.

Then, through pyrosequencing, we observed changes in the phyla, classes and orders of the microbial population. Interestingly, OEA is able to induce alterations in particular in the *Mollicutes* class, belonging to the phylum *Tenericutes*. Interestingly, it has been demonstrated that the class *Mollicutes* is largely influenced by HFD[45], even though its role in the development of obesity needs to be clarified.

These observations, in line with previous studies[45,46], highlight the strong impact that diet has on the gut microbiota, that are at least partially reverted by OEA treatment. For the majority of the populations analyzed, the OEA-induced changes were not observable in the pair-feeding group, suggesting once again the effect that OEA has on the metabolism over the effect on feeding. Moreover, these results reflect only the changes induced on gut microbiota colonizing in the large intestine. Therefore, OEA-induced

changes on the microbiota colonizing the other parts of the intestine[17,47] are yet to be studied.

Lastly, we aimed to investigate whether the peripheral administration of OEA induces changes in gene expression in brain areas involved in the regulation of feeding, the brainstem and the hypothalamus. In fact, through the activation of PPAR-α, OEA induces the transcription of target genes in the liver and small intestine[4]. In the brainstem, we found that among OEA-treated animals of all diet groups, the exposure to HFD induces the expression of PPAR-α. In particular, in OEA-treated animals, the difference between HFD and SHIFT animals is possibly due to the interaction between the change of the diet and the treatment, since the same trend is not observed in veh-treated animals. In the same way, HFD is able to increase the expression of NAPE-PLD compared to other diet groups, in both veh- and OEA-treated animals. As for the PPAR-α, the shift of the diet and the treatment act together in order to induce a statistical reduction of the NAPE-PLD, as shown by the comparison between HFD O and SHIFT O animals. We further analyzed the expression of c-fos in the brainstem. In line with the data obtained with the previous analyses, the exposure to the HFD increases the expression of c-fos mRNA levels in OEA-treated animals, compared to the other diet groups, particularly with the SHIFT group. Moreover, in HFD animals, the treatment with OEA increases c-fos mRNA expression compared to veh-treated animals. This is in line with previous observations, that demonstrate that OEA activates not only the NST and the AP, located in the brainstem, but also the PVN and SON in the hypothalamus[8,9,29]. However, the OEA-induced increase of c-fos expression is not observed in LFD animals. This effect may be in accordance with our behavioral results: on one hand, in LFD animals, the OEA signaling pathway is not disrupted and hence may be exposed to tolerance during the chronic treatment with OEA; on the other hand, in HFD animals, the sensitivity to OEA is being restored, therefore, in these animals, the OEA-induced increase of c-fos expression is still observable.

Lastly, in the hypothalamus, we analyzed the expression of MC4R. In line with the brainstem, the MC4R mRNA levels are increased by HFD in OEA-treated animals, compared to the other diet groups, in particular with the

SHIFT group that may be the result of physiological riadaptations that occur by exposing animals to different diets. In fact, it has been reported that mRNA levels of this receptor significantly change among DIO, DR and LFD rats[22].

Overall, our findings show for the first time how the interaction between the change of the diet and the treatment with OEA modulate gene expression in the brainstem and the hypothalamus. However, these data highlight the complex alterations that occur upon the exposure to HFD, leading to many questions on the underlying mechanisms. Hence, the possible interplay between the diet, the gut microbiota, the gene expression in the CNS, and how OEA can modulate these factors requires further studies.

The data of the present work broaden our knowledge about the biological actions of OEA, that, as has been demonstrated, are not due to the reduction of food intake alone, but to its action on a variety of levels, from gut microbiota to gene expression.

4.5 References

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Chapter 5: General conclusions

Obesity is the world's most widespread chronic pathological conditions, condition that leads to the impairment of the quality of life, and acts as a risk factor for the development of other diseases, such as cardiovascular diseases, diabetes, and hypertension. Therefore, in the past years, research has focused on investigating the mechanisms involved in the control of feeding and energy balance, that has led to the discovery of a variety of signaling pathways, that are organized in a complex network of heterogeneous molecules.

It is well known that the metabolites produced by the gut microbiota directly influence the activity of the liver, of the central nervous system (CNS), of the adipose tissue, of the intestine, and of the skeletal muscle, thus playing a crucial role in the regulation of energy homeostasis and in other physiological processes. In fact, there is evidence supporting a strong link between a dysregulation of the gut microbiota and the onset of pathological conditions, such as obesity, type 2 diabetes, and cardiovascular diseases.

The increasing incidence of obesity and other eating-related disorders highlighted the necessity of a discovery of a novel target for the pharmacological treatment of these diseases and their related comorbidities. In this scenario, oleoylethanolamide (OEA) has rised a great deal of interest due to its effects on feeding: in fact, it is a known pro-satiety factor, leading to a reduction of body weight and food intake.

In the present work, the mechanism of action, the effects on the CNS, and peripheral peripheral organs induced by OEA i.p. administration were investigated. In particular, in the 2nd chapter, it has been demonstrated that, in contrast with previous findings, vagal afferents are not necessary to convey the OEA-induced signal to the CNS. Then, in the 3rd chapter, the effects of the peripheral administration of OEA on gene expression in selected brain areas and peripheral organs were investigated, and, in line with previous findings,

we found that OEA is able to modulate gene expression in almost all the areas analyzed.

All the results mentioned so far were obtained after an acute administration of OEA, from animals fed with standard chow diet. Therefore, we aimed to investigate the anti-obesity effects of chronic administration with OEA on a rat model of diet-induced obesity (DIO). We investigated, in particular, the changes of gene expression and of the gut microbiota, and we found that, overall, the chronic administration with OEA induces beneficial effects in the DIO rats that go beyond the sole reduction of food intake.

A schematic representation of the results obtained in the present work are represented in Fig. 5.1.

Overall, the results of this work show that the anti-obesity effects of peripheral OEA administration have an impact on many different levels, from behavior to gut microbiota.

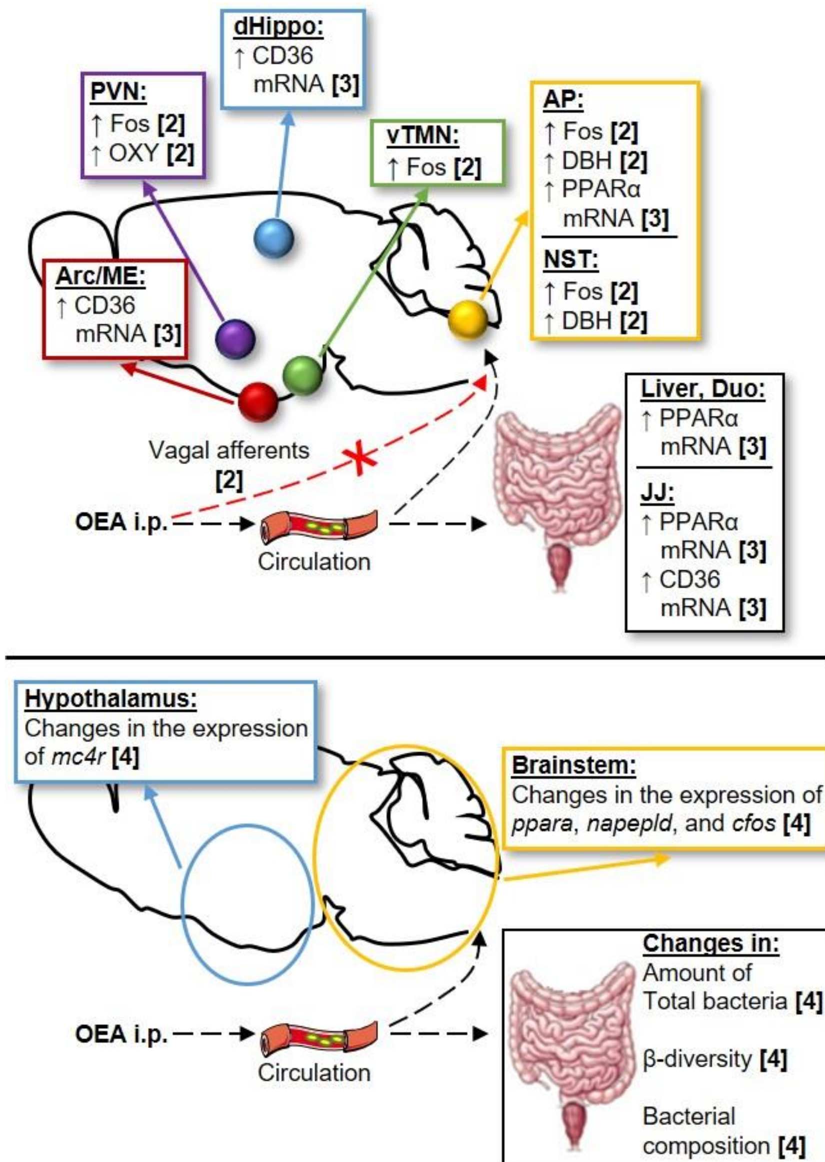

Fig. 5.1: Schematic representation of the results obtained in the present work.
Upper panel: After peripheral administration, OEA enters the bloodstream, reaches the CNS, and activates several brain areas involved in the control of feeding behavior, such as the NST, AP, vTMN and PVN **[chapter 2]**, where it also modulates gene expression **[chapter 3]**, in animals exposed to standard chow diet.
Lower panel: the chronic administration of OEA is able to induce changes in the expression of genes involved in the control of feeding behavior in the brainstem and hypothalamus, and in the composition, β-diversity, and amount of the bacteria colonizing the gut in animals exposed to HFD **[chapter 4]**.

5.1 OEA in clinical practice

Control of food intake and appetite sensation are considered as effective approaches in the control of weight gain[1], and several different nutraceutical and pharmaceutical compounds have been identified with appetite modulator properties, such as n ‐ 3 polyunsaturated fatty acids[2] and zinc[3]. One of the pharmaceutical agents that recently attracted more attention is OEA.

Clinical studies have reported the effects of OEA in the control of weight and obesity: in fact, the daily supplementation with 250mg OEA for 60 days significantly increased the expression of PPARα in 56 obese people (BMI= 30-40 kg/m^2), who showed also decreased appetite and BMI at the end of the study[4]. Moreover, in another study, it has been shown that the supplementation with 120mg epigallocatechin-3-gallate (EGCG) and 170mg N-oleyl-phosphatidylethanolamine (NOPE, a precursor for OEA) for 2 months resulted in a significant reduction in weight and hip circumference in 38 obese people (BMI= 32-41 kg/m^2)[5]. Furthermore, the supplementation with these same substances, but at different dosages (50mg EGCG and 85 mg NOPE) for 2 months induced an increase of satiety and a decrease of body weight in 138 healthy overweight people[6]. However, another study showed no significant effects of the daily supplementation with 120 mg NOPE and 105mg of EGCG on BMI, body composition, feelings of hunger, or binge eating in 50 healthy overweight subjects at the end of an 8 weeks-long study[7]. Inconsistent results of clinical studies can be attributed to the different doses of OEA, differences in the features of the target groups, sample size, duration of supplementation, and type of the supplements (alone or in combination with other compounds).

Recently, the FDA approved a promising OEA supplement (Riduzone), that showed encouraging effects in obese people. In fact, supplementation with Riduzone (200 mg OEA/capsule) 2–3 times/day for 4–12 weeks decreased body mass index about 7–8% in 50 obese people[8].

In conclusion, more future robust human randomized clinical trials are surely required to facilitate the development of apposite nutritional and pharmacological strategies for the control of appetite in obesity. In this

scenario, OEA is one of the novel pharmaceutical bioactive compounds that has attracted more attention thanks to its effects on feeding and weight loss. In fact, by binding PPARα, GPR119, and TRPV1 receptors, it suppresses appetite, and, considering no obvious adverse effects, sufficient efficacy, and safety, OEA supplementation can be recommended to manage obesity and related abnormalities.

5.2 References

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Abbreviations

2-AG, 2-arachidonoylglycerol
2-OG, 2-oleoyl-glycerol
5-HT, serotonin
ACTH, adrenocorticotropic hormone
AEA, N-arachidonoylethanolamide (anandamide)
AgRP, Agouti-related peptide
ANGPTL4, angiopoietin-like protein 4
ANS, autonomic nervous system
AP, area postrema
Arc, arcuate nucleus
ASC, caspase activation and recruitment domain
AVP, vasopressin
BAT, brown adipose tissue
BBB, blood-brain barrier
BMI, body mass index
CART, cocaine- and amphetamine-regulated transcript
CCK, cholecystokinin
CD36, cluster of differentiation 36
CGRP, Calcitonin Gene Related Peptide
CNS, central nervous system
CRH, corticotropin-releasing hormone
DBH, dopamine-β-hydroxylase
DIO, diet-induced obesity
DMN, dorsomedial nucleus
DMV, dorsal motor nucleus of the vagus
Duo, duodenum
DVC, dorsal vagal complex
EASO, European Association for the Study of Obesity
ECS, endocannabinoid system
ECs, endocannabinoids
ENS, enteric nervous system
FAAH, fatty acid amide hydrolase
FABP, fatty acid binding protein
FAO, fatty acid oxidation
FIAF, fasting-induced adipose factor
GH, growth hormone
GHS-R, growth hormone secretagogue receptor
GI, gastrointestinal
GIP, gastric inhibitory peptide
GLP-1, glucagone-like peptide-1
HDAC, histone deacetylase
HDC, histidine-decarboxylase
Hippo, hippocampus
HPA axis, hypothalamus-pituitary-adrenal axis
IGLE, intraganglionic laminar ending
IL- β, interleukin-β
JJ, jejunum

LEA, linoeoylethanolamide
LH, lateral hypothalamus
LHA, lateral hypothalamic area
LPB, lateral parabrachial nucleus
LPL, lipoproteic lipase
MC3R, MC4R, melanocortin receptors 3 and 4
NAAA, N-acylethanilamine-hydrolyzing acid amidase
NAcc, nucleus accumbens
NAE-PLD, NAPE-specific phospholipase D
NAEs, N-acylethanolamines
NANC, non-adrenergic non-cholinergic
NAPEs, N-acylphosphatidylethanolamides
NAT, N-acyltransferase
NEFA, non-esterified fatty acids
NLRPs, NOD receptors containing the pyrin domain
NOPE, N-oleoylphosphatidylethanolamine
NPY, neuropeptide Y
NST, nucleus of the solitary tract
OEA, oleoylethanolamide
OP, obese prone
OR, obese resistant
OXM, oxyntomodulin
OXY, oxytocin
PAMP, pathogen-associated molecular patterns
PEA, palmitoylethanolamide
PFC, prefrontal cortex
PNS, peripheral nervous system
POMC, pro-opiomelanocortin
PP, pancreatic polypetide
PPRE, PPAR response elements
PVN, paraventricular nucleus
PYY, peptide YY
RYGB, Roux-en-Y gastric bypass
SCFAs, short-chain fatty acids
SDA, subdiaphragmatic vagal deafferentation
SEA, stearoylethanolamide
SON, supraoptic nucleus
SRD, sucrose rich diet
TLR4, toll-like receptor 4
TRPV1, transient receptor potential vanilloid type-1
TVX, total subdiaphragmatic vagotomy
TVX, total subdiaphragmatic vagotomy
Ucp-1, uncoupling protein 1
UCPs, uncoupling proteins
VLDL, very low-density lipoproteins
VMH, ventromedial hypothalamus
vTMN, ventral tuberomammillary nucleus
WHO, World Health Organization
α-MSH, alpha melanocyte-stimulating hormone

Δ^9-THC, tetrahydrocannabinol
FAO, fatty acid oxidation
ME, median eminence,
FAT, FA translocase
IL-6, interleukin 6
HFD, high fat diet
LFD, low-fat diet
BW, body weight
EI, energy intake
qPCR, quantitative polymerase chain reaction
CFU, colony forming units
PCoA, principal coordinate analysis
OTUs, operational taxonomic units
RT-qPCR, retrotranscription qPCR
BHB, β-hydroxy-butyrate
EGCG, epigallocatechin-3-gallate