SAPIENZA
UNIVERSITÀ DI ROMA

Prospects for new drugs to treat aberrant eating patterns: possible role of Oleoylethanolamide

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

Marzia Friuli

Department of Physiology and Pharmacology "V.Erspamer"
Sapienza University of Rome

Coordinator of the PhD program:
Prof. Silvana Gaetani

Thesis advisor:
Prof. Silvana Gaetani

ACADEMIC YEAR 2020-2021

INDEX

Chapter 1:

General Introduction

Chapter 2:

Oleoylethanolamide's effects in a rat model of diet-induced obesity: from neuroprotective properties to the modulation of eating behavior, hepatic lipid metabolism and oxidative stress

Chapter 3:

Deranged central oxytocinergic signaling in pre-clinical models of obesity and hedonic feeding: effects of increased Oleoylethanolamide tone

Chapter 4:
General discussion and conclusion

Chapter 1:

General Introduction

1. Obesity and eating related disorders

Obesity is a polygenic and multifactorial condition that represents a very concerning public health issue affecting both developing and developed countries [1,2]. Obesity is characterized by an excess of adipose tissue and occurs when energy homeostasis, reached by balancing the energy intake and the energy expenditure, is impaired [3,4]; it is the result of the interaction between different factors, including genetic, metabolic, behavioral, and environmental ones.

In modern society, the increased abundance and availability of industrialized high-palatable caloric dense food, is an important environmental risk factor for obesity development [5,6]. These foods contain high amounts of sugars, trans and saturated fats, dietary salt, food additives, while present low contents of fiber, carbohydrates accessible to the gut microbiota, polyunsaturated fats, vitamins, minerals, and bioactive compounds [7,8]. Simultaneously, sedentary lifestyle is highly prevalent due to urbanization and technological advancement [9,10].

Obesity represents a severe condition because the excess of adipose tissue, specifically visceral fat depots and deregulates systemic lipid- and glucose-homeostasis contributing to the development of type 2 diabetes (T2DM), non-alcoholic fatty liver disease (NAFLD), cardiovascular and neoplastic diseases, among others [11].

According to the World Health Organization (WHO) the worldwide prevalence of obesity nearly tripled between 1975 and 2016. In 2019, an

estimated 38.2 million children under the age of 5 years were overweight or obese. Overweight and obesity are linked to more deaths worldwide than underweight. Globally there are more people who are obese than underweight; this occurs in every region except parts of sub-Saharan Africa and Asia [12].

If the recent trend in the incidence of obesity will continue, it is estimated that, by 2030, 60% of the world population (3.3 billion people) could be overweight (2.2 billion) or obese (1.1 billion) [13].

The most used parameter to identify excessive weight with respect to height and age 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 WHO categorizes obesity in terms of BMI: underweight (BMI less than 18.5 kg/m^2), normal weight (range from 18.5 to 25kg/m^2), overweight (range from 26 to 30 kg/m^2), and obese (greater than 30 kg/m^2) [12,14] (Fig.1.1).

Fig.1.1: BMI categories according to the World Health Organization

However, the use of BMI as an index of overweight or obesity is not reliable for all individuals; BMI has been mainly used in adults, although it is now

being used in children and elderly individuals. In the case of children and adolescents, BMI z-scores are used because in this population, BMI varies with age and sex [15]; BMI z-score is defined as an index of relative weight adjusted for child age and sex in relation to a reference population [16]. Even though it is the most used index to describe body mass and obesity, BMI does not consider other factors that influence weight, such as percentage of lean/fat mass. Therefore, this index is usually accompanied by the measurement of abdominal circumference since the accumulation of fat in the visceral area is correlated to cardiovascular and metabolic disorders [17].

As above mentioned, obesity can be considered, among other aspects, the consequence of an energy imbalance because of a lack of balance between food intake and energy consumption [18]. Energy expenditure includes the energy required to maintain vital functions (resting metabolic rate), perform physical activity, and provide diet-induced thermogenesis [16]. Therefore, when energy intake exceeds the energy expenditure, the excessive energy can be stored as fat, laying the basis for the development of obesity [19,20]. Energy balance is controlled by multiple physiological mechanisms, which involve a plethora of signals that from the periphery communicate with the brain, and vice versa [21]. Many organs participate to this intricate interplay, including the adipose tissue (that acts as storage), the liver (the center for lipid and glucose metabolism) [22] 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 [23]. However, several studies have expanded the conventional view of the homeostatic regulation of body weight to include also the non-homeostatic control of appetite. The hedonic system has close connections to the hypothalamus (HYPO) and to the areas controlling the homeostatic

functions [24]. A reward deficiency is considered to cause an imbalance between homeostatic and hedonic regulation. A large body of literature documents that beyond the food overconsumption leading to overweight and obesity, also eating disorders, such as anorexia nervosa (AN), bulimia nervosa (BN) and binge eating disorder (BED) have been pathophysiologically linked to dysfunctions of reward mechanisms. Moreover, changes in body weight are frequently accompanied by psychological and psychosocial disturbances thus leading to a persistent alteration of eating behavior. BED, according to the fifth edition of Diagnostic and Statistical Manual of mental disorders (DSM-5), is the most frequent eating disorder occurring in 2–5% of the adult population, with a higher prevalence among women than men [25–27]; it is characterized by uncontrollable and compulsive episodes of excessive consumption of highly palatable food (HPF) accompanied by a strong sense of loss of control, feeling of shame, guilt, disgust, and anxiety. The combination of dieting and stress is a common trigger for BED [28,29], which shares a variety of commonalities with drug addiction [30]. Indeed, excessive intake of palatable energy-rich foods is associated with addiction-like induced deficits in brain reward system that, in turn, might drive overconsumption of these foods to compensate for reward hyposensitivity. Such a self-sustaining mechanism might gradually lead to a wearing off of the acute rewarding properties of food, and a rise of a "pseudo-withdrawal phase" characterized by a negative emotional state (anxiety, depression, irritability and possibly somatic symptoms), when such preferred food is not readily available or when the environmental conditions are stressful [24,31].

2. Neural control of eating behaviors and metabolism

The mechanism behind food consumption is highly complex. The understanding of the biological substrates regulating feeding behavior and metabolism is relevant to address the health problems related to food overconsumption. As mentioned above, several studies have expanded the conventional view of the homeostatic regulation of body weight mainly orchestrated by the HYPO, to include also the non-homeostatic control of appetite. Such processes include food reward and are mainly coordinated by the activation of the central mesolimbic dopaminergic pathway [32]. Indeed, the decision to eat, the choice of a particular food and its quantity can be strongly influenced by the mental state, the environment, by its taste, color, and consistency, to the point that exposure to highly palatable food stimuli can hijack homeostatic/satiety signals and lead to overeating [33,34]. Therefore, the identification of endogenous systems acting as a bridge between homoeostatic and non-homeostatic pathways might represent a significant step toward the development of drugs for the treatment of obesity and aberrant eating patterns [32].

2.1.Homeostatic versus non-homeostatic endogenous system

The CNS regulates energy intake and expenditure not only to provide a healthy nutritional status and meet the body energy needs, but also to process the pleasure deriving from the ingestion of palatable and preferred kinds of food [32].

Homeostatic mechanisms control eating in a state of insufficient energy or in presence of a specific metabolic necessity, whereas non-homeostatic mechanisms include processes such as memory, learning and cognition that might influence feeding based on its hedonic properties or on the bases of

previous experiences [35]. Therefore, we refer to a homeostatic system, when the body weight of an individual is generally maintained constant; slight fluctuations are sometimes present but merely within a limited range around the body weight 'set point'. Some exceptions occur in people, who are in a non-steady state body weight: those people increase their body weights and develop obesity. A large body of evidence has been collected on the concept that the "homeostatic network" interacts with the hedonic system responding to food rewards, thus highlighting the importance of reward processes in motivated behaviors, such as eating. Cues linked to the consumption of a tasty food, promote, indeed, food-seeking, food-attractiveness and eating [36].

The dopaminergic neurotransmission is crucially implicated in the rewarding and motivational aspects of feeding. Dopaminergic neurons from the ventral tegmental area (VTA) project to the nucleus accumbens (NAc), which in turn transmits the signals to other brain regions of the limbic system, including the prefrontal cortex (PFC), the amygdala (AMY), and the hippocampus (HIPPO); this above-mentioned circuit is called "reward-associated mesolimbic dopamine pathway" [37]. For example, decreased dopaminergic signaling, which typically transmits the rewarding aspects of food-related stimuli, promotes overconsumption of tasty foods beyond homeostatic needs to compensate for lower sensitivity to the reward [37].

In addition, evidence emerged that cognitive processes, such as learning and memory, may indirectly influence eating behavior. The memory of a taste, the expectation, and the sight of a certain food have been demonstrated to determine if an individual will start eating and what kind of food will consume [38].

Interestingly, the two homeostatic and non-homeostatic systems, are not independent from each other, on the contrary they reciprocally modulate [39,40] in a plastic fashion that takes also environmental conditions into account.

2.2.Principal peptides and hormones involved in the control of energy metabolism

The HYPO is the most important brain region involved in the central homeostatic regulation of feeding and energy expenditure. It consists of different nuclei, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral HYPO (LH), the dorsomedial HYPO (DMH) and the ventromedial HYPO (VMH). These aforementioned nuclei cooperate to sense neuronal, nutrient and endocrine signals to either suppress or promote eating [41]. Neurons in the HYPO synthesize and release a variety of both anorexigenic and orexigenic neuropeptides including oxytocin (OXY), the agouti-related protein (AgRP), the neuropeptide Y (NPY) [42–44]and the neuropeptide precursor pro-opiomelanocorticotropin (POMC). One subset of neurons concentrated in the ARC co-express and co-release NPY and AgRP [43], which stimulate feeding [42,44]. Another subset of neurons in the ARC expresses POMC, which is cleaved in different anorexigenic peptides including the α-melanocyte-stimulating hormone (α-MSH) produced by the intermedial lobe of the hypophysis[45]; moreover, at the level of the DMH, PVN, LH and ARC, the anorexigenic peptides cocaine- and amphetamine-regulated transcript (CART) is also synthetized [46–48].

However, the HYPO, is not only the brain region responsible for the secretion of both anorexigenic and orexigenic peptides; in fact, it also

integrates signals arising from both gut and adipose tissue that are key elements of the neuroendocrine control of eating [32]. For instance, enteroendocrine L cells synthesize the glucagon-like peptide 1 (GLP-1), which reduces food intake and body weight by stimulating the anorexigenic oxytocinergic neurons located in the PVN [49]. Also, cholecystokinin (CCK), which is secreted by the gastrointestinal (GI) tract and ghrelin, secreted by the stomach, are implicated in gut-brain signaling and feeding control. In particular, CCK acts as a satiety factor, whereas ghrelin increases the meal size and the number of meals [50,51]. Interestingly, it has been suggested that polymorphisms of the gene encoding ghrelin may have a relevant role in the pathogenesis of Prader-Willi syndrome, a rare genetic disorder of childhood that is a predisposing factor for serious pathological obesity [52].

Aside from hormones released by GI tract also leptin, produced by the adipose tissue partakes to eating control, by targeting specific neurons within the HYPO. Leptin, like ghrelin, also acts on the ARC nucleus. A decrease in leptin levels, in fact, induces the activation of the orexigenic neurons of the ARC, with an increase in food intake, stimulation of lipogenesis and fat storage processes and a decrease in energy consumption. On the contrary, the increase in leptin levels stimulates different ARC neurons that produce opposite effects, i.e., of anorexigenic and catabolic type [50,51].

Finally, also insulin, secreted from pancreatic β cells, is able to reduce appetite by engaging the hypothalamic ARC/POMC pathway [53]. Table 1 summarizes the evidence reported in this subparagraph.

Mediator	Site of synthesis	Effects on food intake
Hormone		
Leptin	Adipose tissue	Reduction of long-term food intake
Ghrelin	Stomach	Increase of meal size and number of meals
Insulin	Pancreas	Reduction of meal size
CCK	Gastro-intestinal tract	Reduction of meal size
α-MSH	Hypophysis	Reduction of meal size
GLP-1	Gut	Increase of meal latency
Peptide		
NPY	Arcuate nucleus	Increase of the number of meals
AgRP	Arcuate nucleus	Increase of long term food intake
POMC	Arcuate nucleus	Reduction of long term food intake
CART	Hypothalamus	Reduction of meal size

Table 1: Principal hormones and neuropeptides that regulate eating CCK, cholecystokinin; α-MSH, α-melanocyte-stimulating hormone; GLP-1, glucagon-like peptide 1; NPY, neuropeptide Y; AgRP, agouti-related protein; POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-regulated transcript. For review see: Kim et al., 2018 Nat Rev Neurosci; Valassi et al., 2008 Nutr Metab Cardiovasc Dis.

2.3. The oxytocinergic system: at the crossroad between homeostatic and non-homeostatic signals

OXY is a nine-amino acid neuropeptide hormone, mainly produced in the supraoptic nucleus (SON) and PVN of the HYPO. Its main synthesis occurs within magnocellular neurons of the PVN and SON although, albeit in a small part, also the smallest parvocellular neurons of the PVN contribute to its synthesis [54,55]. Once synthetized from magnocellular neurons, OXY is released both locally within the HYPO through a somato-dendritic release to facilitate autocrine and paracrine regulation under specific demands [55], and distally, through axon terminals, within a variety of brain nuclei such as the ARC, the VTA, the NAc, the parabrachial nucleus, the AMY, the HIPPO, and the PFC [55,56]. Moreover, oxytocinergic magnocellular neurons, differently from parvocellular neurons, possess axonal projections to the posterior lobe of the pituitary gland, which releases OXY into the blood system. Oxytocinergic parvocellular neurons project toward various brainstem areas (e.g., nucleus of the solitary tract, dorsal motor nucleus of the vagus, area postrema) and spinal cord [57] and, relatively recently, it

has been suggested that they also project to magnocellular OXY neurons of the supraoptic nucleus, to modulate the release of OXY into the blood [53,57,58] (Fig. 1.2).

The multitude OXY function both as hormone and neurotransmitter, are mediated by the specific oxytocin receptor (OXY-R), which is a member of the G-protein coupled receptor family. In particular, this receptor has been traditionally identified as being associated with a $G_{q/11}$ protein [60], although recent studies have shown that the OXY-R can also be coupled to inhibitory (G_i) or stimulatory (G_s) G protein, according to the specific localization of the receptor and the physiological state of the animal [55,61]. Therefore, it involves several signaling cascades, including the mitogen-activated protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, phospholipase C pathways, that regulate transcription factors such as CREB or MEF-2 [55]. The expression of the OXY-R was detected in a variety of brain areas where it participates in the regulation of different functions, e.g., AMY (social behavior and fear), HIPPO (spatial memory and neurogenesis), PFC (maternal, socio-sexual, and anxiety behavior), HYPO (homeostatic feeding), periaqueductal gray (anxiety-related behaviors), olfactory bulb (social behavior), striatum, VTA, and NAc (non-homeostatic food intake and reward) [32,62–65]. Furthermore, it has been proposed that OXY released from the axonal terminals of parvocellular neurons projecting to the brainstem and spinal cord contributes to the modulation of cardiovascular functions, breathing, and nociception [66–68]. Outside the CNS, the expression of OXY-R has been identified in different districts, including cardiomyocytes (regulation of ionotropic and chronotropic negative cardiac regulation) [69], adipocytes (stimulation of glucose-oxidation and lipogenesis) [60], and nociceptive ganglion neurons of the dorsal root (modulation of nociception targeting C-

fibers) [70]. OXY-Rs have also been found in osteoblasts and osteoclasts, where OXY exerts an anabolic action[71], in the GI tract, specifically in enteric neurons and enterocytes where it has been seen to modulate GI motility and permeability [72], and in fibroblasts and in keratinocytes of the skin, with a role in the regulation of skin processes such as proliferation, inflammation, and oxidative stress responses [73].

The central oxytocinergic neurotransmission is implicated in a variety of processes that directly or indirectly involve the activation of brain areas belonging to both homeostatic and non-homeostatic circuits; such processes include social cognition, emotionality, dietary choices and food intake [74]. In particular, both preclinical and clinical studies suggest that, apart from its well-known peripheral roles in lactation and parturition, OXY plays also a central role in the regulation of food intake and body weight [75]. In fact, in laboratory rodents and primates, chronic OXY administration was able to induce clinically relevant and sustained weight loss [76,77].

In the homeostatic control of eating, the oxytocinergic system has been reported to have a complex downstream signaling pathway, which comprise the involvement of a variety of different neuropeptides and hormones. In this context, OXY neurons of the PVN receive projections from ARC POMC neurons and consistent data demonstrate interplay between the POMC-derived peptide α-MSH and OXY (anorexigenic pathway). For example, the administration of a melanocortin receptor antagonist abolishes the anorexigenic effect induced by the central administration of OXY [78]. Moreover, OXY secretion can be stimulated by α-MSH application to brain slices containing the PVN [41]; in keeping with such observation, intracerebral ventricular (i.c.v.) administration of α-MSH activates magnocellular oxytocinergic neurons within the PVN and inhibits feeding in rodents; such inhibition is blunted by the administration of an

OXY-R antagonist [79]. On the other side, OXY neurons of the PVN are also in contact with the orexigenic NPY/AgRP neurons arising from the ARC nucleus (orexigenic pathway). In this context, results obtained by optogenetic studies, indicate that the activation of AgRP neurons in the ARC promote eating by exerting an inhibition of the PVN oxytocinergic tone; interestingly, the activation of both AgRP and oxytocinergic neurons has no effect on eating, while the intra-PVN injection of a NPY antagonist for Y1 receptor or a GABA-A receptor antagonist blunt the activation of AgRP-induced feeding [56]. This evidence underlies the importance of the projection of ARC POMC and AgRP/NPY first order neurons to PVN oxytocinergic second order neurons in the regulation of homeostatic feeding (Fig. 1.2). Furthermore, OXY might affect food intake also indirectly, by modulating the brain response to appetite-regulating hormones. Oxytocinergic neurons of the PVN modulate inputs arising from the vagus nerve and projecting to the nucleus of solitary tract (NST). This vagal afferent pathway is triggered by the secretion of a variety of GI hormones, such as CCK and GLP-1, and conveys information to neurons of the NST, a brainstem nucleus that receive parvocellular oxytocinergic axons from the PVN [49,80]. Several experimental observations support the interaction between OXY and CCK; thus, parvocellular oxytocinergic projections within the descending pathway from the PVN to the NST are anatomically located in the proximity of neurons responding to vagally mediate peripheral CCK signals such as those activated by the ingestion of a meal. OXY induces a stimulation of such neurons that leads to an amplification of the CCK-inducing reduction of the meal size [81].

OXY neurons in the PVN and SON, have also been showed to be activated by both peripheral and central administration of the anorectic neuropeptide GLP-1 [82,83]. Rinaman and coworkers demonstrated that the anorexigenic

effect of OXY is absent in rats pretreated with the GLP-1 receptor antagonist and that the central infusion of OXY induces c-fos expression in GLP-1 positive neurons [84]. Moreover, Katsurada and collaborators recently demonstrated that the anorexigenic effect of GLP-1 is accompanied by the activation of a variety of neuropeptidergic neurons within the PVN, including those releasing OXY [49]. These findings suggest that GLP-1 signaling is an important mediator of anorexia induced by OXY. Finally, a large body of evidence suggest the presence of an interplay between the oxytocinergic and the ghrelinergic system. For instance, peripheral ghrelin administration increases plasma OXY levels in rats [85] and central ghrelin administration increases c-fos in oxytocinergic neurons of the rat PVN [86]. These observations corroborate very recent research conducted by Wallace Fitzsimons and collaborators [87] highlighting the existence of a functional crosstalk between the oxytocinergic and the ghrelinergic system. The authors demonstrate that such interaction occurs via the formation of an OXY-R/ghrelin receptor heterocomplex with relevant down-stream signaling consequences (Fig. 1.2-Panel A).

Moreover, OXY not only reduces eating, but also participates to energy and glucose homeostasis and to lipid metabolism. However, the variety of both preclinical and clinical studies investigating the effect of OXY in these contexts reported conflicting results, therefore, further studies are warranted.

In the non-homeostatic control of eating there are, although still sparse, encouraging experimental evidence suggesting that OXY participates to the control of reward-related behavior, by interacting with the central dopaminergic system. The mesolimbic system is well known to play a pivotal role in the regulation of emotion, instinct, and reward-related behavior. Within this system, dopaminergic projections from the VTA to

the NAc are crucially involved in the reward processes associated to feeding behavior [88,89]. In this context, it has been shown that OXY neurons of the PVN regulate the firing of dopaminergic neurons by projecting to the VTA and the NAc [90–94]. Central OXY administration into the VTA [91] and the NAc [95] induces a reduction of sucrose intake after food- deprivation [96]. On the same line, it has been reported that OXY attenuates consumption of fructose-sweetened beverages in monkeys [77]. These results are in keeping with the observation that OXY knockout (KO) mice display a higher palatable sucrose intake both in the dark and in the light phase. In 2013 Mullis and collaborators provided important evidence linking OXY to feeding reward [91]. The authors demonstrated that intra-VTA administration of OXY reduced deprivation-induced eating and palatability-driven sucrose intake; these effects were significantly abolished by the pretreatment with the selective OXY-R antagonist L-368,899. These results strongly support the hypothesis that OXY receptors in the VTA are crucially involved in the reward mechanisms related to food intake. Encouraging results also derive from clinical studies; in particular, intranasal (i.n.) OXY treatment was effective in reducing the consumption of HPF by reducing the reward associated to palatable food [97]. Moreover, a very recent study performed in obese and overweight people suggests that i.n. administration of OXY reduces the functional connectivity between the VTA and a variety of brain areas implicated in the motivation to eat (such as insula, AMY and oral somatosensory cortex) [98]. These studies support the hypothesis that the anorexigenic effect exerted by OXY includes an inhibition of brain reward nuclei activated by the ingestion of HPF. Therefore, it might be hypothesized that OXY reduces the reward value of HPF by dampening dopamine release in the VTA and NAc. Finally, different studies suggest that among the non-homeostatic mechanisms

influencing food intake, OXY might affect eating also on the bases of the social context and the choices among different nutrients. In particular, the central oxytocinergic system is stimulated by consumption of a sucrose-rich meal [99]. At a more general level, OXY-KO mice develop hyperphagia for saccharin and for both sweet and non-sweet carbohydrate [100]. In keeping with this latter result, it has been demonstrated also a functional relationship between OXY and the intake of sugar; in fact, Herisson and collaborators [101] demonstrated that the administration of a selective OXY-R antagonist affects sucrose consumption in mice. However, it should not be ignored, that eating is sometime a consequence of a certain social behavior; thus, it has been demonstrated that the social context is able to influence the initiation and the termination of a meal, as well as the meal duration and the food choices. Herisson [95] and collaborators demonstrated that the social environment impacts the ability of OXY to regulate eating via the involvement of the NAc. In particular, the authors demonstrated that the hypophagic effect of intra-NAc administration of OXY, is abolished when the animals were in a social setting; these results suggest that OXY acting via the NAc reduces food intake in rats that consume a meal in a non-social context. The regulation of feeding behavior also requires the involvement of higher-order brain centers regulating goal-directed behavior, such as cortical regions [35]. For instance, the medial PFC (mPFC) participates to the mechanisms regulating eating on the bases of a conditioned response arising from previous eating-acquired experiences about the consequence to eat. In this case eating behavior is not anymore cue-driven but is a goal-directed action, which takes into consideration the cognitive expectations about that food. These expectations include taste, calorie content of the meal (hypocaloric vs hypercaloric), the satiating properties, and the healthy aspects. OXY has been proposed to reduce

eating by activating cognitive functions and shifting the attention from the short-term benefits derived from eating (for example rewarding properties) to the long-term consequences (such as increase of body weight and health problems) [102] (Fig. 1.2-Panel B).

The evidence reported in this paragraph suggests that OXY affects both the sensing of energy abundance/deficiency (homoeostatic-aspects), and the motivation, as well as the salience and value of food (non-homeostatic aspects). In fact, OXY integrates homeostatic signals derived from the HYPO with hedonic signals arising from the mesolimbic system and with inputs from superordinate decision-making centers such as the mPFC, to coordinate a harmonized response on feeding (Fig. 1.2).

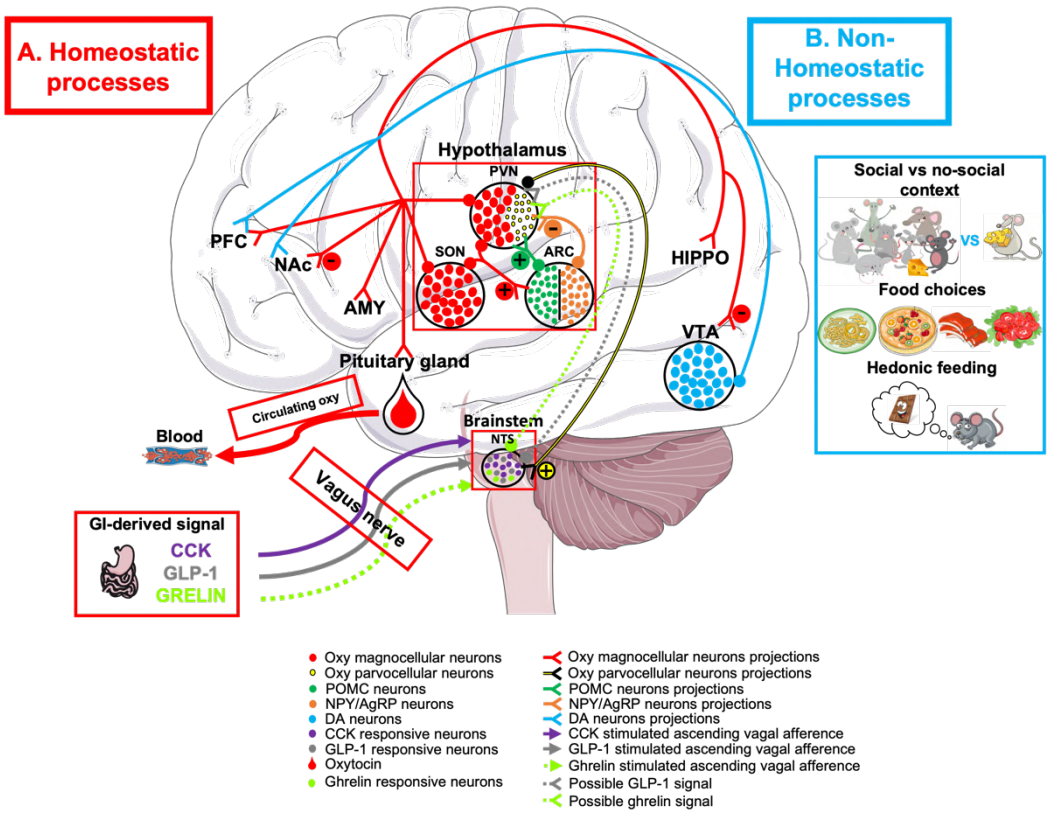

Fig. 1.2: Oxytocin modulation of homeostatic and non-homeostatic processes involved in the regulation of food intake

3. Central and systemic alterations related to obesity

A well-described feature of obesity is chronic, unresolved tissue inflammation that differs from the classical inflammatory response since it is characterized by a chronic low-intensity reaction [102]. Obesity is defined as an excessive accumulation of adipose tissue that is a key factor of systemic inflammation. Particularly, excessive calorie intake and increased fat accumulation trigger the production of effector molecules such as cytokines [104]. This production leads to the chronic, low-grade inflammatory status that induces, in the metabolic tissues, such as liver and skeletal musculature, the recruitment and activation of many mature immune cells (including monocytes that differentiate into macrophages and express the classical proinflammatory phenotype, such as Kupffer cells in the liver) and of other cells, such as adipocytes, that modify the tissue environment [104,105]and amplify tissue altered homeostasis (Fig. 1.3).
Moreover, inflammation resulting from obesity can affect brain structures, such as the HYPO, HIPPO, cerebral cortex, brainstem, and AMY [106]. Low-grade inflammation characteristic for obesity can lead to neuroinflammation through several mechanisms, including the choroid plexuses and disruption of the blood brain barrier (BBB) [107]. The brain is a privileged organ for immunity; however, transitions between peripheral and central inflammation have been reported. Adipokines are produced by the adipose tissue and can also be expressed in the CNS, where receptors for these factors are present. Adipokines produced in the periphery can cross the BBB or modify its physiology by acting on the cells that form the BBB to affect the CNS. Therefore, central inflammation in obesity leads to disruption of hypothalamic satiety signals and perpetuation of overeating [16] (Fig. 1.3).

Central and hepatic alterations, related to obesity, will be extensively discussed in the following paragraphs.

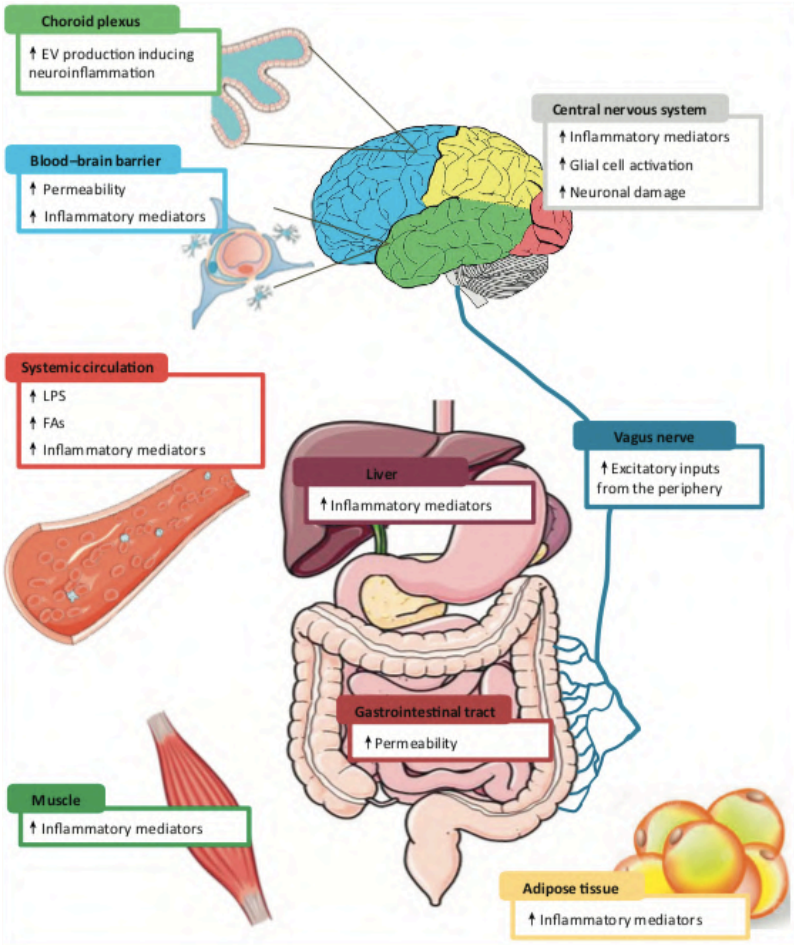

Fig. 1.3: Central and systemic alteration related to obesity; ↑ increased; CNS: central nervous system; EVs: extracellular vesicles; FAs: fatty acids; LPS: lipopolysaccharide.

3.1.Neuroinflammation and altered neurogenesis

Evidence suggests that obesity adversely affects brain function. Indeed, in overweight and obese individuals, adipocytes and macrophages of the metabolic tissues, secrete cytokines and chemokines that cross the BBB and activate, in the CNS, several cell types. In particular, glial cells play a crucial role in the context of neuroinflammation. Microglial cells are considered the resident immune cells of the CNS because when homeostatic disruption is

detected, they can be activated, undergo phenotypic and morphological changes and are able to produce several inflammatory mediators to phagocytize and to present antigens[108].

Astrocytes are the most abundant cell type in the CNS; they are involved in the control and regulation of multiple mechanisms in the CNS such as: neuronal survival, BBB regulation, synapses formation, neuroprotective effect by excessive neurotransmitters removal, secretion of trophic factors, etc. These cells are also important in a pathophysiological context. Indeed, reactive astrocytes are encountered during CNS trauma and neuroinflammation [108]. Therefore, a bidirectional communication between astrocytes and microglia modulates CNS-related inflammation by multiple cytokines and inflammatory mediators [109,110].

In the context of obesity-related neuroinflammation, the HYPO was the brain area mainly studied for its pivotal role in regulating energy homeostasis. The study published in 2005 [111] by De Souza and colleagues was the first to demonstrate that long-term feeding of a lard-based high fat diet (HFD) to rats increased mediobasal hypothalamic activation of the inflammatory signaling intermediates c-Jun N-terminal kinase and nuclear factor (NF)-κβ, resulting in the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 and impairment in insulin and leptin signaling. This initial description of hypothalamic inflammation has been reproduced by many other investigators, with extension of the finding to obese mice and non-human primates [112–119] and models of neonatal overfeeding, type 1 diabetes, and aging [120–123]. In addition, HFD-induced inflammation has been associated with hypothalamic resistance to growth hormone and ghrelin signaling [124,125] and the development of obesity-associated diseases including cognitive dysfunction [126,127], hypertension [128], hepatic

steatosis [129] and β-cell dysfunction [130]. Interestingly, it has also been demonstrated that HYPO inflammation appears to precede low-grade chronic systemic inflammation in obesity as the diet itself could affect inflammatory biomarkers related to neuroinflammation [131]. Thaler et al. reported that, in rodent models, markers of hypothalamic inflammation are elevated within 24 hours of HFD exposure, while in peripheral tissues, inflammation process develops over weeks to months of HFD feeding. Within the first week of HFD exposure, markers of neuron injury also become evident in the ARC of HYPO and in the adjacent nucleus, the median eminence (ME), in association with an increase of markers of reactive gliosis. Although inflammation and gliosis are initially transient, suggesting an effective neuroprotective response, they return and become established with continued HFD exposure. Moreover, they also reported, using an established MRI method, an increased gliosis in the mediobasal HYPO of obese humans [119].

However, the neuroinflammation derived from obesity is not restricted to the HYPO; indeed, during obesity, different evidence supports the presence of neuroinflammation in many areas of the CNS including the cortex, the HIPPO, the AMY, and the cerebellum [132–135]. In particular, other than the HYPO, the HIPPO is best-studied CNS structure in the context of obesity-induced neuroinflammation. Indeed, numerous experimental studies, using animal models of HFD-induced obesity, have revealed modifications in the hippocampal structure and function. For example, in HIPPO, obesity-driven neuroinflammation is characterized by increased expression of cytokines, such as IL-1β, IL-6, and TNF-α and of enzymes that are involved in proinflammatory processes [127,132,136–140]. Moreover, HFD was shown to induce the activation of astrocytes (astrogliosis) as well as the activation of microglia (microgliosis) [127,132,141,142] (Fig. 1.4)

During obesity, also neurons result to be affected. Neurogenesis is a process involving the proliferation, migration, differentiation, survival, and integration of new neurons into existing circuitry, playing a role in brain plasticity [143].

Before the discovery of neural stem cells (NSCs) [144], it was thought that the differentiated cellular elements that form neural circuits in the adult remain substantially unchanged, in terms of number and type, due to the low rate of cell renewal/addition in the CNS. After the discovery of NSCs it was shown that the aforementioned CNS stability has a main exception in two brain regions: the forebrain subventricular zone (SVZ) [145] and the sub-granular zone (SGZ) of the dentate gyrus (DG) within the HIPPO [146]. These "adult neurogenic sites" are fragments of the embryonic germinal layers which maintain stem/progenitor cells within a special microenvironment, a "niche," allowing and regulating NSC activity [147]. In addition, the areas of destination (such as the DG) reached by neuroblasts generated within these neurogenic sites harbor specific, not fully identified yet, environmental signals allowing the integration of young, new-born neurons. These two "canonical" sites of adult neurogenesis have been found in all animal species studied so far, including humans [148–150].

Metabolic diseases, such as obesity and T2DM, are associated with dysregulated neurogenesis [151–156].

Indeed, many researchers have reported that neurogenesis in the hippocampal DG is decreased in rodents with a chronic intake of obesogenic diets, including a HFD [142,157–169] For example, mice administered a HFD show impaired neurogenesis and decreased brain-derived neurotrophic factor, as well as increased lipid peroxidation [151]. Similarly, mice fed with HFD for 7 weeks displayed fewer newly generated

neurons (decreased neurogenesis) in the DG as well as in the dorsal and ventral regions of the HIPPO [170] (Fig 1.4)

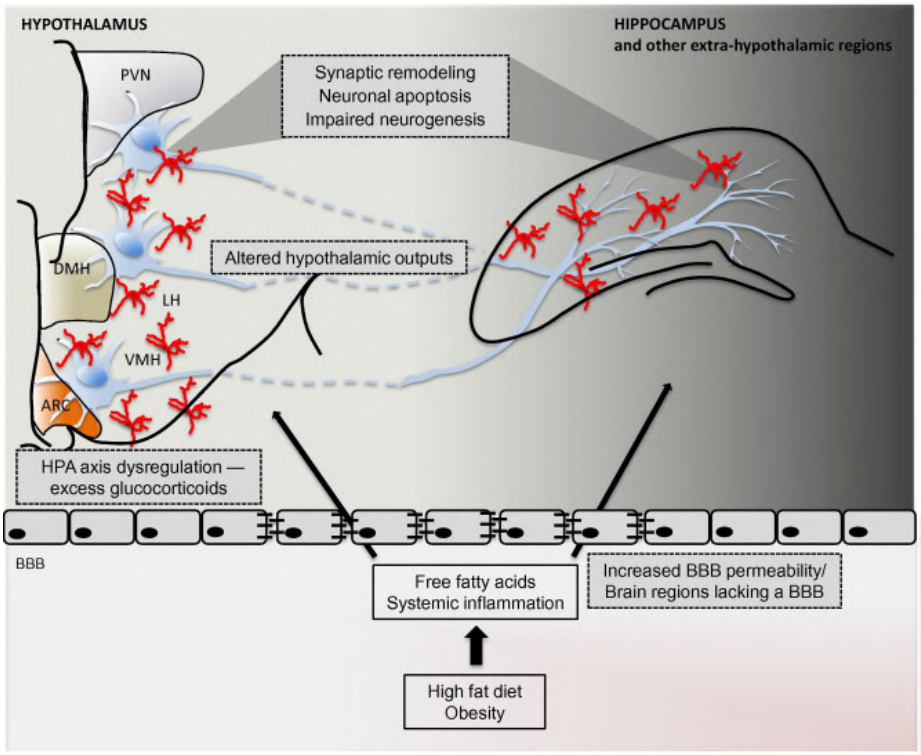

Fig. 1.4: Neuroinflammation and altered neurogenesis related to obesity

3.2. Blood Brain Barrier: structure, functions and changes related to obesity

As discussed above, obesity is characterized by a low-grade inflammation affecting the whole organism. The CNS is an immune-privileged organ due to the presence of the BBB, a barrier tightly controlling exchanges with the periphery [171].

The term BBB is used to describe the distinctive microvasculature of the CNS. Indeed, CNS vessels are continuous non-fenestrated vessels, but also contain a series of additional properties that allow them to strictly regulate the movement of ions, molecules and cells between the blood and the CNS [172,173]. These properties are due to CNS **endothelial cells (ECs)** that form the walls of the blood vessels. Moreover, besides maintaining the BBB

function, ECs have some other roles, such as remaining to attach onto the matrix as a monolayer, maintaining a flat endothelial morphology to prevent turbulent flow, and covering the damaged area by proliferating and migrating rapidly when damage occurs in the vascular structure [174,175]. To perform all these functions ECs need cytoskeletal proteins that, like other mammalian cells, consist of three filamentous proteins: actin microfilaments, microtubules and intermediate filaments [175–177]. Among intermediate filaments protein, the most abundant in ECs is vimentin (VIM) [178,179].

Structurally, ECs of the BBB are held together by **tight junctions (TJs)**, which greatly limit the paracellular flux of solutes [180–182].

TJs are cellular adhesions formed on the apical part of the lateral membrane of ECs [183]. The TJ consists of three integral membrane proteins, namely, claudin (paracellular BBB formation), occludin (resistance of the BBB), and junction adhesion molecules (JAMs, leukocyte extravasation and paracellular permeability of BBB), and several cytoplasmic accessory proteins including zona occludens (ZO)-1,2 and 3, cingulin, and others [183,184]. Cytoplasmic proteins link membrane proteins to actin, which is the primary cytoskeleton protein for the maintenance of structural and functional integrity of the endothelium [184]. Moreover, the TJs interact with basal **adherens junctions (AJs)**, consisting of the membrane protein cadherin that joins the actin cytoskeleton via intermediary proteins, namely, catenins, to form adhesive contacts between cells [183,184].

In addition to ECs, the BBB is composed of the vascular **basement membrane (BM)**, astrocyte end-feet escheating the vessels, and **pericytes (PCs)** embedded within the BM [184].

The vascular tube is surrounded by two **BMs**, the inner vascular BM and the outer parenchymal BM [185,186]. These BMs provide an anchor for

many signaling processes at the vasculature, but also provide an additional barrier for molecules and cells to cross before accessing the neural tissue. The disruption of these BMs is linked to BBB dysfunction and leukocyte infiltration [183].

Astrocytes are a major glial cell type, which extend polarized cellular processes that envelop either neuronal processes or blood vessels [187]. The end feet of the basal process almost completely envelop the vascular tube and contain a discrete array of proteins including aquaporin 4. Astrocytes provide a cellular link between the neuronal circuitry and blood vessels. This neurovascular coupling enables astrocytes to relay signals that regulate blood flow in response to neuronal activity [188,189].

The other main cell types of which blood vessels are made up are the mural cells that sit on the basal surface of the EC layer and include vascular smooth muscle cells that surround the large vessels and PCs, which incompletely cover the endothelial walls of the microvasculature. **PCs** are cells that sit on the basal surface of the microvascular endothelial tube and are embedded in the vascular basement membrane (BM) [190]. PCs play important roles in regulating angiogenesis, deposition of extracellular matrix, wound healing, regulating immune cell infiltration, and regulation of blood flow in response to neural activity, and reports suggest that they also can be multipotent stem cells of the CNS [191]. In addition, these cells have been shown to be important for regulating the formation of the BBB during development, as well as maintaining its function in adulthood and aging [192,193].

Moreover, CNS blood vessels interact with different **immune cell** populations both within the blood as well as within the CNS. The two main cell populations within the CNS are perivascular macrophages and microglial cells [183] (Fig. 1.5)

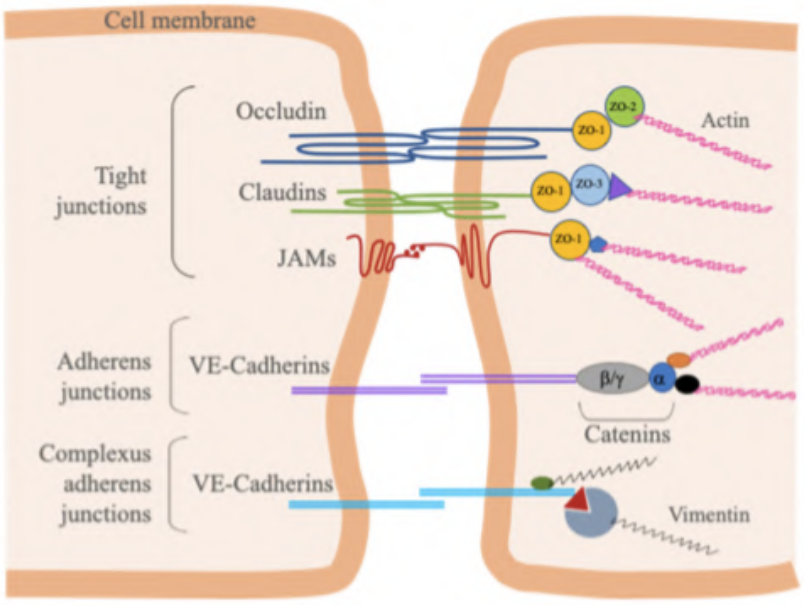

Fig. 1.5: Schematic representation of major tight junction and adherens junction proteins between adjacent endothelial cells of brain microvasculature; zona occludens (ZO)-1,2,3, junctional adhesion molecules (JAMs), vascular endothelial (VE)-cadherin.

The BBB is present throughout the CNS except for the circumventricular organs (CVOs), located around the third and fourth cerebral ventricles, such as area postrema (AP) and ME, in addition to the choroid plexuses [194].

CVOs are characterized by their extensive fenestrated vasculature and lack of the usual BBB [195]. CVOs are in persistent contact with signaling molecules circulating in the bloodstream. Neurons at the CVOs have a variety of receptors for hormones and other signaling molecules, and they have extensive connections to hypothalamic and brainstem nuclei. Therefore, lying at the blood-brain interface, the sensory CVOs are able to detect and integrate humoral and neural information and relay the resulting signals to autonomic control centers of the HYPO [196]. However, tanycytes, which are considered as specialized ependymal cells that are

connected by tight junctions and are immunohistochemical positive to ZO-1 and VIM [194], form a complex network that seal the CNS from the CVOs, creating a distinct blood-cerebrospinal fluid (CSF) barrier. This barrier is detected in the floor and walls of the third ventricle [197–199] as well as in the floor of the fourth ventricle [195].

Therefore, it is evident as disruption of the barriers between the periphery and the CNS can represent an important and deleterious mechanism leading to altered CNS homeostasis and consequently to neuroinflammation.

In this scenario, several studies have assessed the consequences of DIO on BBB permeability. For instance, by using evans blue dye, it was found increased passage of the dye into the CNS of mice fed a HFD for 16 weeks [200]. Moreover, both 10 weeks and 36 weeks of HFD resulted in increased BBB permeability in the cortex and HIPPO [201,202]. Similarly, in a mouse model of DIO, the exposure for 2 months to HFD induces the downregulation of 47 proteins in the cerebral microvessels, including cytoskeletal proteins (such as VIM) [203] (Fig. 1.6)

Moreover, other cell types potentially involved in the context of obesity-induced neuroinflammation and BBB integrity, such as tanycytes, have been studied. For example, in a recent study, Severi and colleagues have demonstrated that after 6 weeks on an HFD, tanycytes of mice's ME, and in particular β_2-tanycytes (prevention of uncontrolled diffusion of substances from the ME environment to the CSF), undergo profound degenerative changes [204].

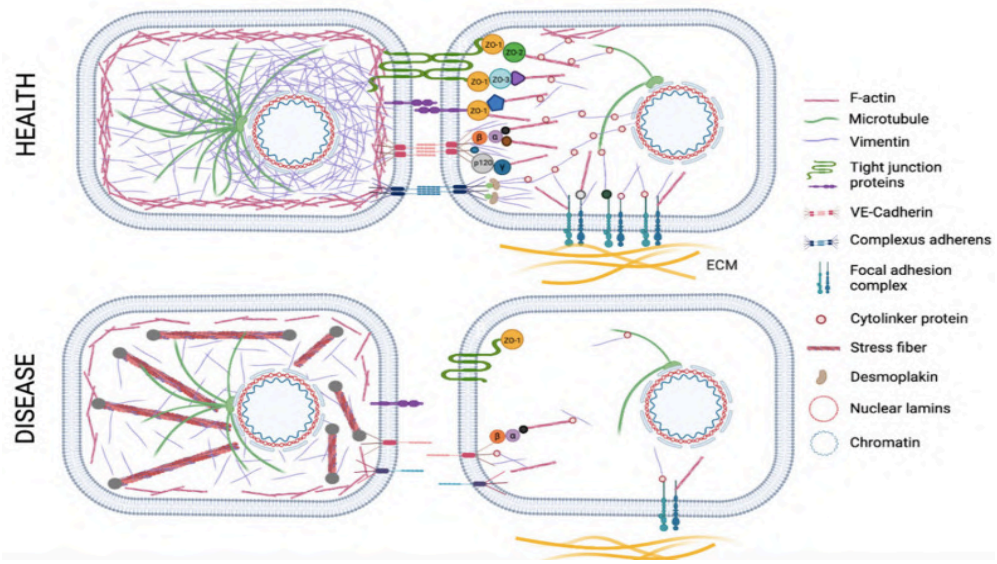

Fig. 1.6: Schematic representation of the cytoskeleton and the interactions of transmembrane proteins and the nucleus in brain microvascular endothelial cells in health and disease.

3.3. Hepatic alterations: aberrant lipid metabolism and oxidative and endoplasmic reticulum stress

As previously mentioned, obesity appears to be associated with the development of both metabolic diseases, such as NAFLD and T2DM, and of non-metabolic diseases, such as asthma, cardiovascular diseases and some forms of cancer [205]. In particular, HFD-associated obesity is very common in patients affected by NAFLD, which represents one of the most frequent causes of liver disease, particularly in Western countries [5,6,206]. Therefore, the increasing prevalence rate of NAFLD parallels the incidence of obesity worldwide [207,208].

NAFLD is a complex systemic disease and its hallmark feature is steatosis, a condition chemically defined as intrahepatic triacylglycerol (TAG) content >5% of liver volume or liver weight [209] or histologically defined when 5% or more of hepatocytes contain visible intracellular TAG [210] In addition to hepatic lipid accumulation NAFLD is characterized by

lipotoxicity, insulin resistance, gut dysbiosis and inflammation and is recognized as the typical hepatic manifestation of metabolic syndrome [211].

The liver is responsible of a wide range of biochemical functions necessary for the metabolic homeostasis of the whole-body [11].

The amount of TAG present in hepatocytes represents a complex interaction between: hepatic fatty acid uptake, derived from plasma free fatty acid (FFA) released from hydrolysis of adipose tissue TAG and FFA released from hydrolysis of circulating TAG; de novo fatty acid synthesis (lipogenesis); fatty acid oxidation (FAO); fatty acid export within very low-density lipoprotein (VLDL) [11].

NAFLD development and progression is strictly dependent on how fat metabolism is regulated. In general, an increase in lipogenesis and fatty acid uptake by hepatocytes, together with a decrease FAO and VLDL release in hepatocytes, cause or exacerbate fatty liver disease [212]. Therefore, any interference and/or alteration in hepatic fat metabolism, in terms of fat storage and release, may represent a risk factor for the onset of NAFLD.

For example, it was reported that ceramide can influence TAG homeostasis and consequently hepatic steatosis throughout the peroxisome proliferator-activated receptor (PPAR)-γ [213], a member of a nuclear hormone superfamily. Ceramide and ceramide-derived sphingolipids are structural components of membranes and have been linked to insulin resistance, oxidative stress, inflammation [214–216] and then to hepatic steatosis. Aberrant hepatic PPAR-γ expression can stimulate hepatic lipogenesis [217] and induce steatosis in mice hepatocytes [218,219], by up-regulating proteins involved in lipid uptake, and TAG storage such as cluster of differentiation (CD)36, monoacylglycerol O-acyltransferase 1, and stearoyl-CoA desaturase 1 (SCD1) [217,220].

PPAR-γ and CD36 mRNA expression are up-regulated in high-fat diet-induced liver steatosis in mice [221]. CD36 expression has been associated with insulin resistance in humans with T2DM [222,223] and increased hepatic *Cd36* gene expression was reported to increase fatty uptake, TAG accumulation and fatty liver [224,225].

Moreover, several lines of evidence suggest that a plethora of dysfunctional processes occur during the progression of NAFLD including inflammation, mitochondrial and endoplasmic reticulum (ER) impairment and oxidative stress [226–229]

Inflammation represents a critical pathophysiological process in liver injury and the severity of inflammation, that is correlated with steatosis, may increase the risk of NAFLD progression [230,231]; both cells residing in the liver (Kupffer cells), and cells recruited from extrahepatic sources (monocytes and natural killer cells) may produce pro-inflammatory factors that lead to the apoptosis and/or the necrosis of hepatocytes [232]. Moreover, during obesity, inflammation in adipose tissue has the potential to be expanded to the liver and, consequently, is associated with NAFLD. Indeed, an excessive amount of fat deposition in hepatocytes, induces TNF-α production and the release of reactive oxygen species (ROS), which in turn contribute to the development and progression of liver inflammation [233]. In addition, excessive deposition of fatty acids (FAs) also induces lipotoxicity, which promotes apoptosis and inflammation [234]. Therefore, a dysfunctional crosstalk between adipose tissue and liver represents a key mechanism underlying the development and progression of NAFLD [212]. Oxidative stress is also crucial is such processes and is characterized by increased production of ROS and/or reduction in the antioxidant body defenses. Excessive levels of ROS can damage different cellular components, including protein, membrane lipids, and nucleic acids, thus

affecting the whole body [235]. The main sites of ROS production comprise the mitochondrial electron transport chain, ER, peroxisomes and enzymatic sources such as nicotinamide adenine dinucleotide phosphate oxidase and uncoupled endothelial nitric oxide synthase [236]. The pathways for ROS production and oxidative stress have been reported to be upregulated in the liver of mice consuming an HFD, and this event precedes the onset of insulin resistance [237]. Moreover, both animal and human studies have reported that long- term HFD feeding leads to increased oxidative stress and dysfunctional mitochondria in many organs [238–241] and induces ER stress in vitro [226–229]. The functional crosstalk between oxidative stress and ER stress is well described. Alterations in the protein folding process and enhanced production of misfolded proteins can exacerbate oxidative stress [242].

4. N-acylethanolamides as lipid mediators

Nowadays, the lack of effective treatment for obesity and eating-related disorders highlights the necessity to discover new targets for the pharmacological treatment of these diseases and their related comorbidities.

In this scenario, in the recent years great attention has been dedicated to a class of endogenous lipid molecules, the N-acyl-ethanolamides (NAEs), to which belong the endocannabinoid anandamide (AEA) and other endocannabinoid-like compounds (structurally similar to endocannabinoids but which do not bind the endocannabinoid receptors) such as palmitoylthanolamide (PEA), oleoylethanolamide (OEA), stearoylethanolamide (SEA) and linoleoylethanolamide (LEA). More than 50 years ago, NAEs were isolated from plant and animal tissues [243,244]

and a strong anti-inflammatory activity has been observed [245]. However, only after the discovery of AEA as an endocannabinoid neurotransmitter that bound cannabinoid receptors, NAEs family gained a great deal of interest [246] for their role in regulating food intake [247]. In the following years the NAEs were increasingly considered as a class of multifunctional lipid mediators for their involvement in a variety of physiological and pathological processes in addition to the regulation of feeding behavior, including pain [248–250], innate immunity [251,252] and reward regulation [253].

For example, PEA by binding different receptors, including PPARs and G-protein coupled receptor (GPR)-55, exerts different biological functions such as anti-inflammatory, analgesic and neuro-protective ones [254,255].

SEA, on the other hand, shows pro-apoptotic and anorexic activity [256,257].

Moreover, AEA has been the most studied molecule among the NAEs and, as an endogenous agonist of the cannabinoid receptors CB1 and CB2, is involved in the induction of analgesia, neuroprotection, hypotension, appetite stimulation, apoptosis in inflammatory and tumor cells and reduction of depression and anxiety [258–263]

In the next paragraphs the biosynthesis, degradation and main biological properties of the OEA will be extensively treated.

4.1.Oleoylethanolamide biosynthesis and degradation

OEA and NAEs are synthesized on-demand from a phospholipid precursor within the membrane lipid bilayer.

OEA is synthesized in the enterocytes of the small intestine from the oleic acid, obtained upon the ingestion of dietary fat [253]. Fatty acids are absorbed from the lumen through CD36, also known as fatty acid transporter, which is an integral membrane glycoprotein expressed on the apical surface of enterocytes in rodent duodenum and jejunum [265]. 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 the administration of carbohydrate, protein, or saline solutions [266,267].

OEA biosynthesis is under the control of the sympathetic nervous system [268,269] and is mediated by two reactions. The first step is represented by the transfer of an acyl group, oleic acid, from the stereospecific numbering-1 (sn-1) position of a membrane phospholipid, such as phosphatidylcholine, to the amine group of phosphatidylethanolamines (PEs) [246]. The enzyme responsible for catalyzing this reaction, named as N-acyltransferase (NAT), is a calcium-dependent enzyme involved in the production of a chemically heterogeneous family of N-acyl-phosphatidylethanolamines (NAPEs) [270,271]. The second step is the release of OEA from NAPE, which contains oleic acid at the amine position. This reaction is catalyzed by a NAPE-selective phospholipase D (NAPE-PLD) that hydrolyzes the distal phosphodiester bond of NAPE [270,271]. All the other NAEs may be produced by a similar biosynthetic pathway that involves the synthesis of the respective NAPEs as precursor [272]; indeed, NAPE-PLD, purified and

molecularly cloned by Dr. Ueda and his collaborators [273], is able to hydrolyze, with similar efficiency, a variety of analogs of the NAPEs family [271].

However, it has been demonstrated that the genetic deletion of the NAPE-PLD does not impair OEA synthesis, suggesting that there are NAPE-PLD-independent mechanisms for OEA synthesis [274]. Moreover, the genetic deletion of NAPE-PLD induces the obese phenotype, with insulin resistance and adipose tissue inflammation, suggesting that the synthesis of NAEs is crucial for the homeostatic regulation of energy balance [275]. It is known that feeding induces OEA formation in the proximal intestine [247,276], due to the increased levels of oleic acid-containing NAPEs, and to the activation of the NAPE-PLD [264]. Interestingly, food intake increases specifically the synthesis of OEA (and the analogue LEA) without affecting the levels of other NAEs, such as PEA and SEA [264]. Moreover, food intake does not change OEA level in the stomach, colon, and submucosa of small intestine, but exclusively in the lumen [264]. Finally, 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 [247,264,267,276].

The main mechanism by which biological OEA effects are terminated is represented by the enzymatic hydrolysis of OEA to oleic acid and ethanolamine [270]. The intracellular enzymes involved in this process are amidases named as: fatty acid amide hydrolase (FAAH) [277] and N-acylethanolamine acid amidase (NAAA) [278].

FAAH belongs to the class of hydrolases, particularly to the family of amidases, and uses an unusual catalytic triad, serine-serine-lysine, to hydrolyze, reversibly and with high efficiency, both starches and esters of

fatty acids [279]. Although it is widely distributed in all mammalian tissues, it is predominantly expressed in the CNS, liver and small intestine [280]. Moreover, a FAAH isozyme, later called FAAH-2, has been observed to be expressed in humans but not in rodents [281]. Between FAAH (also called FAAH-1) and FAAH-2 it has been observed a different subcellular distribution, as the first is localized both at the level of the external mitochondrial membrane and at the level of the ER while the second is localized at level of lipid droplets [282,283].

Finally, NAAA, which belongs to the family of fatty acid cysteine hydrolases, is mainly expressed in the periphery rather than in the brain. This enzyme has been found in high concentrations in lung, spleen, thymus, intestine and in alveolar macrophages of rats [284] and has no homology with FAAH [278]. NAAA also differs from FAAH, since it mostly prefers PEA as a substrate for hydrolysis [250,252,278]. Moreover, it has been demonstrated that NAAA might play a role in regulating NAEs levels during inflammation processes [250,285]. However, its role in OEA metabolism remains poorly understood (Fig. 1.7)

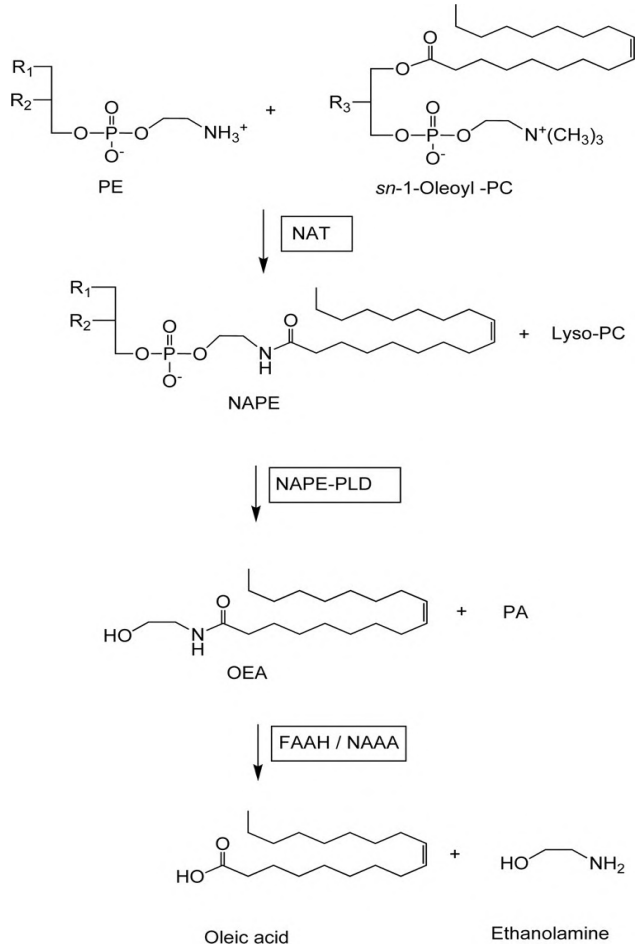

Fig. 1.7: Biosynthesis and degradation pathways of OEA

4.2.Oleoylethanolamide receptors and biological properties

There is accumulating evidence showing that the pro-satiety effect of OEA is mediated by the activation of intestinal PPAR-α [286], which belongs to the family of peroxisome proliferator activated receptors (PPARs).

PPARs are a family of transcription factors constituted by three different isoforms (α, β/δ, and γ), widely expressed in tissues with a higher oxidative capacity such as the cardiovascular system, particularly cardiomyocytes, endothelial cells, and vascular smooth muscle cells [287], but also in several brain areas and in peripheral tissues such as kidney, liver and intestine [288,289]. After being activated by a ligand, PPARs stimulate gene

expression by creating heterodimers with the retinoid X receptor (RXR), thereby binding to specific peroxisome proliferator response elements (PPREs) in the promotor region of target genes [290]. They are involved in different biological processes, such as energy homeostasis, lipid and lipoprotein metabolism, cell proliferation and inflammation, blood pressure control and hypertensive-related complications, such as stroke and renal damage [291,292].

The OEA-induced activation of PPAR-α, and the resulting transcription of the PPAR-α-controlled genes (such as PPAR-α itself, CD36 and fatty acid transport protein 1), 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 on feeding and on lipolysis [286,293]. Moreover, even though OEA activates PPAR-β/δ [286], it has been demonstrated that the effects on 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 PPAR-γ failed to exert these effects [286]. Moreover, beyond the well-known transcriptional effects of OEA, PPAR-α exerts also non transcriptional effects: 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 [294], and that PPAR-α can modulate the firing-rate of neurons by acting on nicotinic receptors [295].

There is increasing pharmacological evidence for additional receptor targets in mediating OEA biological action [270,296], such as the transient receptor potential vanilloid 1 (TRPV1) and the orphan G-protein coupled receptor (GPR)-119 [297,298]. TRPV1 is a member of the vanilloid transient receptor potential cation channel subfamily, abundantly expressed in the

cardiovascular system, peripheral nervous system, CNS and in epithelial cells of the bladder and the GI tract. It is known to act by activating protein kinase A and the endothelial nitric oxide synthase, thus stimulating the production of nitrogen monoxide and the release of calcitonin gene-related peptide and substance P, which, in turn, lead to an altered ion permeability [299,300]. TRPV1 has been demonstrated to be involved in mediating inflammation mechanisms and pain [297]. In accordance with this latter information, intraperitoneal (i.p.) or intradermic OEA injections cause short-lasting nociceptive responses in wild-type mice, but not in mice lacking TRPV1 [297]. 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 [297].

Overton and colleagues have shown that OEA also acts as a medium-potency agonist for GPR119 [298]. The $G_{\alpha s}$ coupled-GPR119, primarily expressed in human and rodent pancreas, fetal liver, GI tract and in rodent brain, stimulates adenylyl cyclase leading to increased intracellular adenosine 3′,5′-cyclic monophosphate levels, thus regulating incretin and insulin hormone secretion [301]. In the GI tract, GPR119 is expressed in enteroendocrine L-cells, which release GLP-1 in response to food ingestion. It has been demonstrated that, after an intraluminal infusion, OEA enhances GLP-1 secretion from intestinal L-cells through a GPR119-dependent mechanism [302]. 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 peripheral administration of OEA [303], thus further confirming the crucial role played by PPAR-α receptors in mediating the effects of OEA.

4.3. Effects of Oleoylethanolamide on feeding and lipid metabolism

Growing evidence accumulated over the last 20 years demonstrated that the principal effect of OEA, after peripheral administration, is a long-lasting and dose-dependent reduction of food intake [304,305]. It is important to point out that this anorexic effect of OEA is not accompanied by stress, malaise, and nausea. In fact, OEA does not change plasma corticosterone levels and does not induce taste aversion in rats [247]. Moreover, the behavioral effects of OEA are selective to feeding since its peripheral administration does not alter water intake or sodium appetite [247].

The hypophagic action of OEA depends on the feeding state of the animal. Particularly, rodent feeding activity that occurs at dark onset, is organized into episodes called "meals" corresponding to a variable amount of food eaten (meal size) [271]. The time interval between the dark onset and the moment in which the animal starts eating is defined as "latency", while the gap occurring between a meal and the subsequent is called "inter-meal interval" and has a variable duration [305–307]. Therefore, in free-feeding rats, 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 [307]. Furthermore, OEA decreases gastric emptying in a dose-dependent manner, an effect that could contribute to the reduction of food intake [308].

Among NAEs, PEA and LEA also induce satiety [309]. 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

[276]. On the other hand, PEA induces satiety to a lesser extent compared to OEA [247].

Additionally, several results reported that beyond reducing appetite, OEA, by recruiting PPAR-α, can regulate several aspects of lipid metabolism [213]. In keeping with this, it has been demonstrated that OEA shows lipolytic effects and increases lipid mobilization from storage sites. In fact, 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 [293]. In the same way, OEA concentration-dependently induced the release of NEFA and glycerol in a rat adipose cells culture, even though, interestingly, the incubation with the highest concentration inhibited this same effect [310]. Furthermore, OEA-induced NEFA and glycerol release has been demonstrated also in vivo, paralleled by an increase of 3-hydroxybutyrate [293], and another study demonstrated the decrease of lipid content in the liver of Zucker rats [304].

Moreover, it has been demonstrated that OEA reduces serum cholesterol and TAG levels [311]. Indeed, rats that underwent a 1-week OEA treatment showed lower circulating levels of TAG compared to the pair-feeding group [247]. Moreover, peripheral OEA administration decreases circulating levels of cholesterol and TAH after one [310], two [304], and four [312] weeks, without affecting HDL cholesterol (393) or glucose [304] circulating levels. Finally, it has also been demonstrated that OEA treatment has beneficial effects on HFD-induced NAFLD in rats, by stimulating fatty acid β-oxidation, and by inhibiting lipogenesis [313].

4.4. Effects of Oleoylethanolamide on the central nervous system

Beyond the regulation of satiety and lipid metabolism, a variety of effects are attributed to exogenous administered OEA spanning in different domains, from neuroprotection [215,216,314] to memory [315] and inflammation. Interestingly, it has also been demonstrated that OEA treatment is able to restore a physiologic sensitivity to the rewarding properties of fat in DIO mice [316], and it is able to exert anti-depressant-like effect in different animal laboratory models [317,318], by regulating the level of both 5-HT and NA in the brain [319].

Regarding the pro-satiety effect evoked by OEA, a large body of evidence revealed the involvement of the activation of selected brain areas, which actively participate in the control of energy homeostasis and feeding behavior. Particularly, it has been observed that after being systemically administered, OEA (10mg/kg i.p.) significantly increases the transcription of the early gene c-fos (marker of neuronal activation) in the NST and AP of brainstem and in the PVN, SON and TMN hypothalamic nuclei [247,320–322]. Moreover, the OEA-induced neurochemical effects evoked by its systemic administration are highly selective, as demonstrated by findings revealing that the levels of additional hypothalamic peptides implicated in energy balance (such as POMC in the ARC) are not affected by OEA i.p. injection [320].

Evidence accumulated over the years highlight, also, the necessary role of different neuronal circuits, such as oxytocinergic, noradrenergic and histaminergic neurotransmission, in mediating OEA action as appetite suppressor [271] (Fig. 1.8). For example, the i.c.v. infusion of the OXY antagonist, L-368,889, which selective blocks the cerebral OXY-R, has been

demonstrated to prevent OEA anorexic effects thus confirming the pivotal role of oxytocinergic transmission in OEA-induced feeding suppression [320].

Moreover, the requirement of hindbrain noradrenergic fibers (that from NST project to the PVN) in mediating OEA's hypophagic effects has been demonstrated in rats subjected to the intra-PVN administration of the toxin saporin (DSAP) able to selectively destroy hindbrain NST-PVN noradrenergic neurons [322].

Finally, it has also been demonstrated that OEA requires the integrity of the brain histaminergic system to fully exert its pro-satiety effect [323]. The interplay between OEA and the histaminergic system will be extensively discussed in the next paragraph.

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

After being systemically administered, OEA quickly reaches the bloodstream, however it cannot readily permeate the brain most probably because of the high expression of its main degrading enzyme, FAAH, in the BBB [324].

Circumventricular organs, such as the AP in the brainstem, characterized by the absence of a functional BBB and particularly enriched in PPAR-α receptors, are attracting a great deal of attention for their possible role in allowing the direct access to the brain for circulating peptides and other peripheral signals, such as OEA. In this background, previous results demonstrated that the systemic administration of OEA at a dose and at a time-point that causes a significant inhibition of eating (10 mg/kg i.p.), strongly activates neurons of the AP and significantly stimulates c-fos

transcription in the SolC, a nucleus of the NST in close contact with the AP [321] (Fig. 1.8)

Finally, the ablation of AP completely prevented both behavioral and neurochemical effects of OEA, thus suggesting a crucial role of this brain area in OEA mechanism of action [325].

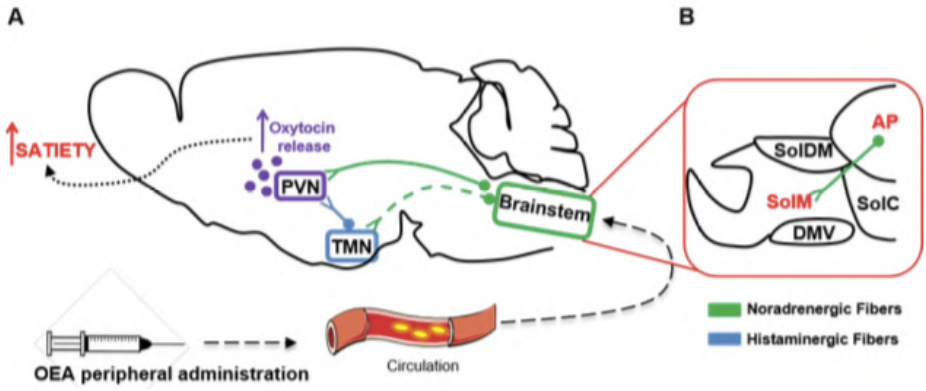

Fig. 1.8: Schematic representation showing the central mechanism mediating OEA's hypophagic action. Panel A: Peripherally administered OEA activates the AP, a brainstem region with fenestrated capillaries that lacks a functional blood-brain barrier (BBB). At this level, OEA may cause the activation of noradrenergic neurons projecting to the nucleus of the solitary tract (NST) and especially to the medial part (SolM) (panel B). Noradrenergic neurons within the NST could, in turn, activate oxytocinergic neurons of the paraventricular nucleus (PVN), stimulating oxytocin expression and release, and, in addition, could presumably activate the tuberomamillary nucleus (TMN), evoking histamine release from neurons projecting to the PVN. Finally, oxytocin released from neurons of the PVN can centrally act to inhibit eating.

4.4.1. Role of the brain histaminergic system

Histamine is an organic nitrogenous compound that is produced from the amino acid L-histidine by the enzyme histidine decarboxylase (HDC) [326]. The most prevalence of histaminergic neurons are located in the TMN of the HYPO; from here these neurons project to the whole CNS through three major pathways: two ascending fiber groups reaching the forebrain structures and one ascending way that innervates the spinal cord [327,328].

Histamine is known to be involved in local immune responses and, as a neurotransmitter, it regulates many physiological functions [329] including arousal [330], release of hypothalamic hormones [331–334], glucose and lipid metabolism [335], and food intake suppression [336,337].

Although histamine can activate both GPR and ligand-gated ion channels, its physiological functions are mainly mediated by the binding of the former referred to as H_1R [338], H_2R [339], H_3R [340], H_4R [341]. Among histamine biological functions, a crucial role is represented by the regulation of eating behavior. Histamine, indeed, induces satiety being released during the appetitive phase of food intake [342]. Moreover, the observation of increased hypothalamic histamine levels during the feeding state of rats that have been fasted for 24 hours, demonstrated a role for histamine also in regulating the consummatory phase of eating behavior [343,344]. Preclinical data collected over the years concerning the administration of histamine within the brain of laboratory rats [345,346] or cats [347] revealed the histamine ability to induce a long-term inhibition of food intake. Moreover, additional evidence of the pro-satiety action of histamine comes from the observation that both the activation of H_1R within the PVN and VMH and the blockade of brain H_3R auto-receptors (that increases histamine release) suppress food consumption [348]. These receptors have been recognized to regulate energy intake and expenditure and their activation has been demonstrated to modulate feeding circadian rhythms [336,349].

Furthermore, there is consistent evidence indicating that a variety of regulatory peptides involved in the homeostatic control of food intake, including leptin, GLP-1 and OEA, require an intact histaminergic system to exert their orexigenic or anorexigenic effect [350,351]. Indeed, Provensi and colleagues demonstrated that mice lacking the enzyme involved in

histamine synthesis, such as HDC, or mice pharmacologically deprived of releasable brain histamine did not respond to the hypophagic effect of exogenously administered OEA as normal mice did [323]. Moreover, also an interplay between OEA and the oxytocinergic and histaminergic systems has been extensively demonstrated. Indeed, it has been demonstrated that the stimulation of the central oxytocinergic system, sustaining the satiety effect of OEA, is linked to the activation of the central histaminergic system [323]. In addition, there is extensive evidence that histaminergic neurons detect acute stress-induced signals [352–354]. In the same way, also OXY peptide is involved in the neuromodulation of a broad variety of behavioral functions related and unrelated to social behavior, including sexual behavior, maternal care, aggression, pair bonding, social memory [355] and modulation of stress, anxiety [356–358], and fear responses [359–361].

Therefore, in collaboration with Prof.ssa Beatrice Passani of University of Florence, we recently published an article in which we aimed to explore the relationship between histaminergic system and OEA on the modulation of OXY immunoreactivity in the PVN of chronic social defeat stress (CSDS) animal model. Both histidine decarboxylase null ($HDC^{-/-}$) and $HDC^{+/+}$ mice were subjected to CSDS for 21 days and treated with either OEA or vehicle daily, starting 10 days after CSDS initiation, until sacrifice. Undisturbed mice served as controls.

It has been found that CSDS increased the number of oxytocin immunofluorescent neurons in the PVN of $HDC^{+/+}$ mice, but not in the PVN of $HDC^{-/-}$ mice, which is surprising and need further investigation. OEA further increased the immunofluorescence of stressed $HDC^{+/+}$ mice but failed to affect $HDC^{-/-}$ mice. It therefore appears that in a disturbed situation as following chronic social defeat, OEA and presumably OXY do not exert their beneficial effect if the histaminergic system is impaired [362] (Fig. 1.9)

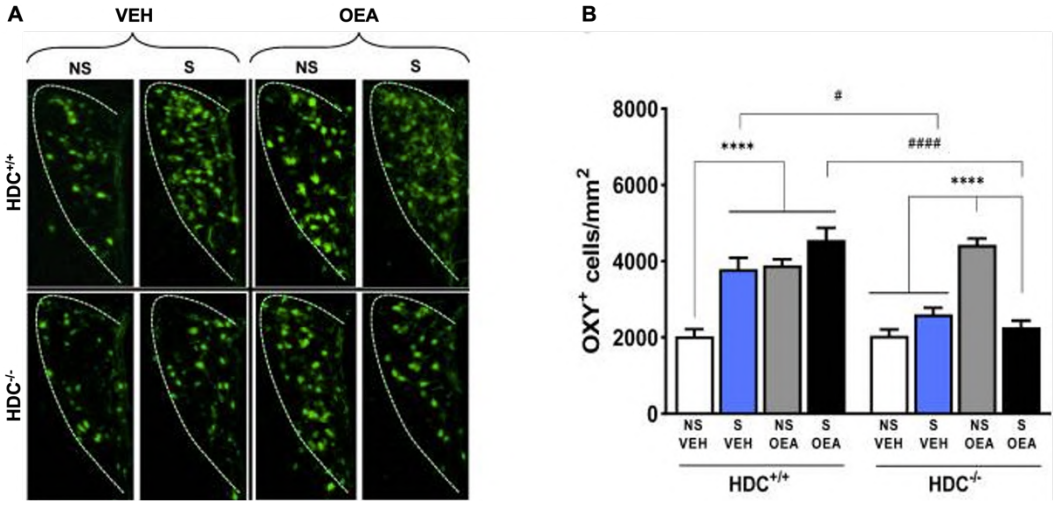

Fig 1.9: Effect of stress and OEA on oxytocin immunofluorescence in the PVN. A) Representative images showing oxytocin immune-positive cell in the PVN of $HDC^{+/+}$ and $HDC^{-/-}$ mice. (B) The bar graphs represent the quantitative data shown in A. Results are expressed as means ± SEM. n= 4-6 slices/brain of 3 mice/group; ****$p<0.0001$, within genotypes; $^{\#\#\#\#}p<0.0001$; $^{\#}p<0.05$ between genotypes. NS, non-stressed mice; S, Stressed mice; VEH, vehicle-treated mice; OEA, oleoylethanolamide (10 mg/kg i.p.).

5. Aim of the study

The increased prevalence of overweight, obesity and eating related disorders is a serious medical and public health problem, and the overconsumption of readily available high-palatable caloric-dense food is the most primary cause for its development [5,6].

Obesity is defined as an excessive accumulation of adipose tissue that is a key factor of systemic inflammation; indeed, a well-described feature of obesity is chronic, unresolved tissue inflammation that differs from the classical inflammatory response since it is characterized by a chronic low-intensity reaction [103]. Therefore, obesity, far from being limited to weight gain, is associated with a cluster of central and peripheral alterations including neuroinflammation, modification in the integrity and functionality of the BBB and fatty liver diseases [105,106]. In this scenario, OEA, a naturally occurring bioactive lipid belonging to the family of NAEs, has received great attention for its biological properties [270,271]. It has been demonstrated that OEA exerts a plethora of protective effects including anti-obesity, anti-inflammatory, and antioxidant properties thus supporting its potential use for the treatment of obesity and eating-related disorders [271,362,363]. In fact, as a drug, OEA reduces food intake and body weight gain [247,304,364] in both lean and obese rodents, reduces lipid synthesis and lipoprotein secretion in hepatocytes [365] and improves HFD-induced liver steatosis in rats [313] and humans [366,367].

Therefore, by using a rat model of diet-induced obesity (DIO), the aim of the **second chapter** of this thesis was to evaluate first whether rats with obese phenotype showed brain and liver alterations, related to obesity, and then whether chronic peripheral administration of OEA might revert such

alterations; moreover, also the effect of OEA on food intake and body weight was evaluated.

To this aim, DIO rats were obtained by exposing male rats to a HFD (60% of the Kcal from fats) for 11 weeks; control rats received a low-fat diet (LFD: 10% of the Kcal from fats). This preclinical model is a useful tool to study the obesity state, sharing several common features with human conditions. Indeed, the availability of HFD and the resulting overconsumption represents the etiology of obesity in modern societies [368]. Subsequently, OEA was intraperitoneally (i.p.) administered daily to rats, for 2 weeks, at the dose of 10 mg/kg. Through different molecular biology techniques were evaluated in the brain of diet-induced obesity (DIO) rats: the expression of glial fibrillary acidic protein (GFAP) in the HYPO, HIPPO, and DG as a marker of neuroinflammation, the number of doublecortin-positive neurons in the DG of the HIPPO as a marker of neurogenesis and the expression of protein VIM and ZO-1 as markers of the integrity and functionality of the BBB. Moreover, in the liver of HFD-fed rats, were evaluated the adipogenesis and different parameters of oxidative and ER stress.

In the last few years several studies have expanded the conventional view of the homeostatic regulation of body weight to include also the non-homeostatic control of appetite [32]. The hedonic system has close connections to the HYPO and homeostatic system [24]. A reward deficiency is considered to cause an imbalance between homeostatic and hedonic regulation [16]. Indeed, a large body of literature documents that beyond the food overconsumption leading to overweight and obesity, also eating disorders, such as BED, have been pathophysiologically linked to dysfunctions of reward mechanisms [32]. In this scenario, the neuropeptide OXY, by binding its receptors (OXY-R), played a role in the control of both

homeostatic and non-homeostatic eating, within cognitive, metabolic and reward mechanisms [32]. Moreover, several studies have demonstrated an interplay between the oxytocinergic system and the endogenous NAEs (such as anandamide, oleoylethanolamide and palmitoylethanolamide) in the modulation of both homeostatic and non-homeostatic systems alterations related to obesity and eating disorders [320,369].

Therefore, the aim of the **third chapter** of this thesis was to evaluate first a possible alteration of the central oxytocinergic system in different preclinical models of aberrant eating pattern (binge eating, DIO and cafeteria models) and second if the direct or indirect pharmacological manipulation of OEA system might positively affect such alterations.

The attention was focused on the study of both the OXY-R and the OXY peptide expressions within: the mPFC that participates to the mechanisms regulating eating on the bases of a conditioned response arising from previous eating-acquired experiences (superordinate decision-making); putamen caudate (CPU) and NAc involved in the non-homeostatic regulation of food intake as part of the reward system; PVN, HYPO, AP and NST, areas involved in the synthesis of OXY and/or strictly involved in the OEA pro-satiety effect.

In particular, 3 different animal models of aberrant eating were used:

1. a rat model of binge-like eating, in which, after cycles of intermittent food restrictions/refeeding and palatable food consumptions, female rats showed a binge-like intake of palatable food, following a 15-min exposure to their sight and smell ("frustration stress")
2. a rat model of DIO, that, as previously described, was obtained by exposing male rats to a HFD (60% of the Kcal from fats) for 11 weeks; control rats received a low-fat diet (LFD: 10% of the Kcal from fats).

3. a rat model of diet-induced obesity based on hedonic overfeeding, induced by the exposure to a cafeteria-diet (CAF rats) for 40 days and subsequently to an abstinence period from this diet.

The impact of the stimulation of the endogenous OEA tone on the central oxytocinergic system was evaluated pharmacologically either directly by peripherally administering OEA (10 mg/kg i.p.) to bingeing and to DIO rats or indirectly by administering the fatty acid amide hydrolase (FAAH, the enzyme involved in the catabolism of the acylethanolamides) inhibitor, PF-3845 (10 mg/kg subcutaneously), to CAF rats.

In conclusion, the main aim of this thesis was to explore in depth the effect of exogenous administration of OEA on both molecular mechanisms involved in obesity-related alterations and endogenous systems which might act as a bridge between homoeostatic and non-homeostatic pathways in the regulation of aberrant food intake.

Therefore, the results reported in this work have the potential to unravel new targets for the development of pharmacological treatments for obesity and eating disorders, two major health problems worldwide.

6. References

1. Kopelman, P.G. Obesity as a Medical Problem. *Nature* 2000.
2. Yach, D.; Stuckler, D.; Brownell, K.D. Epidemiologic and Economic Consequences of the Global Epidemics of Obesity and Diabetes. *Nature Medicine* 2006, *12*.
3. Rogero, M.M.; Calder, P.C. Obesity, Inflammation, Toll-like Receptor 4 and Fatty Acids. *Nutrients* 2018.
4. Hill, J.O.; Wyatt, H.R.; Peters, J.C. Energy Balance and Obesity. *Circulation* **2012**, doi:10.1161/CIRCULATIONAHA.111.087213.
5. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome with Liver and Adipose Inflammation: Comparison to High-Fat Diet. *Obesity* **2011**, *19*, doi:10.1038/oby.2011.18.
6. Hebestreit, A.; Börnhorst, C.; Barba, G.; Siani, A.; Huybrechts, I.; Tognon, G.; Eiben, G.; Moreno, L.A.; Fernández Alvira, J.M.; Loit, H.M.; et al. Associations between Energy Intake, Daily Food Intake and Energy Density of Foods and BMI z-Score in 2-9-Year-Old European Children. *European Journal of Nutrition* **2014**, *53*, doi:10.1007/s00394-013-0575-x.
7. Popkin, B.M. Global Nutrition Dynamics: The World Is Shifting Rapidly toward a Diet Linked with Noncommunicable Diseases. *American Journal of Clinical Nutrition* 2006, *84*.
8. Christ, A.; Latz, E. The Western Lifestyle Has Lasting Effects on Metaflammation. *Nature Reviews Immunology* 2019, *19*.

9. Swinburn, B.A.; Sacks, G.; Hall, K.D.; McPherson, K.; Finegood, D.T.; Moodie, M.L.; Gortmaker, S.L. The Global Obesity Pandemic: Shaped by Global Drivers and Local Environments. *The Lancet* 2011, *378*.
10. Ekelund, U.; Steene-Johannessen, J.; Brown, W.J.; Fagerland, M.W.; Owen, N.; Powell, K.E.; Bauman, A.; Lee, I.M.; Ding, D.; Heath, G.; et al. Does Physical Activity Attenuate, or Even Eliminate, the Detrimental Association of Sitting Time with Mortality? A Harmonised Meta-Analysis of Data from More than 1 Million Men and Women. *The Lancet* **2016**, *388*, doi:10.1016/S0140-6736(16)30370-1.
11. Fabbrini, E.; Sullivan, S.; Klein, S. Obesity and Nonalcoholic Fatty Liver Disease: Biochemical, Metabolic, and Clinical Implications. *Hepatology* 2010, *51*.
12. World Health Organization *Obesity and Overweight*; 2021;
13. Kelly, T.; Yang, W.; Chen, C.S.; Reynolds, K.; He, J. Global Burden of Obesity in 2005 and Projections to 2030. *International Journal of Obesity* **2008**, *32*, doi:10.1038/ijo.2008.102.
14. Mueller, K.; Anwander, A.; Möller, H.E.; Horstmann, A.; Lepsien, J.; Busse, F.; Mohammadi, S.; Schroeter, M.L.; Stumvoll, M.; Villringer, A.; et al. Sex-Dependent Influences of Obesity on Cerebral White Matter Investigated by Diffusion-Tensor Imaging. *PLoS ONE* **2011**, *6*, doi:10.1371/journal.pone.0018544.
15. Must, A.; Anderson, S.E. Body Mass Index in Children and Adolescents: Considerations for Population-Based Applications. *International Journal of Obesity* 2006, *30*.
16. Gómez-Apo, E.; Mondragón-Maya, A.; Ferrari-Díaz, M.; Silva-Pereyra, J. Structural Brain Changes Associated with Overweight and Obesity. *Journal of Obesity* 2021, *2021*.

17. Zhu, S.K.; Wang, Z.M.; Heshka, S.; Heo, M.; Faith, M.S.; Heymsfield, S.B. Waist Circumference and Obesity-Associated Risk Factors among Whites in the Third National Health and Nutrition Examination Survey: Clinical Action Thresholds. *American Journal of Clinical Nutrition* **2002**, *76*, doi:10.1093/ajcn/76.4.743.
18. Ravussin, E.; Ryan, D.H. Three New Perspectives on the Perfect Storm: What's Behind the Obesity Epidemic? *Obesity* 2018, *26*.
19. Duca, F.A.; Lam, T.K.T. Gut Microbiota, Nutrient Sensing and Energy Balance. *Diabetes, Obesity and Metabolism* 2014, *16*.
20. Alcock, J.; Lin, H.C. Fatty Acids from Diet and Microbiota Regulate Energy Metabolism. *F1000Research* 2015, *4*.
21. Monteiro, M.P.; Batterham, R.L. The Importance of the Gastrointestinal Tract in Controlling Food Intake and Regulating Energy Balance. *Gastroenterology* **2017**, *152*, doi:10.1053/j.gastro.2017.01.053.
22. Berthoud, H.R. Multiple Neural Systems Controlling Food Intake and Body Weight. *Neuroscience and Biobehavioral Reviews* 2002, *26*.
23. Boughton, C.K.; Murphy, K.G. Can Neuropeptides Treat Obesity? A Review of Neuropeptides and Their Potential Role in the Treatment of Obesity. *British Journal of Pharmacology* 2013, *170*.
24. Kenny, P.J. Reward Mechanisms in Obesity: New Insights and Future Directions. *Neuron* 2011.
25. Hudson, J.I.; Hiripi, E.; Pope, H.G.; Kessler, R.C. The Prevalence and Correlates of Eating Disorders in the National Comorbidity Survey Replication. *Biological Psychiatry* **2007**, *61*, doi:10.1016/j.biopsych.2006.03.040.
26. Swanson, S.A.; Crow, S.J.; le Grange, D.; Swendsen, J.; Merikangas, K.R. Prevalence and Correlates of Eating Disorders in Adolescents:

Results from the National Comorbidity Survey Replication Adolescent Supplement. *Archives of General Psychiatry* **2011**, *68*, doi:10.1001/archgenpsychiatry.2011.22.

27. Kessler, R.C.; Berglund, P.A.; Chiu, W.T.; Deitz, A.C.; Hudson, J.I.; Shahly, V.; Aguilar-Gaxiola, S.; Alonso, J.; Angermeyer, M.C.; Benjet, C.; et al. The Prevalence and Correlates of Binge Eating Disorder in the World Health Organization World Mental Health Surveys. *Biological Psychiatry* **2013**, *73*, doi:10.1016/j.biopsych.2012.11.020.
28. Sobik, L.; Hutchison, K.; Craighead, L. Cue-Elicited Craving for Food: A Fresh Approach to the Study of Binge Eating. *Appetite* **2005**, *44*, doi:10.1016/j.appet.2004.12.001.
29. Curtis, C.; Davis, C. A Qualitative Study of Binge Eating and Obesity From an Addiction Perspective. *Eating Disorders* **2014**, *22*, doi:10.1080/10640266.2014.857515.
30. Schulte, E.M.; Grilo, C.M.; Gearhardt, A.N. Shared and Unique Mechanisms Underlying Binge Eating Disorder and Addictive Disorders. *Clinical Psychology Review* 2016, *44*.
31. Valdivia, S.; Patrone, A.; Reynaldo, M.; Perello, M. Acute High Fat Diet Consumption Activates the Mesolimbic Circuit and Requires Orexin Signaling in a Mouse Model. *PLoS ONE* **2014**, doi:10.1371/journal.pone.0087478.
32. Romano, A.; Friuli, M.; Cifani, C.; Gaetani, S. Oxytocin in the Neural Control of Eating: At the Crossroad between Homeostatic and Non-Homeostatic Signals. *Neuropharmacology* 2020.
33. D'Addario, C.; Micioni Di Bonaventura, M. v.; Pucci, M.; Romano, A.; Gaetani, S.; Ciccocioppo, R.; Cifani, C.; Maccarrone, M. Endocannabinoid Signaling and Food Addiction. *Neuroscience and Biobehavioral Reviews* 2014, *47*.

34. Saper, C.B.; Chou, T.C.; Elmquist, J.K. The Need to Feed: Homeostatic and Hedonic Control of Eating. *Neuron* 2002, *36*.
35. Liu, C.M.; Kanoski, S.E. Homeostatic and Non-Homeostatic Controls of Feeding Behavior: Distinct vs. Common Neural Systems. *Physiology and Behavior* **2018**, doi:10.1016/j.physbeh.2018.02.011.
36. Berridge, K.C. Food Reward: Brain Substrates of Wanting and Liking. *Neuroscience and Biobehavioral Reviews* **1996**, doi:10.1016/0149-7634(95)00033-B.
37. Baik, J.H. Dopamine Signaling in Reward-Related Behaviors. *Frontiers in Neural Circuits* 2013.
38. Higgs, S.; Spetter, M.S.; Thomas, J.M.; Rotshtein, P.; Lee, M.; Hallschmid, M.; Dourish, C.T. Interactions between Metabolic, Reward and Cognitive Processes in Appetite Control: Implications for Novel Weight Management Therapies. *Journal of Psychopharmacology* **2017**, doi:10.1177/0269881117736917.
39. Lutter, M.; Nestler, E.J. Homeostatic and Hedonic Signals Interact in the Regulation of Food Intake. *The Journal of Nutrition* **2009**, doi:10.3945/jn.108.097618.
40. Murray, S.; Tulloch, A.; Gold, M.S.; Avena, N.M. Hormonal and Neural Mechanisms of Food Reward, Eating Behaviour and Obesity. *Nature Reviews Endocrinology* 2014.
41. Schwartz, M.W.; Woods, S.C.; Porte, D.; Seeley, R.J.; Baskin, D.G. Central Nervous System Control of Food Intake. *Nature* 2000.
42. Chambers, A.P.; Woods, S.C. The Role of Neuropeptide y in Energy Homeostasis. *Handbook of Experimental Pharmacology* **2012**, doi:10.1007/978-3-642-24716-3_2.
43. Hagan, M.M.; Rushing, P.A.; Pritchard, L.M.; Schwartz, M.W.; Strack, A.M.; van der Ploeg, L.H.T.; Woods, S.C.; Seeley, R.J. Long-Term

Orexigenic Effects of AgRP-(83-132) Involve Mechanisms Other than Melanocortin Receptor Blockade. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2000**, doi:10.1152/ajpregu.2000.279.1.r47.

44. Stanley, B.G.; Anderson, K.C.; Grayson, M.H.; Leibowitz, S.F. Repeated Hypothalamic Stimulation with Neuropeptide Y Increases Daily Carbohydrate and Fat Intake and Body Weight Gain in Female Rats. *Physiology and Behavior* **1989**, doi:10.1016/0031-9384(89)90251-5.
45. Dietrich, M.O.; Horvath, T.L. Feeding Signals and Brain Circuitry. *European Journal of Neuroscience* 2009.
46. Battistoni, S.; Kloting, I.; Cifani, C.; Massi, M.; Polidori, C. Gender Differences in Nociceptin/Orphanin FQ-Induced Food Intake in Strains Derived from Rats Prone (WOKW) and Resistant (Dark Agouti) to Metabolic Syndrome: A Possible Involvement of the Cocaine- and Amphetamine-Regulated Transcript System. *Genes and Nutrition* **2011**, doi:10.1007/s12263-010-0189-3.
47. Cifani, C.; Kloting, I.; Morini, G.; Grandi, D.; Massi, M.; Polidori, C. Nociceptin/Orphanin FQ-Induced Food Intake and Cocaine Amphetamine Regulated Transcript Gene Expression in Strains Derived from Rats Prone (WOKW) and Resistant (Dark Agouti) to Metabolic Syndrome. *Peptides* **2009**, doi:10.1016/j.peptides.2008.11.012.
48. Filippetti, R.; Kloting, I.; Massi, M.; Cifani, C.; Polidori, C. Involvement of Cocaine-Amphetamine Regulated Transcript in the Differential Feeding Responses to Nociceptin/Orphanin FQ in Dark Agouti and Wistar Ottawa Karlsburg W Rats. *Peptides* **2007**.
49. Katsurada, K.; Maejima, Y.; Nakata, M.; Kodaira, M.; Suyama, S.; Iwasaki, Y.; Kario, K.; Yada, T. Endogenous GLP-1 Acts on

Paraventricular Nucleus to Suppress Feeding: Projection from Nucleus Tractus Solitarius and Activation of Corticotropin-Releasing Hormone, Nesfatin-1 and Oxytocin Neurons. *Biochemical and Biophysical Research Communications* **2014**, doi:10.1016/j.bbrc.2014.07.116.

50. Kim, K.S.; Seeley, R.J.; Sandoval, D.A. Signalling from the Periphery to the Brain That Regulates Energy Homeostasis. *Nature Reviews Neuroscience* 2018.
51. Valassi, E.; Scacchi, M.; Cavagnini, F. Neuroendocrine Control of Food Intake. *Nutrition, Metabolism and Cardiovascular Diseases* 2008.
52. Haqq, A.M.; Grambow, S.C.; Muehlbauer, M.; Newgard, C.B.; Svetkey, L.P.; Carrel, A.L.; Yanovski, J.A.; Purnell, J.Q.; Freemark, M. Ghrelin Concentrations in Prader-Willi Syndrome (PWS) Infants and Children: Changes during Development. *Clinical Endocrinology* **2008**, *69*, doi:10.1111/j.1365-2265.2008.03385.x.
53. Benoit, S.C.; Air, E.L.; Coolen, L.M.; Strauss, R.; Jackman, A.; Clegg, D.J.; Seeley, R.J.; Woods, S.C. The Catabolic Action of Insulin in the Brain Is Mediated by Melanocortins. *Journal of Neuroscience* **2002**, doi:10.1523/jneurosci.22-20-09048.2002.
54. Althammer, F.; Grinevich, V. Diversity of Oxytocin Neurones: Beyond Magno- and Parvocellular Cell Types? *Journal of Neuroendocrinology* 2018.
55. Jurek, B.; Neumann, I.D. The Oxytocin Receptor: From Intracellular Signaling to Behavior. *Physiological Reviews* **2018**, doi:10.1152/physrev.00031.2017.
56. Maejima, Y.; Yokota, S.; Nishimori, K.; Shimomura, K. The Anorexigenic Neural Pathways of Oxytocin and Their Clinical Implication. *Neuroendocrinology* **2018**, doi:10.1159/000489263.

57. Spetter, M.S.; Hallschmid, M. Current Findings on the Role of Oxytocin in the Regulation of Food Intake. *Physiology and Behavior* 2017.

58. Swanson, L.W.; Sawchenko, P.E. Hypothalamic Integration: Organization of the Paraventricular and Supraoptic Nuclei. *Annual Review of Neuroscience* **1983**, doi:10.1146/annurev.ne.06.030183.001413.

59. Sawchenko, P.E.; Swanson, L.W. Immunohistochemical Identification of Neurons in the Paraventricular Nucleus of the Hypothalamus That Project to the Medulla or to the Spinal Cord in the Rat. *Journal of Comparative Neurology* **1982**, doi:10.1002/cne.902050306.

60. Gimpl, G.; Fahrenholz, F. The Oxytocin Receptor System: Structure, Function, and Regulation. *Physiological Reviews* 2001.

61. Zhou, X.B.; Lutz, S.; Steffens, F.; Korth, M.; Wieland, T. Oxytocin Receptors Differentially Signal via Gq and G i Proteins in Pregnant and Nonpregnant Rat Uterine Myocytes: Implications for Myometrial Contractility. *Molecular Endocrinology* **2007**, doi:10.1210/me.2006-0220.

62. Atasoy, D.; Nicholas Betley, J.; Su, H.H.; Sternson, S.M. Deconstruction of a Neural Circuit for Hunger. *Nature* **2012**, doi:10.1038/nature11270.

63. Gil, M.; Bhatt, R.; Picotte, K.B.; Hull, E.M. Sexual Experience Increases Oxytocin Receptor Gene Expression and Protein in the Medial Preoptic Area of the Male Rat. *Psychoneuroendocrinology* **2013**, doi:10.1016/j.psyneuen.2013.02.002.

64. Figueira, R.J.; Peabody, M.F.; Lonstein, J.S. Oxytocin Receptor Activity in the Ventrocaudal Periaqueductal Gray Modulates Anxiety-Related Behavior in Postpartum Rats. *Behavioral Neuroscience* **2008**, doi:10.1037/0735-7044.122.3.618.

65. Banki, L.; Büki, A.; Horvath, G.; Kekesi, G.; Kis, G.; Somogyvári, F.; Jancsó, G.; Vécsei, L.; Varga, E.; Tuboly, G. Distinct Changes in Chronic Pain Sensitivity and Oxytocin Receptor Expression in a New Rat Model (Wisket) of Schizophrenia. *Neuroscience Letters* **2020**, doi:10.1016/j.neulet.2019.134561.
66. Mack, S.O.; Kc, P.; Wu, M.; Coleman, B.R.; Tolentino-Silva, F.P.; Haxhiu, M.A. Paraventricular Oxytocin Neurons Are Involved in Neural Modulation of Breathing. *Journal of Applied Physiology* **2002**, doi:10.1152/japplphysiol.00839.2001.
67. Petersson, M. Cardiovascular Effects of Oxytocin. In Proceedings of the Progress in Brain Research; 2002.
68. Condés-Lara, M.; González, N.M.; Martínez-Lorenzana, G.; Delgado, O.L.; Freund-Mercier, M.J. Actions of Oxytocin and Interactions with Glutamate on Spontaneous and Evoked Dorsal Spinal Cord Neuronal Activities. *Brain Research* **2003**, doi:10.1016/S0006-8993(03)02690-8.
69. Gutkowska, J.; Jankowski, M. Oxytocin Revisited: Its Role in Cardiovascular Regulation. *Journal of Neuroendocrinology* 2012.
70. Juif, P.E.; Poisbeau, P. Neurohormonal Effects of Oxytocin and Vasopressin Receptor Agonists on Spinal Pain Processing in Male Rats. *Pain* **2013**, doi:10.1016/j.pain.2013.05.003.
71. Copland, J.A.; Ives, K.L.; Simmons, D.J.; Soloff, M.S. Functional Oxytocin Receptors Discovered in Human Osteoblasts. *Endocrinology* **1999**, doi:10.1210/endo.140.9.7130.
72. Welch, M.G.; Margolis, K.G.; Li, Z.; Gershon, M.D. Oxytocin Regulates Gastrointestinal Motility, Inflammation, Macromolecular Permeability, and Mucosal Maintenance in Mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **2014**, doi:10.1152/ajpgi.00176.2014.

73. Deing, V.; Roggenkamp, D.; Kühnl, J.; Gruschka, A.; Stäb, F.; Wenck, H.; Bürkle, A.; Neufang, G. Oxytocin Modulates Proliferation and Stress Responses of Human Skin Cells: Implications for Atopic Dermatitis. *Experimental Dermatology* **2013**, doi:10.1111/exd.12155.
74. Plessow, F.; Eddy, K.T.; Lawson, E.A. The Neuropeptide Hormone Oxytocin in Eating Disorders. *Current Psychiatry Reports* 2018.
75. Romano, A.; Tempesta, B.; di Bonaventura, M.V.M.; Gaetani, S. From Autism to Eating Disorders and More: The Role of Oxytocin in Neuropsychiatric Disorders. *Frontiers in Neuroscience* 2016.
76. Blevins, J.E.; Graham, J.L.; Morton, G.J.; Bales, K.L.; Schwartz, M.W.; Baskin, D.G.; Havel, P.J. Chronic Oxytocin Administration Inhibits Food Intake, Increases Energy Expenditure, and Produces Weight Loss in Fructose-Fed Obese Rhesus Monkeys. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2015**, doi:10.1152/ajpregu.00441.2014.
77. Blevins, J.E.; Baskin, D.G. Translational and Therapeutic Potential of Oxytocin as an Anti-Obesity Strategy: Insights from Rodents, Nonhuman Primates and Humans. *Physiology and Behavior* **2015**, doi:10.1016/j.physbeh.2015.05.023.
78. Maejima, Y.; Sedbazar, U.; Suyama, S.; Kohno, D.; Onaka, T.; Takano, E.; Yoshida, N.; Koike, M.; Uchiyama, Y.; Fujiwara, K.; et al. Nesfatin-1-Regulated Oxytocinergic Signaling in the Paraventricular Nucleus Causes Anorexia through a Leptin-Independent Melanocortin Pathway. *Cell Metabolism* **2009**, doi:10.1016/j.cmet.2009.09.002.
79. Yosten, G.L.C.; Samson, W.K. The Anorexigenic and Hypertensive Effects of Nesfatin-1 Are Reversed by Pretreatment with an Oxytocin Receptor Antagonist. *American Journal of Physiology - Regulatory*

Integrative and Comparative Physiology **2010**, doi:10.1152/ajpregu.00804.2009.

80. Affleck, V.S.; Coote, J.H.; Pyner, S. The Projection and Synaptic Organisation of NTS Afferent Connections with Presympathetic Neurons, GABA and NNOS Neurons in the Paraventricular Nucleus of the Hypothalamus. *Neuroscience* **2012**, doi:10.1016/j.neuroscience.2012.05.070.
81. Blevins, J.E.; Eakin, T.J.; Murphy, J.A.; Schwartz, M.W.; Baskin, D.G. Oxytocin Innervation of Caudal Brainstem Nuclei Activated by Cholecystokinin. *Brain Research* **2003**, doi:10.1016/j.brainres.2003.08.036.
82. Larsen, P.J.; Tang-Christensen, M.; Jessop, D.S. Central Administration of Glucagon-like Peptide-1 Activates Hypothalamic Neuroendocrine Neurons in the Rat. *Endocrinology* **1997**, doi:10.1210/endo.138.10.5270.
83. Saito, R.; So, M.; Motojima, Y.; Matsuura, T.; Yoshimura, M.; Hashimoto, H.; Yamamoto, Y.; Kusuhara, K.; Ueta, Y. Activation of Nesfatin-1-Containing Neurones in the Hypothalamus and Brainstem by Peripheral Administration of Anorectic Hormones and Suppression of Feeding via Central Nesfatin-1 in Rats. *Journal of Neuroendocrinology* **2016**, doi:10.1111/jne.12400.
84. Rinaman, L.; Rothe, E.E. GLP-1 Receptor Signaling Contributes to Anorexigenic Effect of Centrally Administered Oxytocin in Rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2002**, doi:10.1152/ajpregu.00008.2002.
85. Pekary, A.E.; Sattin, A. Rapid Modulation of TRH and TRH-like Peptide Release in Rat Brain and Peripheral Tissues by Ghrelin and 3-TRP-Ghrelin. *Peptides* **2012**, doi:10.1016/j.peptides.2012.04.021.

86. Olszewski, P.K.; Bomberg, E.M.; Martell, A.; Grace, M.K.; Levine, A.S. Intraventricular Ghrelin Activates Oxytocin Neurons: Implications in Feeding Behavior. *NeuroReport* **2007**, doi:10.1097/WNR.0b013e328058684e.

87. Wallace Fitzsimons, S.E.; Chruścicka, B.; Druelle, C.; Stamou, P.; Nally, K.; Dinan, T.G.; Cryan, J.F.; Schellekens, H. A Ghrelin Receptor and Oxytocin Receptor Heterocomplex Impairs Oxytocin Mediated Signalling. *Neuropharmacology* **2019**, doi:10.1016/j.neuropharm.2018.12.022.

88. Adamantidis, A.R.; Tsai, H.C.; Boutrel, B.; Zhang, F.; Stuber, G.D.; Budygin, E.A.; Touriño, C.; Bonci, A.; Deisseroth, K.; de Lecea, L. Optogenetic Interrogation of Dopaminergic Modulation of the Multiple Phases of Reward-Seeking Behavior. *Journal of Neuroscience* **2011**, doi:10.1523/JNEUROSCI.2246-11.2011.

89. Watabe-Uchida, M.; Zhu, L.; Ogawa, S.K.; Vamanrao, A.; Uchida, N. Whole-Brain Mapping of Direct Inputs to Midbrain Dopamine Neurons. *Neuron* **2012**, doi:10.1016/j.neuron.2012.03.017.

90. Hung, L.W.; Neuner, S.; Polepalli, J.S.; Beier, K.T.; Wright, M.; Walsh, J.J.; Lewis, E.M.; Luo, L.; Deisseroth, K.; Dölen, G.; et al. Gating of Social Reward by Oxytocin in the Ventral Tegmental Area. *Science* **2017**, doi:10.1126/science.aan4994.

91. Mullis, K.; Kay, K.; Williams, D.L. Oxytocin Action in the Ventral Tegmental Area Affects Sucrose Intake. *Brain Research* **2013**, doi:10.1016/j.brainres.2013.03.026.

92. Peris, J.; MacFadyen, K.; Smith, J.A.; de Kloet, A.D.; Wang, L.; Krause, E.G. Oxytocin Receptors Are Expressed on Dopamine and Glutamate Neurons in the Mouse Ventral Tegmental Area That Project to

Nucleus Accumbens and Other Mesolimbic Targets. *Journal of Comparative Neurology* **2017**, doi:10.1002/cne.24116.

93. Song, Z.; Borland, J.M.; Larkin, T.E.; O'Malley, M.; Albers, H.E. Activation of Oxytocin Receptors, but Not Arginine-Vasopressin (AVP) Receptors, in the Ventral Tegmental Area of Male Syrian Hamsters Is Essential for the Reward-like Properties of Social Interactions. *Psychoneuroendocrinology* **2016**, doi:10.1016/j.psyneuen.2016.09.001.
94. Tang, Y.; Chen, Z.; Tao, H.; Li, C.; Zhang, X.; Tang, A.; Liu, Y. Oxytocin Activation of Neurons in Ventral Tegmental Area and Interfascicular Nucleus of Mouse Midbrain. *Neuropharmacology* **2014**, doi:10.1016/j.neuropharm.2013.10.004.
95. Herisson, F.M.; Waas, J.R.; Fredriksson, R.; Schiöth, H.B.; Levine, A.S.; Olszewski, P.K. Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake. *Journal of Neuroendocrinology* **2016**, doi:10.1111/jne.12381.
96. Klockars, A.; Levine, A.S.; Olszewski, P.K. Central Oxytocin and Food Intake: Focus on Macronutrient-Driven Reward. *Frontiers in Endocrinology* 2015.
97. Ott, V.; Finlayson, G.; Lehnert, H.; Heitmann, B.; Heinrichs, M.; Born, J.; Hallschmid, M. Oxytocin Reduces Reward-Driven Food Intake in Humans. *Diabetes* **2013**, doi:10.2337/db13-0663.
98. Kerem, L.; Hadjikhani, N.; Holsen, L.; Lawson, E.A.; Plessow, F. Oxytocin Reduces the Functional Connectivity between Brain Regions Involved in Eating Behavior in Men with Overweight and Obesity. *International Journal of Obesity* **2019**, doi:10.1038/s41366-019-0489-7.

99. Olszewski, P.K.; Klockars, A.; Olszewska, A.M.; Fredriksson, R.; Schiöth, H.B.; Levine, A.S. Molecular, Immunohistochemical, and Pharmacological Evidence of Oxytocin's Role as Inhibitor of Carbohydrate but Not Fat Intake. *Endocrinology* **2010**, doi:10.1210/en.2010-0151.

100. Sclafani, A.; Rinaman, L.; Vollmer, R.R.; Amico, J.A. Oxytocin Knockout Mice Demonstrate Enhanced Intake of Sweet and Nonsweet Carbohydrate Solutions. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2007**, doi:10.1152/ajpregu.00826.2006.

101. Herisson, F.M.; Brooks, L.L.; Waas, J.R.; Levine, A.S.; Olszewski, P.K. Functional Relationship between Oxytocin and Appetite for Carbohydrates versus Saccharin. *NeuroReport* **2014**, doi:10.1097/WNR.0000000000000201.

102. Striepens, N.; Schröter, F.; Stoffel-Wagner, B.; Maier, W.; Hurlemann, R.; Scheele, D. Oxytocin Enhances Cognitive Control of Food Craving in Women. *Human Brain Mapping* **2016**, doi:10.1002/hbm.23308.

103. Ying, W.; Fu, W.; Lee, Y.S.; Olefsky, J.M. The Role of Macrophages in Obesity-Associated Islet Inflammation and β-Cell Abnormalities. *Nature Reviews Endocrinology* 2020.

104. Boulangé, C.L.; Neves, A.L.; Chilloux, J.; Nicholson, J.K.; Dumas, M.E. Impact of the Gut Microbiota on Inflammation, Obesity, and Metabolic Disease. *Genome Medicine* 2016.

105. Klöting, N.; Blüher, M. Adipocyte Dysfunction, Inflammation and Metabolic Syndrome. *Reviews in Endocrine and Metabolic Disorders* 2014.

106. Guillemot-Legris, O.; Muccioli, G.G. Obesity-Induced Neuroinflammation: Beyond the Hypothalamus. *Trends in Neurosciences* 2017, *40*.
107. Novo, A.M.; Batista, S. Multiple Sclerosis: Implications of Obesity in Neuroinflammation. In *Advances in Neurobiology*; 2017; Vol. 19.
108. Jha, M.K.; Lee, W.H.; Suk, K. Functional Polarization of Neuroglia: Implications in Neuroinflammation and Neurological Disorders. *Biochemical Pharmacology* 2016, *103*.
109. Colombo, E.; Farina, C. Astrocytes: Key Regulators of Neuroinflammation. *Trends in Immunology* 2016, *37*.
110. Linnerbauer, M.; Wheeler, M.A.; Quintana, F.J. Astrocyte Crosstalk in CNS Inflammation. *Neuron* 2020, *108*.
111. de Souza, C.T.; Araujo, E.P.; Bordin, S.; Ashimine, R.; Zollner, R.L.; Boschero, A.C.; Saad, M.J.A.; Velloso, L.A. Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus. *Endocrinology* **2005**, *146*, doi:10.1210/en.2004-1520.
112. Milanski, M.; Degasperi, G.; Coope, A.; Morari, J.; Denis, R.; Cintra, D.E.; Tsukumo, D.M.L.; Anhe, G.; Amaral, M.E.; Takahashi, H.K.; et al. Saturated Fatty Acids Produce an Inflammatory Response Predominantly through the Activation of TLR4 Signaling in Hypothalamus: Implications for the Pathogenesis of Obesity. *Journal of Neuroscience* **2009**, *29*, doi:10.1523/JNEUROSCI.2760-08.2009.
113. Posey, K.A.; Clegg, D.J.; Printz, R.L.; Byun, J.; Morton, G.J.; Vivekanandan-Giri, A.; Pennathur, S.; Baskin, D.G.; Heinecke, J.W.; Woods, S.C.; et al. Hypothalamic Proinflammatory Lipid Accumulation, Inflammation, and Insulin Resistance in Rats Fed a

High-Fat Diet. *American Journal of Physiology - Endocrinology and Metabolism* **2009**, *296*, doi:10.1152/ajpendo.90377.2008.

114. Kleinridders, A.; Schenten, D.; Könner, A.C.; Belgardt, B.F.; Mauer, J.; Okamura, T.; Wunderlich, F.T.; Medzhitov, R.; Brüning, J.C. MyD88 Signaling in the CNS Is Required for Development of Fatty Acid-Induced Leptin Resistance and Diet-Induced Obesity. *Cell Metabolism* **2009**, *10*, doi:10.1016/j.cmet.2009.08.013.
115. Grayson, B.E.; Levasseur, P.R.; Williams, S.M.; Smith, M.S.; Marks, D.L.; Grove, K.L. Changes in Melanocortin Expression and Inflammatory Pathways in Fetal Offspring of Nonhuman Primates Fed a High-Fat Diet. *Endocrinology* **2010**, *151*, doi:10.1210/en.2009-1019.
116. Valdearcos, M.; Robblee, M.M.; Benjamin, D.I.; Nomura, D.K.; Xu, A.W.; Koliwad, S.K. Microglia Dictate the Impact of Saturated Fat Consumption on Hypothalamic Inflammation and Neuronal Function. *Cell Reports* **2014**, *9*, doi:10.1016/j.celrep.2014.11.018.
117. Weissmann, L.; Quaresma, P.G.F.; Santos, A.C.; de Matos, A.H.B.; Pascoal, V.D.Á.B.; Zanotto, T.M.; Castro, G.; Guadagnini, D.; Silva, J.M. da; Velloso, L.A.; et al. IKKε Is Key to Induction of Insulin Resistance in the Hypothalamus, and Its Inhibition Reverses Obesity. *Diabetes* **2014**, *63*, doi:10.2337/db13-1817.
118. Maric, T.; Woodside, B.; Luheshi, G.N. The Effects of Dietary Saturated Fat on Basal Hypothalamic Neuroinflammation in Rats. *Brain, Behavior, and Immunity* **2014**, *36*, doi:10.1016/j.bbi.2013.09.011.
119. Thaler, J.P.; Yi, C.X.; Schur, E.A.; Guyenet, S.J.; Hwang, B.H.; Dietrich, M.O.; Zhao, X.; Sarruf, D.A.; Izgur, V.; Maravilla, K.R.; et al. Obesity Is Associated with Hypothalamic Injury in Rodents and Humans. *Journal of Clinical Investigation* **2012**, *122*, doi:10.1172/JCI59660.

120. Ziko, I.; de Luca, S.; Dinan, T.; Barwood, J.M.; Sominsky, L.; Cai, G.; Kenny, R.; Stokes, L.; Jenkins, T.A.; Spencer, S.J. Neonatal Overfeeding Alters Hypothalamic Microglial Profiles and Central Responses to Immune Challenge Long-Term. *Brain, Behavior, and Immunity* **2014**, *41*, doi:10.1016/j.bbi.2014.06.014.

121. Wee, Y.S.; Weis, J.J.; Gahring, L.C.; Rogers, S.W.; Weis, J.H. Age-Related Onset of Obesity Corresponds with Metabolic Dysregulation and Altered Microglia Morphology in Mice Deficient for Ifitm Proteins. *PLoS ONE* **2015**, *10*, doi:10.1371/journal.pone.0123218.

122. Hu, P.; Thinschmidt, J.S.; Yan, Y.; Hazra, S.; Bhatwadekar, A.; Caballero, S.; Salazar, T.; Miyan, J.A.; Li, W.; Derbenev, A.; et al. CNS Inflammation and Bone Marrow Neuropathy in Type 1 Diabetes. *American Journal of Pathology* **2013**, *183*, doi:10.1016/j.ajpath.2013.07.009.

123. Hu, P.; Thinschmidt, J.S.; Caballero, S.; Adamson, S.; Cole, L.; Chan-Ling, T.; Grant, M.B. Loss of Survival Factors and Activation of Inflammatory Cascades in Brain Sympathetic Centers in Type 1 Diabetic Mice. *American Journal of Physiology - Endocrinology and Metabolism* **2015**, *308*, doi:10.1152/ajpendo.00504.2014.

124. Baquedano, E.; Ruiz-Lopez, A.M.; Sustarsic, E.G.; Herpy, J.; List, E.O.; Chowen, J.A.; Frago, L.M.; Kopchick, J.J.; Argente, J. The Absence of GH Signaling Affects the Susceptibility to High-Fat Diet-Induced Hypothalamic Inflammation in Male Mice. *Endocrinology* **2014**, *155*, doi:10.1210/en.2014-1367.

125. Naznin, F.; Toshinai, K.; Waise, T.M.Z.; NamKoong, C.; Md Moin, A.S.; Sakoda, H.; Nakazato, M. Diet-Induced Obesity Causes Peripheral and Central Ghrelin Resistance by Promoting

Inflammation. *Journal of Endocrinology* **2015**, *226*, doi:10.1530/JOE-15-0139.

126. Pistell, P.J.; Morrison, C.D.; Gupta, S.; Knight, A.G.; Keller, J.N.; Ingram, D.K.; Bruce-Keller, A.J. Cognitive Impairment Following High Fat Diet Consumption Is Associated with Brain Inflammation. *Journal of Neuroimmunology* **2010**, *219*, doi:10.1016/j.jneuroim.2009.11.010.
127. Jeon, B.T.; Jeong, E.A.; Shin, H.J.; Lee, Y.; Lee, D.H.; Kim, H.J.; Kang, S.S.; Cho, G.J.; Choi, W.S.; Roh, G.S. Resveratrol Attenuates Obesity-Associated Peripheral and Central Inflammation and Improves Memory Deficit in Mice Fed a High-Fat Diet. *Diabetes* **2012**, *61*, doi:10.2337/db11-1498.
128. Purkayastha, S.; Zhang, G.; Cai, D. Uncoupling the Mechanisms of Obesity and Hypertension by Targeting Hypothalamic IKK-Î 2 and NF-Î°B. *Nature Medicine* **2011**, *17*, doi:10.1038/nm.2372.
129. Milanski, M.; Arruda, A.P.; Coope, A.; Ignacio-Souza, L.M.; Nunez, C.E.; Roman, E.A.; Romanatto, T.; Pascoal, L.B.; Caricilli, A.M.; Torsoni, M.A.; et al. Inhibition of Hypothalamic Inflammation Reverses Diet-Induced Insulin Resistance in the Liver. *Diabetes* **2012**, *61*, doi:10.2337/db11-0390.
130. Calegari, V.C.; Torsoni, A.S.; Vanzela, E.C.; Araú, E.P.; Morari, J.; Zoppi, C.C.; Sbragia, L.; Boschero, A.C.; Velloso, L.A. Inflammation of the Hypothalamus Leads to Defective Pancreatic Islet Function. *Journal of Biological Chemistry* **2011**, *286*, doi:10.1074/jbc.M110.173021.
131. Marques, C.G.; dos Santos Quaresma, M.V.L.; Nakamoto, F.P.; Magalhães, A.C.O.; Lucin, G.A.; Thomatieli-Santos, R.V. Does Modern Lifestyle Favor Neuroimmunometabolic Changes? A Path to Obesity. *Frontiers in Nutrition* 2021, *8*.

132. Lu, J.; Wu, D. mei; Zheng, Y. lin; Hu, B.; Cheng, W.; Zhang, Z. feng; Shan, Q. Ursolic Acid Improves High Fat Diet-Induced Cognitive Impairments by Blocking Endoplasmic Reticulum Stress and IκB Kinase β/Nuclear Factor-KB-Mediated Inflammatory Pathways in Mice. *Brain, Behavior, and Immunity* **2011**, *25*, doi:10.1016/j.bbi.2011.06.009.

133. Beilharz, J.E.; Maniam, J.; Morris, M.J. Short-Term Exposure to a Diet High in Fat and Sugar, or Liquid Sugar, Selectively Impairs Hippocampal-Dependent Memory, with Differential Impacts on Inflammation. *Behavioural Brain Research* **2016**, *306*, doi:10.1016/j.bbr.2016.03.018.

134. Guillemot-Legris, O.; Masquelier, J.; Everard, A.; Cani, P.D.; Alhouayek, M.; Muccioli, G.G. High-Fat Diet Feeding Differentially Affects the Development of Inflammation in the Central Nervous System. *Journal of Neuroinflammation* **2016**, *13*, doi:10.1186/s12974-016-0666-8.

135. Tapia-González, S.; García-Segura, L.M.; Tena-Sempere, M.; Frago, L.M.; Castellano, J.M.; Fuente-Martín, E.; García-Cáceres, C.; Argente, J.; Chowen, J.A. Activation of Microglia in Specific Hypothalamic Nuclei and the Cerebellum of Adult Rats Exposed to Neonatal Overnutrition. *Journal of Neuroendocrinology* **2011**, *23*, doi:10.1111/j.1365-2826.2011.02113.x.

136. Dutheil, S.; Ota, K.T.; Wohleb, E.S.; Rasmussen, K.; Duman, R.S. High-Fat Diet Induced Anxiety and Anhedonia: Impact on Brain Homeostasis and Inflammation. *Neuropsychopharmacology* **2016**, *41*, doi:10.1038/npp.2015.357.

137. Kang, E.B.; Koo, J.H.; Jang, Y.C.; Yang, C.H.; Lee, Y.; Cosio-Lima, L.M.; Cho, J.Y. Neuroprotective Effects of Endurance Exercise

Against High-Fat Diet-Induced Hippocampal Neuroinflammation. *Journal of Neuroendocrinology* **2016**, *28*, doi:10.1111/jne.12385.

138. Miao, Y.; Ren, J.; Jiang, L.; Liu, J.; Jiang, B.; Zhang, X. α-Lipoic Acid Attenuates Obesity-Associated Hippocampal Neuroinflammation and Increases the Levels of Brain-Derived Neurotrophic Factor in Ovariectomized Rats Fed a High-Fat Diet. *International Journal of Molecular Medicine* **2013**, *32*, doi:10.3892/ijmm.2013.1482.
139. Cai, M.; Wang, H.; Li, J. jing; Zhang, Y.L.; Xin, L.; Li, F.; Lou, S. jie The Signaling Mechanisms of Hippocampal Endoplasmic Reticulum Stress Affecting Neuronal Plasticity-Related Protein Levels in High Fat Diet-Induced Obese Rats and the Regulation of Aerobic Exercise. *Brain, Behavior, and Immunity* **2016**, *57*, doi:10.1016/j.bbi.2016.05.010.
140. Tucsek, Z.; Toth, P.; Sosnowska, D.; Gautam, T.; Mitschelen, M.; Koller, A.; Szalai, G.; Sonntag, W.E.; Ungvari, Z.; Csiszar, A. Obesity in Aging Exacerbates Blood-Brain Barrier Disruption, Neuroinflammation, and Oxidative Stress in the Mouse Hippocampus: Effects on Expression of Genes Involved in Beta-Amyloid Generation and Alzheimer's Disease. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* **2014**, *69*, doi:10.1093/gerona/glt177.
141. Grayson, B.E.; Fitzgerald, M.F.; Hakala-Finch, A.P.; Ferris, V.M.; Begg, D.P.; Tong, J.; Woods, S.C.; Seeley, R.J.; Davidson, T.L.; Benoit, S.C. Improvements in Hippocampal-Dependent Memory and Microglial Infiltration with Calorie Restriction and Gastric Bypass Surgery, but Not with Vertical Sleeve Gastrectomy. *International Journal of Obesity* **2014**, *38*, doi:10.1038/ijo.2013.100.
142. Rivera, P.; Pérez-Martín, M.; Pavón, F.J.; Serrano, A.; Crespillo, A.; Cifuentes, M.; López-Ávalos, M.D.; Grondona, J.M.; Vida, M.;

Fernández-Llebrez, P.; et al. Pharmacological Administration of the Isoflavone Daidzein Enhances Cell Proliferation and Reduces High Fat Diet-Induced Apoptosis and Gliosis in the Rat Hippocampus. *PLoS ONE* **2013**, *8*, doi:10.1371/journal.pone.0064750.

143. Christie, B.R.; Cameron, H.A. Neurogenesis in the Adult Hippocampus. *Hippocampus* **2006**, *16*, doi:10.1002/hipo.20151.
144. Reynolds, B.A.; Weiss, S. Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System. *Science* **1992**, *255*, doi:10.1126/science.1553558.
145. Lois, C.; Alvarez-Buylla, A. Long-Distance Neuronal Migration in the Adult Mammalian Brain. *Science* **1994**, *264*, doi:10.1126/science.8178174.
146. Gage, F.H. Mammalian Neural Stem Cells. *Science* 2000, *287*.
147. Kriegstein, A.; Alvarez-Buylla, A. The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annual Review of Neuroscience* 2009, *32*.
148. Lindsey, B.W.; Tropepe, V. A Comparative Framework for Understanding the Biological Principles of Adult Neurogenesis. *Progress in Neurobiology* 2006, *80*.
149. Bonfanti, L.; Ponti, G. Adult Mammalian Neurogenesis and the New Zealand White Rabbit. *Veterinary Journal* 2008, *175*.
150. Grandel, H.; Brand, M. Comparative Aspects of Adult Neural Stem Cell Activity in Vertebrates. *Development Genes and Evolution* 2013, *223*.
151. Park, H.R.; Park, M.; Choi, J.; Park, K.Y.; Chung, H.Y.; Lee, J. A High-Fat Diet Impairs Neurogenesis: Involvement of Lipid Peroxidation and Brain-Derived Neurotrophic Factor. *Neuroscience Letters* **2010**, *482*, doi:10.1016/j.neulet.2010.07.046.

152. Ho, N.; Sommers, M.S.; Lucki, I. Effects of Diabetes on Hippocampal Neurogenesis: Links to Cognition and Depression. *Neuroscience and Biobehavioral Reviews* 2013, *37*.

153. Nam, S.M.; Kim, J.W.; Yoo, D.Y.; Jung, H.Y.; Choi, J.H.; Hwang, I.K.; Seong, J.K.; Yoon, Y.S. Reduction of Adult Hippocampal Neurogenesis Is Amplified by Aluminum Exposure in a Model of Type 2 Diabetes. *Journal of Veterinary Science* **2016**, *17*, doi:10.4142/jvs.2016.17.1.13.

154. Nam, S.M.; Kim, J.W.; Kwon, H.J.; Yoo, D.Y.; Jung, H.Y.; Kim, D.W.; Hwang, I.K.; Seong, J.K.; Yoon, Y.S. Differential Effects of Low- and High-Dose Zinc Supplementation on Synaptic Plasticity and Neurogenesis in the Hippocampus of Control and High-Fat Diet-Fed Mice. *Neurochemical Research* **2017**, *42*, doi:10.1007/s11064-017-2353-2.

155. Tang, S.S.; Ren, Y.; Ren, X.Q.; Cao, J.R.; Hong, H.; Ji, H.; Hu, Q.H. ERα and/or ERβ Activation Ameliorates Cognitive Impairment, Neurogenesis and Apoptosis in Type 2 Diabetes Mellitus Mice. *Experimental Neurology* **2019**, *311*, doi:10.1016/j.expneurol.2018.09.002.

156. Tanokashira, D.; Kurata, E.; Fukuokaya, W.; Kawabe, K.; Kashiwada, M.; Takeuchi, H.; Nakazato, M.; Taguchi, A. Metformin Treatment Ameliorates Diabetes-Associated Decline in Hippocampal Neurogenesis and Memory via Phosphorylation of Insulin Receptor Substrate 1. *FEBS Open Bio* **2018**, *8*, doi:10.1002/2211-5463.12436.

157. Boitard, C.; Etchamendy, N.; Sauvant, J.; Aubert, A.; Tronel, S.; Marighetto, A.; Layé, S.; Ferreira, G. Juvenile, but Not Adult Exposure to High-Fat Diet Impairs Relational Memory and Hippocampal Neurogenesis in Mice. *Hippocampus* **2012**, *22*, doi:10.1002/hipo.22032.

158. Gault, V.A.; Lennox, R.; Flatt, P.R. Sitagliptin, a Dipeptidyl Peptidase-4 Inhibitor, Improves Recognition Memory, Oxidative Stress and

Hippocampal Neurogenesis and Upregulates Key Genes Involved in Cognitive Decline. *Diabetes, Obesity and Metabolism* **2015**, *17*, doi:10.1111/dom.12432.

159. Hwang, I.K.; Kim, I.Y.; Kim, D.W.; Yoo, K.Y.; Kim, Y.N.; Yi, S.S.; Won, M.H.; Lee, I.S.; Yoon, Y.S.; Seong, J.K. Strain-Specific Differences in Cell Proliferation and Differentiation in the Dentate Gyrus of C57BL/6N and C3H/HeN Mice Fed a High Fat Diet. *Brain Research* **2008**, *1241*, doi:10.1016/j.brainres.2008.08.024.
160. Pathak, N.M.; Pathak, V.; Lynch, A.M.; Irwin, N.; Gault, V.A.; Flatt, P.R. Stable Oxyntomodulin Analogues Exert Positive Effects on Hippocampal Neurogenesis and Gene Expression as Well as Improving Glucose Homeostasis in High Fat Fed Mice. *Molecular and Cellular Endocrinology* **2015**, *412*, doi:10.1016/j.mce.2015.05.035.
161. Vinuesa, A.; Pomilio, C.; Menafra, M.; Bonaventura, M.M.; Garay, L.; Mercogliano, M.F.; Schillaci, R.; Lux Lantos, V.; Brites, F.; Beauquis, J.; et al. Juvenile Exposure to a High Fat Diet Promotes Behavioral and Limbic Alterations in the Absence of Obesity. *Psychoneuroendocrinology* **2016**, *72*, doi:10.1016/j.psyneuen.2016.06.004.
162. Yoo, D.Y.; Choi, J.H.; Kim, W.; Yoo, K.Y.; Lee, C.H.; Yoon, Y.S.; Won, M.H.; Hwang, I.K. Effects of Melissa Officinalis L. (Lemon Balm) Extract on Neurogenesis Associated with Serum Corticosterone and GABA in the Mouse Dentate Gyrus. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-010-0312-2.
163. Yoo, D.Y.; Kim, W.; Kim, D.W.; Yoo, K.Y.; Chung, J.Y.; Youn, H.Y.; Yoon, Y.S.; Choi, S.Y.; Won, M.H.; Hwang, I.K. Pyridoxine Enhances Cell Proliferation and Neuroblast Differentiation by Upregulating the

GABaergic System in the Mouse Dentate Gyrus. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-010-0385-y.

164. Yoo, D.Y.; Kim, W.; Nam, S.M.; Yoo, K.Y.; Lee, C.H.; Choi, J.H.; Won, M.H.; Hwang, I.K.; Yoon, Y.S. Reduced Cell Proliferation and Neuroblast Differentiation in the Dentate Gyrus of High Fat Diet-Fed Mice Are Ameliorated by Metformin and Glimepiride Treatment. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-011-0566-3.
165. Yoo, D.Y.; Nam, S.M.; Kim, W.; Lee, C.H.; Won, M.H.; Hwang, I.K.; Yoon, Y.S. N-Acetylserotonin Increases Cell Proliferation and Differentiating Neuroblasts with Tertiary Dendrites through Upregulation of Brain-Derived Neurotrophic Factor in the Mouse Dentate Gyrus. *Journal of Veterinary Medical Science* **2011**, *73*, doi:10.1292/jvms.11-0123.
166. Yoo, D.Y.; Kim, W.; Kim, D.W.; Nam, S.M.; Jung, H.Y.; Kim, J.W.; Lee, C.H.; Choi, J.H.; Won, M.H.; Yoon, Y.S.; et al. Cell Proliferation and Neuroblast Differentiation in the Dentate Gyrus of High-Fat Diet-Fed Mice Are Increased after Rosiglitazone Treatment. *Journal of Veterinary Science* **2014**, *15*, doi:10.4142/jvs.2014.15.1.27.
167. Yoo, D.Y.; Kim, W.; Yoo, K.Y.; Nam, S.M.; Chung, J.Y.; Yoon, Y.S.; Won, M.H.; Hwang, I.K. Effects of Pyridoxine on a High-Fat Diet-Induced Reduction of Cell Proliferation and Neuroblast Differentiation Depend on Cyclic Adenosine Monophosphate Response Element Binding Protein in the Mouse Dentate Gyrus. *Journal of Neuroscience Research* **2012**, *90*, doi:10.1002/jnr.23035.
168. Klein, C.; Jonas, W.; Iggena, D.; Empl, L.; Rivalan, M.; Wiedmer, P.; Spranger, J.; Hellweg, R.; Winter, Y.; Steiner, B. Exercise Prevents High-Fat Diet-Induced Impairment of Flexible Memory Expression in the Water Maze and Modulates Adult Hippocampal Neurogenesis in

Mice. *Neurobiology of Learning and Memory* **2016**, *131*, doi:10.1016/j.nlm.2016.03.002.

169. Hamilton, A.; Patterson, S.; Porter, D.; Gault, V.A.; Holscher, C. Novel GLP-1 Mimetics Developed to Treat Type 2 Diabetes Promote Progenitor Cell Proliferation in the Brain. *Journal of Neuroscience Research* **2011**, *89*, doi:10.1002/jnr.22565.
170. Murata, Y.; Narisawa, Y.; Shimono, R.; Ohmori, H.; Mori, M.; Ohe, K.; Mine, K.; Enjoji, M. A High Fat Diet-Induced Decrease in Hippocampal Newly-Born Neurons of Male Mice Is Exacerbated by Mild Psychological Stress Using a Communication Box. *Journal of Affective Disorders* **2017**, *209*, doi:10.1016/j.jad.2016.11.046.
171. Chow, B.W.; Gu, C. The Molecular Constituents of the Blood-Brain Barrier. *Trends in Neurosciences* 2015, *38*.
172. Daneman, R. The Blood-Brain Barrier in Health and Disease. *Annals of Neurology* **2012**, *72*, doi:10.1002/ana.23648.
173. Zlokovic, B. v. The Blood-Brain Barrier in Health and Chronic Neurodegenerative Disorders. *Neuron* 2008, *57*.
174. Peng, Z.; Shu, B.; Zhang, Y.; Wang, M. Endothelial Response to Pathophysiological Stress. *Arteriosclerosis, thrombosis, and vascular biology* 2019, *39*.
175. Bayir, E.; Sendemir, A. Role of Intermediate Filaments in Blood–Brain Barrier in Health and Disease. *Cells* 2021, *10*.
176. Prasain, N.; Stevens, T. The Actin Cytoskeleton in Endothelial Cell Phenotypes. *Microvascular Research* **2009**, *77*, doi:10.1016/j.mvr.2008.09.012.
177. Shahzad, K.A.; Qin, Z.; Li, Y.; Xia, D. The Roles of Focal Adhesion and Cytoskeleton Systems in Fluid Shear Stress-Induced Endothelial Cell Response. *Biocell* **2020**, *44*, doi:10.32604/biocell.2020.08500.

178. Ramos, I.; Stamatakis, K.; Oeste, C.L.; Pérez-Sala, D. Vimentin as a Multifaceted Player and Potential Therapeutic Target in Viral Infections. *International Journal of Molecular Sciences* 2020, *21*.

179. Päll, T.; Pink, A.; Kasak, L.; Turkina, M.; Anderson, W.; Valkna, A.; Kogerman, P. Soluble CD44 Interacts with Intermediate Filament Protein Vimentin on Endothelial Cell Surface. *PLoS ONE* **2011**, *6*, doi:10.1371/journal.pone.0029305.

180. Reese, T.S.; Karnovsky, M.J. Fine Structural Localization of a Blood-Brain Barrier to Exogenous Peroxidase. *The Journal of cell biology* **1967**, *34*, doi:10.1083/jcb.34.1.207.

181. Brightman, M.W.; Reese, T.S. Junctions between Intimately Apposed Cell Membranes in the Vertebrate Brain. *The Journal of cell biology* **1969**, *40*, doi:10.1083/jcb.40.3.648.

182. Westergaard, E.; Brightman, M.W. Transport of Proteins across Normal Cerebral Arterioles. *Journal of Comparative Neurology* **1973**, *152*, doi:10.1002/cne.901520103.

183. Daneman, R.; Prat, A. The Blood–Brain Barrier. *Cold Spring Harbor Perspectives in Biology* **2015**, *7*, doi:10.1101/cshperspect.a020412.

184. Ballabh, P.; Braun, A.; Nedergaard, M. The Blood-Brain Barrier: An Overview: Structure, Regulation, and Clinical Implications. *Neurobiology of Disease* 2004, *16*.

185. del Zoppo, G.J.; Milner, R.; Mabuchi, T.; Hung, S.; Wang, X.; Koziol, J.A. Vascular Matrix Adhesion and the Blood-Brain Barrier. In Proceedings of the Biochemical Society Transactions; 2006; Vol. 34.

186. Sorokin, L. The Impact of the Extracellular Matrix on Inflammation. *Nature Reviews Immunology* 2010, *10*.

187. Abbott, N.J.; Rönnbäck, L.; Hansson, E. Astrocyte-Endothelial Interactions at the Blood-Brain Barrier. *Nature Reviews Neuroscience* 2006, *7*.

188. Attwell, D.; Buchan, A.M.; Charpak, S.; Lauritzen, M.; MacVicar, B.A.; Newman, E.A. Glial and Neuronal Control of Brain Blood Flow. *Nature* 2010, *468*.

189. Gordon, G.R.J.; Howarth, C.; Macvicar, B.A. Bidirectional Control of Arteriole Diameter by Astrocytes. In Proceedings of the Experimental Physiology; 2011; Vol. 96.

190. Sims, D.E. The Pericyte-A Review. *Tissue and Cell* 1986, *18*.

191. Armulik, A.; Genové, G.; Betsholtz, C. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Developmental Cell* 2011, *21*.

192. Armulik, A.; Genové, G.; Mäe, M.; Nisancioglu, M.H.; Wallgard, E.; Niaudet, C.; He, L.; Norlin, J.; Lindblom, P.; Strittmatter, K.; et al. Pericytes Regulate the Blood-Brain Barrier. *Nature* **2010**, *468*, doi:10.1038/nature09522.

193. Daneman, R.; Zhou, L.; Kebede, A.A.; Barres, B.A. Pericytes Are Required for Bloodĝ€"brain Barrier Integrity during Embryogenesis. *Nature* **2010**, *468*, doi:10.1038/nature09513.

194. Al-Kaabi, M.; Hussam, F.; Al-Marsoummi, S.; Al-Anbaki, A.; Al-Salihi, A.; Al-Aubaidy, H. Expression of ZO1, Vimentin, Pan-Cadherin and AGTR1 in Tanycyte-like Cells of the Sulcus Medianus Organum. *Biochemical and Biophysical Research Communications* **2018**, *502*, doi:10.1016/j.bbrc.2018.05.151.

195. Collins, P. The Sulcus Medianus Organ in the Fourth Cerebral Ventricle of the Rabbit: A Previously Undescribed Circumventricular Organ. *Journal of Anatomy* **1989**, *162*.

196. Fry, M.; Ferguson, A. v. The Sensory Circumventricular Organs: Brain Targets for Circulating Signals Controlling Ingestive Behavior. *Physiology and Behavior* **2007**, *91*, doi:10.1016/j.physbeh.2007.04.003.

197. Langlet, F.; Mullier, A.; Bouret, S.G.; Prevot, V.; Dehouck, B. Tanycyte-like Cells Form a Blood-Cerebrospinal Fluid Barrier in the Circumventricular Organs of the Mouse Brain. *Journal of Comparative Neurology* **2013**, *521*, doi:10.1002/cne.23355.

198. Mullier, A.; Bouret, S.G.; Prevot, V.; Dehouck, B. Differential Distribution of Tight Junction Proteins Suggests a Role for Tanycytes in Blood-Hypothalamus Barrier Regulation in the Adult Mouse Brain. *Journal of Comparative Neurology* **2010**, *518*, doi:10.1002/cne.22273.

199. Mirzadeh, Z.; Kusne, Y.; Duran-Moreno, M.; Cabrales, E.; Gil-Perotin, S.; Ortiz, C.; Chen, B.; Garcia-Verdugo, J.M.; Sanai, N.; Alvarez-Buylla, A. Bi- and Uniciliated Ependymal Cells Define Continuous Floor-Plate-Derived Tanycytic Territories. *Nature Communications* **2017**, *8*, doi:10.1038/ncomms13759.

200. Nerurkar, P. v.; Johns, L.M.; Buesa, L.M.; Kipyakwai, G.; Volper, E.; Sato, R.; Shah, P.; Feher, D.; Williams, P.G.; Nerurkar, V.R. Momordica Charantia (Bitter Melon) Attenuates High-Fat Diet-Associated Oxidative Stress and Neuroinflammation. *Journal of Neuroinflammation* **2011**, *8*, doi:10.1186/1742-2094-8-64.

201. Elahy, M.; Lam, V.; Pallebage-Gamarallage, M.M.; Giles, C.; Mamo, J.C.L.; Takechi, R. Nicotine Attenuates Disruption of Blood-Brain Barrier Induced by Saturated-Fat Feeding in Wild-Type Mice. *Nicotine and Tobacco Research* **2015**, *17*, doi:10.1093/ntr/ntv044.

202. Takechi, R.; Pallebage-Gamarallage, M.M.; Lam, V.; Giles, C.; Mamo, J.C. Nutraceutical Agents with Anti-Inflammatory Properties Prevent Dietary Saturated-Fat Induced Disturbances in Blood-Brain Barrier

Function in Wild-Type Mice. *Journal of Neuroinflammation* **2013**, *10*, doi:10.1186/1742-2094-10-73.

203. Ouyang, S.; Hsuchou, H.; Kastin, A.J.; Wang, Y.; Yu, C.; Pan, W. Diet-Induced Obesity Suppresses Expression of Many Proteins at the Blood-Brain Barrier. *Journal of Cerebral Blood Flow and Metabolism* **2014**, *34*, doi:10.1038/jcbfm.2013.166.
204. Severi, I.; Fosca, M.; Colleluori, G.; Marini, F.; Imperatori, L.; Senzacqua, M.; di Vincenzo, A.; Barbatelli, G.; Fiori, F.; Rau, J. v.; et al. High-Fat Diet Impairs Mouse Median Eminence: A Study by Transmission and Scanning Electron Microscopy Coupled with Raman Spectroscopy. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22158049.
205. Rask-Madsen, C.; Kahn, C.R. Tissue-Specific Insulin Signaling, Metabolic Syndrome, and Cardiovascular Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2012**, *32*, doi:10.1161/ATVBAHA.111.241919.
206. Giudetti, A.M.; Micioni Di Bonaventura, M.V.; Ferramosca, A.; Longo, S.; Micioni Di Bonaventura, E.; Friuli, M.; Romano, A.; Gaetani, S.; Cifani, C. Brief Daily Access to Cafeteria-Style Diet Impairs Hepatic Metabolism Even in the Absence of Excessive Body Weight Gain in Rats. *FASEB Journal* **2020**, doi:10.1096/fj.201902757R.
207. Younossi, Z.M.; Koenig, A.B.; Abdelatif, D.; Fazel, Y.; Henry, L.; Wymer, M. Global Epidemiology of Nonalcoholic Fatty Liver Disease—Meta-Analytic Assessment of Prevalence, Incidence, and Outcomes. *Hepatology* **2016**, *64*, doi:10.1002/hep.28431.
208. Younossi, Z.; Anstee, Q.M.; Marietti, M.; Hardy, T.; Henry, L.; Eslam, M.; George, J.; Bugianesi, E. Global Burden of NAFLD and NASH:

Trends, Predictions, Risk Factors and Prevention. *Nature Reviews Gastroenterology and Hepatology* 2018, *15*.

209. Hoyumpa, A.M.; Greene, H.L.; Dunn, G.D.; Schenker, S. Fatty Liver: Biochemical and Clinical Considerations. *The American Journal of Digestive Diseases* **1975**, *20*, doi:10.1007/BF01070758.
210. Kleiner, D.E.; Brunt, E.M.; van Natta, M.; Behling, C.; Contos, M.J.; Cummings, O.W.; Ferrell, L.D.; Liu, Y.C.; Torbenson, M.S.; Unalp-Arida, A.; et al. Design and Validation of a Histological Scoring System for Nonalcoholic Fatty Liver Disease. *Hepatology* **2005**, *41*, doi:10.1002/hep.20701.
211. Kim, C.H.; Younossi, Z.M. Nonalcoholic Fatty Liver Disease: A Manifestation of the Metabolic Syndrome. *Cleveland Clinic Journal of Medicine* 2008, *75*.
212. Wang, X.; Rao, H.; Liu, F.; Wei, L.; Li, H.; Wu, C. Recent Advances in Adipose Tissue Dysfunction and Its Role in the Pathogenesis of Non-Alcoholic Fatty Liver Disease. *Cells* 2021, *10*.
213. Desvergne, B.; Wahli, W. Peroxisome Proliferator-Activated Receptors: Nuclear Control of Metabolism. *Endocrine Reviews* 1999.
214. Guijarro, A.; Fu, J.; Astarita, G.; Piomelli, D. CD36 Gene Deletion Decreases Oleoylethanolamide Levels in Small Intestine of Free-Feeding Mice. *Pharmacological Research* **2010**, doi:10.1016/j.phrs.2009.09.003.
215. Sun, Y.; Alexander, S.P.H.; Garle, M.J.; Gibson, C.L.; Hewitt, K.; Murphy, S.P.; Kendall, D.A.; Bennett, A.J. Cannabinoid Activation of PPARα; a Novel Neuroprotective Mechanism. *British Journal of Pharmacology* **2007**, *152*, 734–743, doi:10.1038/sj.bjp.0707478.
216. Sayd, A.; Antón, M.; Alén, F.; Caso, J.R.; Pavón, J.; Leza, J.C.; de Fonseca, F.R.; García-Bueno, B.; Orio, L. Systemic Administration of

Oleoylethanolamide Protects from Neuroinflammation and Anhedonia Induced by LPS in Rats. *International Journal of Neuropsychopharmacology* **2015**, doi:10.1093/ijnp/pyu111.

217. Lee, Y.K.; Park, J.E.; Lee, M.; Hardwick, J.P. Hepatic Lipid Homeostasis by Peroxisome Proliferator-Activated Receptor Gamma 2. *Liver Research* 2018.
218. Zhang, Y.L.; Hernandez-Ono, A.; Siri, P.; Weisberg, S.; Conlon, D.; Graham, M.J.; Crooke, R.M.; Huang, L.S.; Ginsberg, H.N. Aberrant Hepatic Expression of PPARγ2 Stimulates Hepatic Lipogenesis in a Mouse Model of Obesity, Insulin Resistance, Dyslipidemia, and Hepatic Steatosis. *Journal of Biological Chemistry* **2006**, doi:10.1074/jbc.M604709200.
219. Yamazaki, T.; Shiraishi, S.; Kishimoto, K.; Miura, S.; Ezaki, O. An Increase in Liver PPARγ2 Is an Initial Event to Induce Fatty Liver in Response to a Diet High in Butter: PPARγ2 Knockdown Improves Fatty Liver Induced by High-Saturated Fat. *Journal of Nutritional Biochemistry* **2011**, doi:10.1016/j.jnutbio.2010.04.009.
220. Lee, Y.J.; Ko, E.H.; Kim, J.E.; Kim, E.; Lee, H.; Choi, H.; Yu, J.H.; Kim, H.J.; Seong, J.K.; Kim, K.S.; et al. Nuclear Receptor PPARγ-Regulated Monoacylglycerol O-Acyltransferase 1 (MGAT1) Expression Is Responsible for the Lipid Accumulation in Diet-Induced Hepatic Steatosis. *Proceedings of the National Academy of Sciences of the United States of America* **2012**, doi:10.1073/pnas.1203218109.
221. Bonen, A.; Parolin, M.L.; Steinberg, G.R.; Calles-Escandon, J.; Tandon, N.N.; Glatz, J.F.C.; Luiken, J.J.F.P.; Heigenhauser, George.J.F.; Dyck, D.J. Triacylglycerol Accumulation in Human Obesity and Type 2 Diabetes Is Associated with Increased Rates of Skeletal Muscle Fatty

Acid Transport and Increased Sarcolemmal FAT/CD36. *The FASEB Journal* **2004**, doi:10.1096/fj.03-1065fje.

222. Su, X.; Abumrad, N.A. Cellular Fatty Acid Uptake: A Pathway under Construction. *Trends in Endocrinology and Metabolism* 2009.
223. Koonen, D.P.Y.; Jacobs, R.L.; Febbraio, M.; Young, M.E.; Soltys, C.L.M.; Ong, H.; Vance, D.E.; Dyck, J.R.B. Increased Hepatic CD36 Expression Contributes to Dyslipidemia Associated with Diet-Induced Obesity. *Diabetes* **2007**, doi:10.2337/db07-0907.
224. Zhou, J.; Febbraio, M.; Wada, T.; Zhai, Y.; Kuruba, R.; He, J.; Lee, J.H.; Khadem, S.; Ren, S.; Li, S.; et al. Hepatic Fatty Acid Transporter Cd36 Is a Common Target of LXR, PXR, and PPARγ in Promoting Steatosis. *Gastroenterology* **2008**, doi:10.1053/j.gastro.2007.11.037.
225. Hui, S.T.; Parks, B.W.; Org, E.; Norheim, F.; Che, N.; Pan, C.; Castellani, L.W.; Charugundla, S.; Dirks, D.L.; Psychogios, N.; et al. The Genetic Architecture of NAFLD among Inbred Strains of Mice. *eLife* **2015**, doi:10.7554/eLife.05607.
226. Kartha, G.K.; Moshal, K.S.; Sen, U.; Joshua, I.G.; Tyagi, N.; Steed, M.M.; Tyagi, S.C. Renal Mitochondrial Damage and Protein Modification in Type-2 Diabetes. *Acta Diabetologica* **2008**, *45*, doi:10.1007/s00592-008-0025-z.
227. Flessa, C.M.; Kyrou, I.; Nasiri-Ansari, N.; Kaltsas, G.; Papavassiliou, A.G.; Kassi, E.; Randeva, H.S. Endoplasmic Reticulum Stress and Autophagy in the Pathogenesis of Non-Alcoholic Fatty Liver Disease (NAFLD): Current Evidence and Perspectives. *Current Obesity Reports* 2021, *10*.
228. Lim, S.; Kim, J.W.; Targher, G. Links between Metabolic Syndrome and Metabolic Dysfunction-Associated Fatty Liver Disease. *Trends in Endocrinology and Metabolism* 2021, *32*.

229. di Meo, S.; Reed, T.T.; Venditti, P.; Victor, V.M. Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxidative Medicine and Cellular Longevity* 2016, *2016*.

230. Horn, C.L.; Morales, A.L.; Savard, C.; Farrell, G.C.; Ioannou, G.N. Role of Cholesterol-Associated Steatohepatitis in the Development of NASH. *Hepatology Communications* 2022, *6*.

231. McPherson, S.; Hardy, T.; Henderson, E.; Burt, A.D.; Day, C.P.; Anstee, Q.M. Evidence of NAFLD Progression from Steatosis to Fibrosing-Steatohepatitis Using Paired Biopsies: Implications for Prognosis and Clinical Management. *Journal of Hepatology* **2015**, *62*, doi:10.1016/j.jhep.2014.11.034.

232. Liao, C.Y.; Song, M.J.; Gao, Y.; Mauer, A.S.; Revzin, A.; Malhi, H. Hepatocyte-Derived Lipotoxic Extracellular Vesicle Sphingosine 1-Phosphate Induces Macrophage Chemotaxis. *Frontiers in Immunology* **2018**, *9*, doi:10.3389/fimmu.2018.02980.

233. Zhang, P.; Wang, W.; Mao, M.; Gao, R.; Shi, W.; Li, D.; Calderone, R.; Sui, B.; Tian, X.; Meng, X. Similarities and Differences: A Comparative Review of the Molecular Mechanisms and Effectors of NAFLD and AFLD. *Frontiers in Physiology* 2021, *12*.

234. Mota, M.; Banini, B.A.; Cazanave, S.C.; Sanyal, A.J. Molecular Mechanisms of Lipotoxicity and Glucotoxicity in Nonalcoholic Fatty Liver Disease. *Metabolism: Clinical and Experimental* **2016**, *65*, doi:10.1016/j.metabol.2016.02.014.

235. Nita, M.; Grzybowski, A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. *Oxidative Medicine and Cellular Longevity* 2016, *2016*.

236. Yoboue, E.D.; Sitia, R.; Simmen, T. Redox Crosstalk at Endoplasmic Reticulum (ER) Membrane Contact Sites (MCS) Uses Toxic Waste to Deliver Messages. *Cell Death and Disease* 2018, *9*.

237. Matsuzawa-Nagata, N.; Takamura, T.; Ando, H.; Nakamura, S.; Kurita, S.; Misu, H.; Ota, T.; Yokoyama, M.; Honda, M.; Miyamoto, K. ichi; et al. Increased Oxidative Stress Precedes the Onset of High-Fat Diet-Induced Insulin Resistance and Obesity. *Metabolism: Clinical and Experimental* **2008**, *57*, doi:10.1016/j.metabol.2008.03.010.

238. Sun, Y.; Ge, X.; Li, X.; He, J.; Wei, X.; Du, J.; Sun, J.; Li, X.; Xun, Z.; Liu, W.; et al. High-Fat Diet Promotes Renal Injury by Inducing Oxidative Stress and Mitochondrial Dysfunction. *Cell Death and Disease* **2020**, *11*, doi:10.1038/s41419-020-03122-4.

239. Marei, W.F.A.; Smits, A.; Mohey-Elsaeed, O.; Pintelon, I.; Ginneberge, D.; Bols, P.E.J.; Moerloose, K.; Leroy, J.L.M.R. Differential Effects of High Fat Diet-Induced Obesity on Oocyte Mitochondrial Functions in Inbred and Outbred Mice. *Scientific Reports* **2020**, *10*, doi:10.1038/s41598-020-66702-6.

240. Abdurrachim, D.; Ciapaite, J.; Wessels, B.; Nabben, M.; Luiken, J.J.F.P.; Nicolay, K.; Prompers, J.J. Cardiac Diastolic Dysfunction in High-Fat Diet Fed Mice Is Associated with Lipotoxicity without Impairment of Cardiac Energetics in Vivo. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **2014**, *1841*, doi:10.1016/j.bbalip.2014.07.016.

241. Mantena, S.K.; Vaughn, D.P.; Andringa, K.K.; Eccleston, H.B.; King, A.L.; Abrams, G.A.; Doeller, J.E.; Kraus, D.W.; Darley-Usmar, V.M.; Bailey, S.M. High Fat Diet Induces Dysregulation of Hepatic Oxygen Gradients and Mitochondrial Function in Vivo. *Biochemical Journal* **2009**, *417*, doi:10.1042/BJ20080868.

242. Cao, S.S.; Kaufman, R.J. Endoplasmic Reticulum Stress and Oxidative Stress in Cell Fate Decision and Human Disease. *Antioxidants and Redox Signaling* 2014, *21*.

243. Kuehl, F.A.; Jacob, T.A.; Ganley, O.H.; Ormond, R.E.; Meisinger, M.A.P. The Identification of N-(2-Hydroxyethyl)- Palmitamide as a Naturally Occurring Anti-Inflammatory Agent. *Journal of the American Chemical Society* 1957, *79*.

244. BACHUR, N.R.; MASEK, K.; MELMON, K.L.; UDENFRIEND, S. FATTY ACID AMIDES OF ETHANOLAMINE IN MAMMALIAN TISSUES. *The Journal of biological chemistry* **1965**, *240*, doi:10.1016/s0021-9258(18)97531-9.

245. Lambert, D.; Vandevoorde, S.; Jonsson, K.-O.; Fowler, C. The Palmitoylethanolamide Family: A New Class of Anti-Inflammatory Agents ? *Current Medicinal Chemistry* **2012**, *9*, doi:10.2174/0929867023370707.

246. Piomelli, D. The Molecular Logic of Endocannabinoid Signalling. *Nature Reviews Neuroscience* **2003**, *4*, doi:10.1038/nrn1247.

247. Rodríguez De Fonseca, F.; Navarro, M.; Gómez, R.; Escuredo, L.; Nava, F.; Fu, J.; Murillo-Rodríguez, E.; Giuffrida, A.; Loverme, J.; Gaetani, S.; et al. An Anorexic Lipid Mediator Regulated by Feeding. *Nature* **2001**, doi:10.1038/35102582.

248. Calignano, A.; la Rana, G.; Giuffrida, A.; Piomelli, D. Control of Pain Initiation by Endogenous Cannabinoids. *Nature* **1998**, *394*, doi:10.1038/28393.

249. LoVerme, J.; Russo, R.; la Rana, G.; Fu, J.; Farthing, J.; Mattace-Raso, G.; Meli, R.; Hohmann, A.; Calignano, A.; Piomelli, D. Rapid Broad-Spectrum Analgesia through Activation of Peroxisome Proliferator-

Activated Receptor-α. *Journal of Pharmacology and Experimental Therapeutics* **2006**, *319*, doi:10.1124/jpet.106.111385.

250. Sasso, O.; Moreno-Sanz, G.; Martucci, C.; Realini, N.; Dionisi, M.; Mengatto, L.; Duranti, A.; Tarozzo, G.; Tarzia, G.; Mor, M.; et al. Antinociceptive Effects of the N-Acylethanolamine Acid Amidase Inhibitor ARN077 in Rodent Pain Models. *Pain* **2013**, *154*, doi:10.1016/j.pain.2012.10.018.
251. Lo, V.J.; Fu, J.; Astarita, G.; la Rana, G.; Russo, R.; Calignano, A.; Piomelli, D. The Nuclear Receptor PPAR {alpha} Mediates the Antiinflammatory Actions of Palmitoylethanolamide. *Molecular Pharmacology* **2004**, .
252. Solorzano, C.; Zhu, C.; Battista, N.; Astarita, G.; Lodola, A.; Rivara, S.; Mor, M.; Russo, R.; Maccarrone, M.; Antonietti, F.; et al. Selective N-Acylethanolamine-Hydrolyzing Acid Amidase Inhibition Reveals a Key Role for Endogenous Palmitoylethanolamide in Inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **2009**, *106*, doi:10.1073/pnas.0907417106.
253. Melis, M.; Pillolla, G.; Luchicchi, A.; Muntoni, A.L.; Yasar, S.; Goldberg, S.R.; Pistis, M. Endogenous Fatty Acid Ethanolamides Suppress Nicotine-Induced Activation of Mesolimbic Dopamine Neurons through Nuclear Receptors. *Journal of Neuroscience* **2008**, *28*, doi:10.1523/JNEUROSCI.3221-08.2008.
254. Alhouayek, M.; Muccioli, G.G. Harnessing the Anti-Inflammatory Potential of Palmitoylethanolamide. *Drug Discovery Today* 2014, *19*, 1632–1639.
255. Keppel Hesselink, J.M.; Costagliola, C.; Fakhry, J.; Kopsky, D.J. Palmitoylethanolamide, a Natural Retinoprotectant: Its Putative

Relevance for the Treatment of Glaucoma and Diabetic Retinopathy. *Journal of Ophthalmology* 2015, *2015*.

256. Maccarrone, M.; Pauselli, R.; di Rienzo, M.; Finazzi-Agro, A. Binding, Degradation and Apoptotic Activity of Stearoylethanolamide in Rat C6 Glioma Cells. *Biochem J* **2002**, *366*, 137–144, doi:10.1042/BJ20020438.
257. Terrazzino, S.; Berto, F.; Dalle Carbonare, M.; Fabris, M.; Guiotto, a; Bernardini, D.; Leon, a Stearoylethanolamide Exerts Anorexic Effects in Mice via Down-Regulation of Liver Stearoyl-Coenzyme A Desaturase-1 MRNA Expression. *Faseb J* **2004**, *18*, 1580–1582, doi:10.1096/fj.03-1080fje.
258. Cravatt, B.F.; Demarest, K.; Patricelli, M.P.; Bracey, M.H.; Giang, D.K.; Martin, B.R.; Lichtman, A.H. Supersensitivity to Anandamide and Enhanced Endogenous Cannabinoid Signaling in Mice Lacking Fatty Acid Amide Hydrolase. *Proceedings of the National Academy of Sciences* **2001**, *98*, 9371–9376, doi:10.1073/pnas.161191698.
259. Devane, W.; Hanus, L.; Breuer, A.; Pertwee, R.; Stevenson, L.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science* **1992**, *258*, 1946–1949, doi:10.1126/science.1470919.
260. Gobbi, G.; Bambico, F.R.; Mangieri, R.; Bortolato, M.; Campolongo, P.; Solinas, M.; Cassano, T.; Morgese, M.G.; Debonnel, G.; Duranti, a; et al. Antidepressant-like Activity and Modulation of Brain Monoaminergic Transmission by Blockade of Anandamide Hydrolysis. *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *102*, 18620–18625, doi:10.1073/pnas.0509591102.
261. Kathuria, S.; Gaetani, S.; Fegley, D.; Valiño, F.; Duranti, A.; Tontini, A.; Mor, M.; Tarzia, G.; la Rana, G.; Calignano, A.; et al. Modulation

of Anxiety through Blockade of Anandamide Hydrolysis. *Nature Medicine* **2003**, *9*, 76–81, doi:10.1038/nm803.

262. Maccarrone, M.; Bab, I.; Bíró, T.; Cabral, G.A.; Dey, S.K.; di Marzo, V.; Konje, J.C.; Kunos, G.; Mechoulam, R.; Pacher, P.; et al. Endocannabinoid Signaling at the Periphery: 50 Years after THC. *Trends in Pharmacological Sciences* **2015**, *36*, 277–296, doi:10.1016/j.tips.2015.02.008.
263. Williams, C.M.; Kirkham, T.C. Anandamide Induces Overeating: Mediation by Central Cannabinoid (CB1) Receptors. *Psychopharmacology* **1999**, *143*, 315–317, doi:10.1007/s002130050953.
264. Fu, J.; Astarita, G.; Gaetani, S.; Kim, J.; Cravatt, B.F.; Mackie, K.; Piomelli, D. Food Intake Regulates Oleoylethanolamide Formation and Degradation in the Proximal Small Intestine. *Journal of Biological Chemistry* **2007**, doi:10.1074/jbc.M607809200.
265. Laugerette, F.; Passilly-Degrace, P.; Patris, B.; Niot, I.; Febbraio, M.; Montmayeur, J.P.; Besnard, P. CD36 Involvement in Orosensory Detection of Dietary Lipids, Spontaneous Fat Preference, and Digestive Secretions. *Journal of Clinical Investigation* **2005**, *115*, doi:10.1172/JCI25299.
266. Schwartz, G.J.; Fu, J.; Astarita, G.; Li, X.; Gaetani, S.; Campolongo, P.; Cuomo, V.; Piomelli, D. The Lipid Messenger OEA Links Dietary Fat Intake to Satiety. *Cell Metabolism* **2008**, doi:10.1016/j.cmet.2008.08.005.
267. Igarashi, M.; DiPatrizio, N. v.; Narayanaswami, V.; Piomelli, D. Feeding-Induced Oleoylethanolamide Mobilization Is Disrupted in the Gut of Diet-Induced Obese Rodents. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **2015**, *1851*, doi:10.1016/j.bbalip.2015.05.006.

268. LoVerme, J.; Guzmám, M.; Gaetani, S.; Piomelli, D. Cold Exposure Stimulates Synthesis of the Bioactive Lipid Oleoylethanolamide in Rat Adipose Tissue. *Journal of Biological Chemistry* **2006**, *281*, doi:10.1074/jbc.M604751200.

269. Fu, J.; di Patrizio, N. v.; Guijarro, A.; Schwartz, G.J.; Li, X.; Gaetani, S.; Astarita, G.; Piomelli, D. Sympathetic Activity Controls Fat-Induced Oleoylethanolamide Signaling in Small Intestine. *Journal of Neuroscience* **2011**, *31*, doi:10.1523/JNEUROSCI.5668-10.2011.

270. Piomelli, D. A Fatty Gut Feeling. *Trends in Endocrinology and Metabolism* 2013.

271. Romano, A.; Tempesta, B.; Provensi, G.; Passani, M.B.; Gaetani, S. Central Mechanisms Mediating the Hypophagic Effects of Oleoylethanolamide and N-Acylphosphatidylethanolamines: Different Lipid Signals? *Frontiers in Pharmacology* 2015.

272. Cadas, H.; Gaillet, S.; Beltramo, M.; Venance, L.; Piomelli, D. Biosynthesis of an Endogenous Cannabinoid Precursor in Neurons and Its Control by Calcium and CAMP. *Journal of Neuroscience* **1996**, *16*, doi:10.1523/jneurosci.16-12-03934.1996.

273. Okamoto, Y.; Morishita, J.; Tsuboi, K.; Tonai, T.; Ueda, N. Molecular Characterization of a Phospholipase D Generating Anandamide and Its Congeners. *Journal of Biological Chemistry* **2004**, *279*, 5298–5305, doi:10.1074/jbc.M306642200.

274. Tsuboi, K.; Okamoto, Y.; Ikematsu, N.; Inoue, M.; Shimizu, Y.; Uyama, T.; Wang, J.; Deutsch, D.G.; Burns, M.P.; Ulloa, N.M.; et al. Enzymatic Formation of N-Acylethanolamines from N-Acylethanolamine Plasmalogen through N-Acylphosphatidylethanolamine-Hydrolyzing Phospholipase D-Dependent and -Independent Pathways. *Biochimica et Biophysica Acta*

- *Molecular and Cell Biology of Lipids* **2011**, *1811*, doi:10.1016/j.bbalip.2011.07.009.

275. Geurts, L.; Everard, A.; van Hul, M.; Essaghir, A.; Duparc, T.; Matamoros, S.; Plovier, H.; Castel, J.; Denis, R.G.P.; Bergiers, M.; et al. Adipose Tissue NAPE-PLD Controls Fat Mass Development by Altering the Browning Process and Gut Microbiota. *Nature Communications* **2015**, *6*, doi:10.1038/ncomms7495.
276. Petersen, G.; Sørensen, C.; Schmid, P.C.; Artmann, A.; Tang-Christensen, M.; Hansen, S.H.; Larsen, P.J.; Schmid, H.H.O.; Hansen, H.S. Intestinal Levels of Anandamide and Oleoylethanolamide in Food-Deprived Rats Are Regulated through Their Precursors. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2006**, doi:10.1016/j.bbalip.2005.12.011.
277. Cravatt, B.F.; Giang, D.K.; Mayfield, S.P.; Boger, D.L.; Lerner, R.A.; Gilula, N.B. Molecular Characterization of an Enzyme That Degrades Neuromodulatory Fatty-Acid Amides. *Nature* **1996**, *384*, 83–87, doi:10.1038/384083a0.
278. Tsuboi, K.; Sun, Y.X.; Okamoto, Y.; Araki, N.; Tonai, T.; Ueda, N. Molecular Characterization of N-Acylethanolamine-Hydrolyzing Acid Amidase, a Novel Member of the Choloylglycine Hydrolase Family with Structural and Functional Similarity to Acid Ceramidase. *Journal of Biological Chemistry* **2005**, *280*, 11082–11092, doi:10.1074/jbc.M413473200.
279. McKinney, M.K.; Cravatt, B.F. Evidence for Distinct Roles in Catalysis for Residues of the Serine-Serine-Lysine Catalytic Triad of Fatty Acid Amide Hydrolase. *The Journal of biological chemistry* **2003**, *278*, 37393–37399, doi:10.1074/jbc.M303922200.

280. Katayama, K.; Ueda, N.; Kurahashi, Y.; Suzuki, H.; Yamamoto, S.; Kato, I. Distribution of Anandamide Amidohydrolase in Rat Tissues with Special Reference to Small Intestine. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* **1997**, *1347*, doi:10.1016/S0005-2760(97)00078-7.

281. Wei, B.Q.; Mikkelsen, T.S.; McKinney, M.K.; Lander, E.S.; Cravatt, B.F. A Second Fatty Acid Amide Hydrolase with Variable Distribution among Placental Mammals. *Journal of Biological Chemistry* **2006**, *281*, 36569–36578, doi:10.1074/jbc.M606646200.

282. Gulyas, A.I.; Cravatt, B.F.; Bracey, M.H.; Dinh, T.P.; Piomelli, D.; Boscia, F.; Freund, T.F. Segregation of Two Endocannabinoid-Hydrolyzing Enzymes into Pre- and Postsynaptic Compartments in the Rat Hippocampus, Cerebellum and Amygdala. *European Journal of Neuroscience* **2004**, *20*, 441–458, doi:10.1111/j.1460-9568.2004.03428.x.

283. Kaczocha, M.; Glaser, S.T.; Chae, J.; Brown, D.A.; Deutsch, D.G. Lipid Droplets Are Novel Sites of N-Acylethanolamine Inactivation by Fatty Acid Amide Hydrolase-2. *Journal of Biological Chemistry* **2010**, *285*, 2796–2806, doi:10.1074/jbc.M109.058461.

284. Tsuboi, K.; Takezaki, N.; Ueda, N. The N-Acylethanolamine-Hydrolyzing Acid Amidase (NAAA). *Chemistry and Biodiversity* 2007, *4*.

285. lo Verme, J.; Fu, J.; Astarita, G.; la Rana, G.; Russo, R.; Calignano, A.; Piomelli, D. The Nuclear Receptor Peroxisome Proliferator-Activated Receptor-α Mediates the Anti-Inflammatory Actions of Palmitoylethanolamide. *Molecular Pharmacology* **2005**, *67*, doi:10.1124/mol.104.006353.

286. Fu, J.; Gaetani, S.; Oveisi, F.; lo Verme, J.; Serrano, A.; de Fonseca, F.R.; Rosengarth, A.; Luecke, H.; di Giacomo, B.; Tarzia, G.; et al. Oleylethanolamide Regulates Feeding and Body Weight through Activation of the Nuclear Receptor PPAR-α. *Nature* **2003**, *425*, doi:10.1038/nature01921.

287. Sierra, S.; Luquin, N.; Navarro-Otano, J. The Endocannabinoid System in Cardiovascular Function: Novel Insights and Clinical Implications. *Clinical Autonomic Research* 2018, *28*.

288. Gallelli, C.A.; Calcagnini, S.; Romano, A.; Koczwara, J.B.; de Ceglia, M.; Dante, D.; Villani, R.; Giudetti, A.M.; Cassano, T.; Gaetani, S. Modulation of the Oxidative Stress and Lipid Peroxidation by Endocannabinoids and Their Lipid Analogues. *Antioxidants* 2018, *7*.

289. Mattace Raso, G.; Simeoli, R.; Russo, R.; Santoro, A.; Pirozzi, C.; D'Emmanuele Di Villa Bianca, R.; Mitidieri, E.; Paciello, O.; Pagano, T.B.; Orefice, N.S.; et al. N-Palmitoylethanolamide Protects the Kidney from Hypertensive Injury in Spontaneously Hypertensive Rats via Inhibition of Oxidative Stress. *Pharmacological Research* **2013**, *76*, doi:10.1016/j.phrs.2013.07.007.

290. Zoete, V.; Grosdidier, A.; Michielin, O. Peroxisome Proliferator-Activated Receptor Structures: Ligand Specificity, Molecular Switch and Interactions with Regulators. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 2007, *1771*.

291. Shin, S.J.; Lim, J.H.; Chung, S.; Youn, D.Y.; Chung, H.W.; Kim, H.W.; Lee, J.H.; Chang, Y.S.; Park, C.W. Peroxisome Proliferator-Activated Receptor-α Activator Fenofibrate Prevents High-Fat Diet-Induced Renal Lipotoxicity in Spontaneously Hypertensive Rats. *Hypertension Research* **2009**, *32*, doi:10.1038/hr.2009.107.

292. Gelosa, P.; Banfi, C.; Gianella, A.; Brioschi, M.; Pignieri, A.; Nobili, E.; Castiglioni, L.; Cimino, M.; Tremoli, E.; Sironi, L. Peroxisome Proliferator-Activated Receptor α Agonism Prevents Renal Damage and the Oxidative Stress and Inflammatory Processes Affecting the Brains of Stroke-Prone Rats. *Journal of Pharmacology and Experimental Therapeutics* **2010**, *335*, doi:10.1124/jpet.110.171090.

293. Guzmán, M.; lo Verme, J.; Fu, J.; Oveisi, F.; Blázquez, C.; Piomelli, D. Oleoylethanolamide Stimulates Lipolysis by Activating the Nuclear Receptor Peroxisome Proliferator-Activated Receptor α (PPAR-α). *Journal of Biological Chemistry* **2004**, *279*, doi:10.1074/jbc.M404087200.

294. Khasabova, I.A.; Xiong, Y.; Coicou, L.G.; Piomelli, D.; Seybold, V. Peroxisome Proliferator-Activated Receptor α Mediates Acute Effects of Palmitoylethanolamide on Sensory Neurons. *Journal of Neuroscience* **2012**, *32*, doi:10.1523/JNEUROSCI.0130-12.2012.

295. Melis, M.; Carta, S.; Fattore, L.; Tolu, S.; Yasar, S.; Goldberg, S.R.; Fratta, W.; Maskos, U.; Pistis, M. Peroxisome Proliferator-Activated Receptors-Alpha Modulate Dopamine Cell Activity through Nicotinic Receptors. *Biological Psychiatry* **2010**, *68*, doi:10.1016/j.biopsych.2010.04.016.

296. Dipasquale, P.; Romano, A.; Cianci, S.; Righetti, L.; Gaetani, S. Oleoylethanolamide: A New Player in Energy Metabolism Control. Role in Food Intake. *Drug Discovery Today: Disease Mechanisms* 2010, *7*.

297. Wang, X.; Miyares, R.L.; Ahern, G.P. Oleoylethanolamide Excites Vagal Sensory Neurones Induces Visceral Pain and Reduces Short-Term Food Intake in Mice via Capsaicin Receptor TRPV1. *Journal of Physiology* **2005**, *564*, doi:10.1113/jphysiol.2004.081844.

298. Overton, H.A.; Babbs, A.J.; Doel, S.M.; Fyfe, M.C.T.; Gardner, L.S.; Griffin, G.; Jackson, H.C.; Procter, M.J.; Rasamison, C.M.; Tang-Christensen, M.; et al. Deorphanization of a G Protein-Coupled Receptor for Oleoylethanolamide and Its Use in the Discovery of Small-Molecule Hypophagic Agents. *Cell Metabolism* **2006**, *3*, doi:10.1016/j.cmet.2006.02.004.

299. Yang, D.; Luo, Z.; Ma, S.; Wong, W.T.; Ma, L.; Zhong, J.; He, H.; Zhao, Z.; Cao, T.; Yan, Z.; et al. Activation of TRPV1 by Dietary Capsaicin Improves Endothelium-Dependent Vasorelaxation and Prevents Hypertension. *Cell Metabolism* **2010**, *12*, doi:10.1016/j.cmet.2010.05.015.

300. Poblete, I.M.; Orliac, M.L.; Briones, R.; Adler-Graschinsky, E.; Huidobro-Toro, J.P. Anandamide Elicits an Acute Release of Nitric Oxide through Endothelial TRPV1 Receptor Activation in the Rat Arterial Mesenteric Bed. *Journal of Physiology* **2005**, *568*, doi:10.1113/jphysiol.2005.094292.

301. Shah, U.; Kowalski, T.J. *GPR119 Agonists for the Potential Treatment of Type 2 Diabetes and Related Metabolic Disorders*; 2010; Vol. 84;.

302. Lauffer, L.M.; Iakoubov, R.; Brubaker, P.L. GPR119 Is Essential for Oleoylethanolamide-Induced Glucagon-like Peptide-1 Secretion from the Intestinal Enteroendocrine L-Cell. *Diabetes* **2009**, *58*, 1058–1066, doi:10.2337/db08-1237.

303. Lan, H.; Vassileva, G.; Corona, A.; Liu, L.; Baker, H.; Golovko, A.; Abbondanzo, S.J.; Hu, W.; Yang, S.; Ning, Y.; et al. GPR119 Is Required for Physiological Regulation of Glucagon-like Peptide-1 Secretion but Not for Metabolic Homeostasis. *Journal of Endocrinology* **2009**, *201*, doi:10.1677/JOE-08-0453.

304. Fu, J.; Oveisi, F.; Gaetani, S.; Lin, E.; Piomelli, D. Oleoylethanolamide, an Endogenous PPAR-α Agonist, Lowers Body Weight and Hyperlipidemia in Obese Rats. *Neuropharmacology* **2005**, doi:10.1016/j.neuropharm.2005.02.013.

305. Azari, E.K.; Ramachandran, D.; Weibel, S.; Arnold, M.; Romano, A.; Gaetani, S.; Langhans, W.; Mansouri, A. Vagal Afferents Are Not Necessary for the Satiety Effect of the Gut Lipid Messenger Oleoylethanolamide. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2014**, doi:10.1152/ajpregu.00067.2014.

306. Romano, A.; Coccurello, R.; Giacovazzo, G.; Bedse, G.; Moles, A.; Gaetani, S. Oleoylethanolamide: A Novel Potential Pharmacological Alternative to Cannabinoid Antagonists for the Control of Appetite. *BioMed Research International* **2014**, doi:10.1155/2014/203425.

307. Gaetani, S.; Oveisi, F.; Piomelli, D. Modulation of Meal Pattern in the Rat by the Anorexic Lipid Mediator Oleoylethanolamide. *Neuropsychopharmacology* **2003**, *28*, doi:10.1038/sj.npp.1300166.

308. Aviello, G.; Matias, I.; Capasso, R.; Petrosino, S.; Borrelli, F.; Orlando, P.; Romano, B.; Capasso, F.; di Marzo, V.; Izzo, A.A. Inhibitory Effect of the Anorexic Compound Oleoylethanolamide on Gastric Emptying in Control and Overweight Mice. *Journal of Molecular Medicine* **2008**, *86*, doi:10.1007/s00109-008-0305-7.

309. Hansen, H.S. Role of Anorectic N-Acylethanolamines in Intestinal Physiology and Satiety Control with Respect to Dietary Fat. *Pharmacological Research* 2014, *86*.

310. Yang, Y.; Chen, M.; Georgeson, K.E.; Harmon, C.M. Mechanism of Oleoylethanolamide on Fatty Acid Uptake in Small Intestine after Food Intake and Body Weight Reduction. *American Journal of*

Physiology - Regulatory Integrative and Comparative Physiology **2007**, *292*, doi:10.1152/ajpregu.00270.2006.

311. Romano, A.; Friuli, M.; del Coco, L.; Longo, S.; Vergara, D.; del Boccio, P.; Valentinuzzi, S.; Cicalini, I.; Fanizzi, F.P.; Gaetani, S.; et al. Chronic Oleoylethanolamide Treatment Decreases Hepatic Triacylglycerol Level in Rat Liver by a Pparγ/Srebp-Mediated Suppression of Fatty Acid and Triacylglycerol Synthesis. *Nutrients* **2021**, *13*, doi:10.3390/nu13020394.
312. Thabuis, C.; Destaillats, F.; Landrier, J.F.; Tissot-Favre, D.; Martin, J.C. Analysis of Gene Expression Pattern Reveals Potential Targets of Dietary Oleoylethanolamide in Reducing Body Fat Gain in C3H Mice. *Journal of Nutritional Biochemistry* **2010**, *21*, doi:10.1016/j.jnutbio.2009.07.006.
313. Li, L.; Li, L.; Chen, L.; Lin, X.; Xu, Y.; Ren, J.; Fu, J.; Qiu, Y. Effect of Oleoylethanolamide on Diet-Induced Nonalcoholic Fatty Liver in Rats. *Journal of Pharmacological Sciences* **2015**, doi:10.1016/j.jphs.2014.12.001.
314. Antón, M.; Alén, F.; Gómez de Heras, R.; Serrano, A.; Pavón, F.J.; Leza, J.C.; García-Bueno, B.; Rodríguez de Fonseca, F.; Orio, L. Oleoylethanolamide Prevents Neuroimmune HMGB1/TLR4/NF-KB Danger Signaling in Rat Frontal Cortex and Depressive-like Behavior Induced by Ethanol Binge Administration. *Addiction Biology* **2017**, doi:10.1111/adb.12365.
315. Campolongo, P.; Roozendaal, B.; Trezza, V.; Cuomo, V.; Astarita, G.; Fu, J.; McGaugh, J.L.; Piomelli, D. Fat-Induced Satiety Factor Oleoylethanolamide Enhances Memory Consolidation. *Proceedings of the National Academy of Sciences of the United States of America* **2009**, doi:10.1073/pnas.0903038106.

316. Tellez, L.A.; Medina, S.; Han, W.; Ferreira, J.G.; Licona-Limón, P.; Ren, X.; Lam, T.K.T.; Schwartz, G.J.; de Araujo, I.E. A Gut Lipid Messenger Links Excess Dietary Fat to Dopamine Deficiency. *Science* **2013**, *341*, doi:10.1126/science.1239275.

317. Costa, A.; Cristiano, C.; Cassano, T.; Gallelli, C.A.; Gaetani, S.; Ghelardini, C.; Blandina, P.; Calignano, A.; Passani, M.B.; Provensi, G. Histamine-Deficient Mice Do Not Respond to the Antidepressant-like Effects of Oleoylethanolamide. *Neuropharmacology* **2018**, doi:10.1016/j.neuropharm.2018.03.033.

318. Jin, P.; Yu, H.L.; Tian-Lan; Zhang, F.; Quan, Z.S. Antidepressant-like Effects of Oleoylethanolamide in a Mouse Model of Chronic Unpredictable Mild Stress. *Pharmacology Biochemistry and Behavior* **2015**, doi:10.1016/j.pbb.2015.04.001.

319. Yu, H.L.; Sun, L.P.; Li, M.M.; Quan, Z.S. Involvement of Norepinephrine and Serotonin System in Antidepressant-like Effects of Oleoylethanolamide in the Mice Models of Behavior Despair. *Neuroscience Letters* **2015**, *593*, doi:10.1016/j.neulet.2015.03.019.

320. Gaetani, S.; Fu, J.; Cassano, T.; Dipasquale, P.; Romano, A.; Righetti, L.; Cianci, S.; Laconca, L.; Giannini, E.; Scaccianoce, S.; et al. The Fat-Induced Satiety Factor Oleoylethanolamide Suppresses Feeding through Central Release of Oxytocin. *Journal of Neuroscience* **2010**, doi:10.1523/JNEUROSCI.0036-10.2010.

321. Romano, A.; Karimian Azari, E.; Tempesta, B.; Mansouri, A.; Micioni Di Bonaventura, M. v.; Ramachandran, D.; Lutz, T.A.; Bedse, G.; Langhans, W.; Gaetani, S. High Dietary Fat Intake Influences the Activation of Specific Hindbrain and Hypothalamic Nuclei by the Satiety Factor Oleoylethanolamide. *Physiology and Behavior* **2014**, doi:10.1016/j.physbeh.2014.04.039.

322. Romano, A.; Potes, C.S.; Tempesta, B.; Cassano, T.; Cuomo, V.; Lutz, T.; Gaetani, S. Hindbrain Noradrenergic Input to the Hypothalamic PVN Mediates the Activation of Oxytocinergic Neurons Induced by the Satiety Factor Oleoylethanolamide. *American Journal of Physiology - Endocrinology and Metabolism* **2013**, doi:10.1152/ajpendo.00411.2013.
323. Provensi, G.; Coccurello, R.; Umehara, H.; Munari, L.; Giacovazzo, G.; Galeotti, N.; Nosi, D.; Gaetani, S.; Romano, A.; Moles, A.; et al. Satiety Factor Oleoylethanolamide Recruits the Brain Histaminergic System to Inhibit Food Intake. *Proceedings of the National Academy of Sciences of the United States of America* **2014**, *111*, doi:10.1073/pnas.1322016111.
324. Egertová, M.; Cravatt, B.F.; Elphick, M.R. Fatty Acid Amide Hydrolase Expression in Rat Choroid Plexus: Possible Role in Regulation of the Sleep-Inducing Action of Oleamide. *Neuroscience Letters* **2000**, *282*, doi:10.1016/S0304-3940(00)00841-7.
325. Romano, A.; Gallelli, C.A.; Koczwara, J.B.; Braegger, F.E.; Vitalone, A.; Falchi, M.; Micioni Di Bonaventura, M.V.; Cifani, C.; Cassano, T.; Lutz, T.A.; et al. Role of the Area Postrema in the Hypophagic Effects of Oleoylethanolamide. *Pharmacological Research* **2017**, doi:10.1016/j.phrs.2017.05.017.
326. Dale, H.H.; Laidlaw, P.P. The Physiological Action of B-iminazolylethylamine. *The Journal of Physiology* **1910**, *41*, doi:10.1113/jphysiol.1910.sp001406.
327. Watanabe, T.; Taguchi, Y.; Shiosaka, S.; Tanaka, J.; Kubota, H.; Terano, Y.; Tohyama, M.; Wada, H. Distribution of the Histaminergic Neuron System in the Central Nervous System of Rats; a Fluorescent Immunohistochemical Analysis with Histidine Decarboxylase as a Marker. *Brain Research* **1984**, *295*, doi:10.1016/0006-8993(84)90811-4.

328. Airaksinen, M.S.; Panula, P. The Histaminergic System in the Guinea Pig Central Nervous System: An Immunocytochemical Mapping Study Using an Antiserum against Histamine. *Journal of Comparative Neurology* **1988**, *273*, doi:10.1002/cne.902730204.

329. Nieto-Alamilla, G.; Márquez-Gómez, R.; García-Gálvez, A.M.; Morales-Figueroa, G.E.; Arias-Montaño, J.A. The Histamine H3 Receptor: Structure, Pharmacology, and Function. *Molecular Pharmacology* 2016, *90*.

330. Lin, J.S.; Sakai, K.; Vanni-Mercier, G.; Arrang, J.M.; Garbarg, M.; Schwartz, J.C.; Jouvet, M. Involvement of Histaminergic Neurons in Arousal Mechanisms Demonstrated with H3-Receptor Ligands in the Cat. *Brain Research* **1990**, *523*, doi:10.1016/0006-8993(90)91508-E.

331. Hatton, G.I.; Yang, Q.Z. Synaptically Released Histamine Increases Dye Coupling among Vasopressinergic Neurons of the Supraoptic Nucleus: Mediation by H1 Receptors and Cyclic Nucleotides. *Journal of Neuroscience* **1996**, *16*, doi:10.1523/jneurosci.16-01-00123.1996.

332. Kjær, A.; Knigge, U.; Rouleau, A.; Garbarg, M.; Warberg, J. Dehydration-Induced Release of Vasopressin Involves Activation of Hypothalamic Histaminergic Neurons. *Endocrinology* **1994**, *135*, doi:10.1210/endo.135.2.8033816.

333. Knigge, U.; Warberg, J. The Role of Histamine in the Neuroendocrine Regulation of Pituitary Hormone Secretion. *Acta Endocrinologica* 1991, *124*.

334. Knigge, U.; Matzen, S.; Bach, F.W.; Bang, P.; Warberg, J. Involvement of Histaminergic Neurons in the Stress-Induced Release of pro-Opiomelanocortin-Derived Peptides in Rats. *Acta Endocrinologica* **1989**, *120*, doi:10.1530/acta.0.1200533.

335. Sakata, T.; Kurokawa, M.; Oohara, A.; Yoshimatsu, H. A Physiological Role of Brain Histamine during Energy Deficiency. *Brain Research Bulletin* **1994**, *35*, doi:10.1016/0361-9230(94)90093-0.

336. Provensi, G.; Blandina, P.; Passani, M.B. The Histaminergic System as a Target for the Prevention of Obesity and Metabolic Syndrome. *Neuropharmacology* 2016, *106*.

337. Sakata, T.; Yoshimatsu, H. Homeostatic Maintenance Regulated by Hypothalamic Neuronal Histamine. In Proceedings of the Methods and Findings in Experimental and Clinical Pharmacology; 1995; Vol. 17.

338. Ash, A.S.F.; Schild, H.O. Receptors Mediating Some Actions of Histamine. *British Journal of Pharmacology* **1997**, *120*, doi:10.1111/j.1476-5381.1997.tb06810.x.

339. Black, J.W.; Duncan, W.A.M.; Durant, C.J.; Ganellin, C.R.; Parsons, E.M. Definition and Antagonism of Histamine H2-Receptors. *Nature* **1972**, *236*, doi:10.1038/236385a0.

340. Arrang, J.M.; Garbarg, M.; Schwartz, J.C. Auto-Inhibition of Brain Histamine Release Mediated by a Novel Class (H3) of Histamine Receptor. *Nature* **1983**, *302*, doi:10.1038/302832a0.

341. Oda, T.; Morikawa, N.; Saito, Y.; Masuho, Y.; Matsumoto, S.I. Molecular Cloning and Characterization of a Novel Type of Histamine Receptor Preferentially Expressed in Leukocytes. *Journal of Biological Chemistry* **2000**, *275*, doi:10.1074/jbc.M006480200.

342. Sakata, T.; Yoshimatsu, H.; Kurokawa, M. Hypothalamic Neuronal Histamine: Implications of Its Homeostatic Control of Energy Metabolism. *Nutrition* **1997**, *13*, doi:10.1016/S0899-9007(97)91277-6.

343. Ishizuka, T.; Yamatodani, A. Integrative Role of the Histaminergic System in Feeding and Taste Perception. *Frontiers in Systems Neuroscience* **2012**, doi:10.3389/fnsys.2012.00044.

344. Itoh, Y.; Oishi, R.; Saeki, K. Feeding-Induced Increase in the Extracellular Concentration of Histamine in Rat Hypothalamus as Measured by in Vivo Microdialysis. *Neuroscience Letters* **1991**, *125*, doi:10.1016/0304-3940(91)90037-T.

345. Lecklin, A.; Tuomisto, L. The Blockade of H1 Receptors Attenuates the Suppression of Feeding and Diuresis Induced by Inhibition of Histamine Catabolism. *Pharmacology Biochemistry and Behavior* **1998**, *59*, doi:10.1016/S0091-3057(97)00465-6.

346. Itowi, N.; Nagai, K.; Nakagawa, H.; Watanabe, T.; Wada, H. Changes in the Feeding Behavior of Rats Elicited by Histamine Infusion. *Physiology and Behavior* **1988**, *44*, doi:10.1016/0031-9384(88)90142-4.

347. Clineschmidt, B. v.; Lotti, V.J. Histamine: Intraventricular Injection Suppresses Ingestive Behavior of the Cat. *Archives Internationales de Pharmacodynamie et de Therapie* **1973**, *206*.

348. Masaki, T.; Yoshimatsu, H. The Hypothalamic H1 Receptor: A Novel Therapeutic Target for Disrupting Diurnal Feeding Rhythm and Obesity. *Trends in Pharmacological Sciences* 2006, *27*.

349. Passani, M.B.; Blandina, P. Histamine Receptors in the CNS as Targets for Therapeutic Intervention. *Trends in Pharmacological Sciences* 2011, *32*.

350. Passani, M.B.; Blandina, P.; Torrealba, F. The Histamine H3 Receptor and Eating Behavior. *Journal of Pharmacology and Experimental Therapeutics* **2011**, *336*, doi:10.1124/jpet.110.171306.

351. Panula, P.; Nuutinen, S. The Histaminergic Network in the Brain: Basic Organization and Role in Disease. *Nature Reviews Neuroscience* 2013, *14*.

352. Taylor, K.M.; Snyder, S.H. Histamine in Rat Brain: Sensitive Assay of Endogenous Levels, Formation in Vivo and Lowering by Inhibitors of Histidine Decarboxylase. *Journal of Pharmacology and Experimental Therapeutics* **1971**, *179*.

353. Haxhiu, M.A.; Tolentino-Silva, F.; Pete, G.; Kc, P.; Mack, S.O. Monoaminergic Neurons, Chemosensation and Arousal. In Proceedings of the Respiration Physiology; 2001; Vol. 129.

354. Miklós, I.H.; Kovács, K.J. Functional Heterogeneity of the Responses of Histaminergic Neuron Subpopulations to Various Stress Challenges. *European Journal of Neuroscience* **2003**, *18*, doi:10.1111/j.1460-9568.2003.03033.x.

355. Baldi, E.; Costa, A.; Rani, B.; Passani, M.B.; Blandina, P.; Romano, A.; Provensi, G. Oxytocin and Fear Memory Extinction: Possible Implications for the Therapy of Fear Disorders? *International Journal of Molecular Sciences* 2021, *22*.

356. Engelmann, M.; Landgraf, R. Microdialysis Administration of Vasopressin into the Septum Improves Social Recognition in Brattleboro Rats. *Physiology and Behavior* **1994**, *55*, doi:10.1016/0031-9384(94)90022-1.

357. Neumann, I.D. Involvement of the Brain Oxytocin System in Stress Coping: Interactions with the Hypothalamo-Pituitary-Adrenal Axis. In Proceedings of the Progress in Brain Research; 2002; Vol. 139.

358. Amico, J.A.; Mantella, R.C.; Vollmer, R.R.; Li, X. Anxiety and Stress Responses in Female Oxytocin Deficient Mice. *Journal of Neuroendocrinology* **2004**, *16*, doi:10.1111/j.0953-8194.2004.01161.x.

359. Hasan, M.T.; Althammer, F.; Silva da Gouveia, M.; Goyon, S.; Eliava, M.; Lefevre, A.; Kerspern, D.; Schimmer, J.; Raftogianni, A.; Wahis, J.; et al. A Fear Memory Engram and Its Plasticity in the Hypothalamic Oxytocin System. *Neuron* **2019**, *103*, doi:10.1016/j.neuron.2019.04.029.

360. Zhu, L.; Onaka, T. Involvement of Medullary A2 Noradrenergic Neurons in the Activation of Oxytocin Neurons after Conditioned Fear Stimuli. *European Journal of Neuroscience* **2002**, *16*, doi:10.1046/j.1460-9568.2002.02285.x.

361. Martinon, D.; Lis, P.; Roman, A.N.; Tornesi, P.; Applebey, S. v.; Buechner, G.; Olivera, V.; Dabrowska, J. Oxytocin Receptors in the Dorsolateral Bed Nucleus of the Stria Terminalis (BNST) Bias Fear Learning toward Temporally Predictable Cued Fear. *Translational Psychiatry* **2019**, *9*, doi:10.1038/s41398-019-0474-x.

362. Rani, B.; Santangelo, A.; Romano, A.; Koczwara, J.B.; Friuli, M.; Provensi, G.; Blandina, P.; Casarrubea, M.; Gaetani, S.; Passani, M.B.; et al. Brain Histamine and Oleoylethanolamide Restore Behavioral Deficits Induced by Chronic Social Defeat Stress in Mice. *Neurobiology of Stress* **2021**, *14*, doi:10.1016/j.ynstr.2021.100317.

363. Gaetani, S.; Romano, A.; Provensi, G.; Ricca, V.; Lutz, T.; Passani, M.B. Eating Disorders: From Bench to Bedside and Back. *Journal of Neurochemistry* 2016.

364. Romano, A.; Micioni Di Bonaventura, M.V.; Gallelli, C.A.; Koczwara, J.B.; Smeets, D.; Giusepponi, M.E.; de Ceglia, M.; Friuli, M.; Micioni Di Bonaventura, E.; Scuderi, C.; et al. Oleoylethanolamide Decreases Frustration Stress-Induced Binge-like Eating in Female Rats: A Novel Potential Treatment for Binge Eating Disorder. *Neuropsychopharmacology* **2020**, doi:10.1038/s41386-020-0686-z.

365. Serrano, A.; del Arco, I.; Javier Pavón, F.; Macías, M.; Perez-Valero, V.; Rodríguez de Fonseca, F. The Cannabinoid CB1 Receptor Antagonist SR141716A (Rimonabant) Enhances the Metabolic Benefits of Long-Term Treatment with Oleoylethanolamide in Zucker Rats. *Neuropharmacology* **2008**, doi:10.1016/j.neuropharm.2007.03.007.
366. Pan, X.; Schwartz, G.J.; Hussain, M.M. Oleoylethanolamide Differentially Regulates Glycerolipid Synthesis and Lipoprotein Secretion in Intestine and Liver. *Journal of Lipid Research* **2018**, doi:10.1194/jlr.M089250.
367. Tutunchi, H.; Naeini, F.; Saghafi-Asl, M.; Farrin, N.; Monshikarimi, A.; Ostadrahimi, A. Effects of Oleoylethanolamide Supplementation on Atherogenic Indices and Hematological Parameters in Patients with Nonalcoholic Fatty Liver Disease: A Clinical Trial. *Health Promotion Perspectives* **2020**, *10*, doi:10.34172/hpp.2020.56.
368. Tutunchi, H.; Ostadrahimi, A.; Saghafi-Asl, M.; Hosseinzadeh-Attar, M.J.; Shakeri, A.; Asghari-Jafarabadi, M.; Roshanravan, N.; Farrin, N.; Naemi, M.; Hasankhani, M. Oleoylethanolamide Supplementation in Obese Patients Newly Diagnosed with Non-Alcoholic Fatty Liver Disease: Effects on Metabolic Parameters, Anthropometric Indices, and Expression of PPAR-α, UCP1, and UCP2 Genes. *Pharmacological Research* **2020**, *156*, doi:10.1016/j.phrs.2020.104770.
369. Lutz, T.A.; Woods, S.C. Overview of Animal Models of Obesity. *Current Protocols in Pharmacology* **2012**, doi:10.1002/0471141755.ph0561s58.
370. Romano, A.; Cassano, T.; Tempesta, B.; Cianci, S.; Dipasquale, P.; Coccurello, R.; Cuomo, V.; Gaetani, S. The Satiety Signal Oleoylethanolamide Stimulates Oxytocin Neurosecretion from Rat

Hypothalamic Neurons. *Peptides* **2013**, *49*, doi:10.1016/j.peptides.2013.08.006.

Chapter 2:

Oleoylethanolamide's effects in a rat model of diet-induced obesity: from neuroprotective properties to the modulation of eating behavior, hepatic lipid metabolism and oxidative stress

1. Abstract

Far from being limited to weight gain, obesity is now associated with a cluster of central and peripheral alterations including systemic low-grade inflammation, modification in the integrity and functionality of the blood-brain-barrier (BBB) and fatty liver diseases. In the last two decades, oleoylethanolamide (OEA), a naturally occurring bioactive lipid belonging to the family of N-acylethanolamides (NAEs), has received great attention for its biological properties which spanning in different domains: from neuroprotection to memory, from inflammation to mood disorders, from the regulation of satiety to lipid metabolism. Therefore, using a rat model of diet-induced obesity (DIO), the aim of the present chapter was to evaluate first whether rats with an obese phenotype showed alterations at both central and peripheral levels and subsequently whether the chronic peripheral administration of OEA might ameliorates such alterations; moreover, also the effect of OEA on food intake and body weight was evaluated.

2. Introduction

Obesity is a polygenic and multifactorial condition that represents a very concerning public health issue affecting both developing and developed countries [1,2]. The accumulation of adipose tissue represents a key factor in the development of the chronic, unresolved tissue inflammation related to obesity, that differs from the classical inflammatory response since it is characterized by a chronic low-intensity reaction [3] associated with a cluster of central and peripheral alterations.

Indeed, evidence suggests that obesity adversely affects brain functions. The central nervous system (CNS) is an immune-privileged organ due to the presence of the blood-brain barrier (BBB), a barrier tightly controlling exchanges with the periphery [4]. The BBB is present throughout the CNS except for the circumventricular organs (CVOs), such as the area postrema (AP) and the median eminence (ME) [5], characterized by extensive fenestrated vasculature through which this CVOs are in persistent contact with signaling molecules circulating in the bloodstream. In the context of obesity-induced neuroinflammation, different cellular types, involved in the functionality of both BBB and CVOs, result to be affected [6–10]; therefore, a disruption of these barriers, between the periphery and the CNS, can represent an important and deleterious mechanism leading to altered CNS homeostasis and consequently to neuroinflammation.

Indeed, in overweight and obese individuals, many mature immune cells, recruited and activated in the periphery, can cross the BBB and activate, in the CNS, several cell types [11–14]. Particularly, glial cells (such as microglia and astrocytes) that are physiologically involved in the control and regulation of multiple mechanisms in the CNS, are recruited during CNS trauma and neuroinflammation to modulate such pathophysiological

alterations [15–17]. Moreover, in the context of obesity-related neuroinflammation, also neurons result to be affected. Indeed, metabolic diseases, such as obesity, are associated with dysregulated neurogenesis [18–23], a process involving the proliferation, migration, differentiation, survival, and integration of new neurons into existing circuitry, playing a role in brain plasticity [24]. Several studies have reported that this altered neurogenesis, related to obesity, is particularly evident in the dentate gyrus (DG) of the hippocampus (HIPPO) [25–30] a brain area characterized by adult neurogenesis processes [31]. Although different evidence supports the presence of neuroinflammation in many areas of the CNS [32–35] in the context of obesity, the best-studied CNS structures are the hypothalamus (HYPO), for its pivotal role in regulating energy homeostasis, and HIPPO.

Concerning peripheral alterations, obesity appears to be associated with the development of metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2DM); NAFLD is a complex disease characterized by steatosis (or hepatic lipid accumulation), lipo-toxicity, insulin resistance, gut dysbiosis and inflammation [36]. NAFLD development and progression is strictly dependent on how fat metabolism is regulated [37]. Therefore, any interference and/or alteration in hepatic fat metabolism, in terms of fat storage and release, may represent a risk factor for the onset of NAFLD. Moreover, several lines of evidence suggest that a plethora of dysfunctional processes occur during the progression of NAFLD including inflammation, mitochondrial and endoplasmic reticulum (ER) impairment and oxidative stress [38–41].

In this scenario, oleoylethanolamide (OEA), a naturally occurring bioactive lipid, has been demonstrated to exert a plethora of protective effects including anti-obesity, anti-inflammatory, and antioxidant properties thus supporting its potential use for the treatment of obesity and eating-related

disorders [42–44]. In fact, as a drug, OEA reduces food intake and body weight gain [45–47] in both lean and obese rodents. Moreover, it has been demonstrated that OEA has beneficial effects on the high fat diet (HFD)-induced NAFLD in rats, by stimulating fatty acid β-oxidation, and by inhibiting lipogenesis [48].

Therefore, using a rat model of diet-induced obesity (DIO), the aim of the present chapter was to evaluate first whether rats with an obese phenotype showed alterations at both central (evaluating markers of neuroinflammation, neurogenesis and BBB proteins expression) and hepatic (evaluating lipid metabolism, oxidative stress and ER stress) levels and subsequently whether the chronic peripheral administration of OEA (10 mg/kg) might ameliorate such alterations. To this aim, DIO rats were obtained by exposing male rats to a HFD (60% of the Kcal from fats) for 11 weeks; control rats received a low-fat diet (LFD: 10% of the Kcal from fats). Subsequently, OEA was intraperitoneally (i.p.) administered daily to rats, for 2 weeks, at the dose of 10 mg/kg. We evaluated first the known effect of OEA on food intake and body weight; second, we evaluated in the brain of DIO rats: the expression of the glial fibrillary acidic protein (GFAP) in the HYPO, HIPPO, and DG as a marker of neuroinflammation, the number of doublecortin (DCX)-positive neurons in the DG of the HIPPO as a marker of neurogenesis and the expression of vimentin (VIM) and zona occludens (ZO)-1 as proteins involved in the integrity and functionality of the BBB (unpublished data).

Finally, in collaboration with Prof.ssa Anna Maria Giudetti of the University of Salento we have evaluated, in HFD-fed rats, the effects of OEA treatment on lipid metabolism and particularly on the expression of key enzymes involved in different steps of fatty acid syntheses, transport and oxidation including diacylglycerol acyltransferase (DGAT)-1, 2,

stearoyl-CoA desaturase (SCD)-1, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), low-density lipoprotein receptor (LDLR) and carnitine palmitoyl-transferase (CPT)-1. Moreover, in HFD-fed rats, we have evaluated the effects of OEA treatment on the activities of key enzymes involved in the balance of the redox state (such as glutathione peroxidase, superoxide dismutase and catalase) and on the expression of different parameters of oxidative and ER stress.

3. Materials and methods

3.1. Animals and diets

A total of 96 male Wistar-Han rats (Charles River, 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 12:12h 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 high-fat diet, 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 European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals and were approved and supervised by a veterinarian.

- **Induction of obesity**

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. 2.1). At the end of the induction of obesity, the HFD animals whose BW gain (BWG, 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.

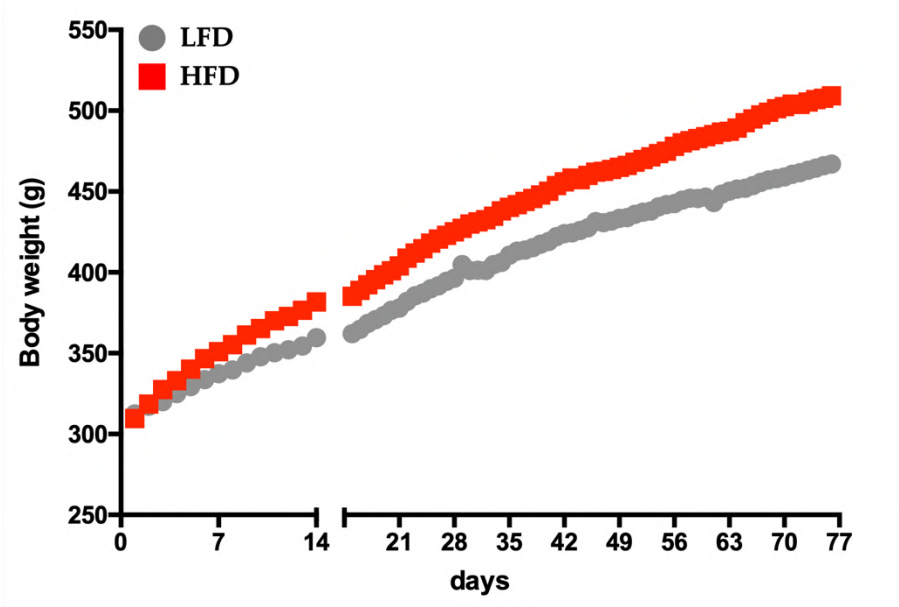

Fig. 2.1: Time course of the body weight gain in rats exposed to LFD or HFD for 11 weeks

3.2. Drugs and treatments

OEA (Sigma-Aldrich) was dissolved in the vehicle (VEH; saline/PEG/Tween80, 90/5/5 v/v/v, 2 ml/kg), and administered 10 mg/kg via intraperitoneal (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.

3.3. Chronic treatments

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 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, 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,

pSHIFT and pLFD). 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 intake 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, to evaluate the effects of chronic administration of OEA (10 mg/kg, i.p.), were evaluated: the food intake (FI) expressed in kcal of each day of treatment, normalized by the weight of the animal (kcal/kg) and the BW gain expressed as percentage of weight of each day of chronic treatment normalized for the weight on day 1.

The FI, 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).

The detailed timeline of the experiment is depicted in Fig. 2.2.

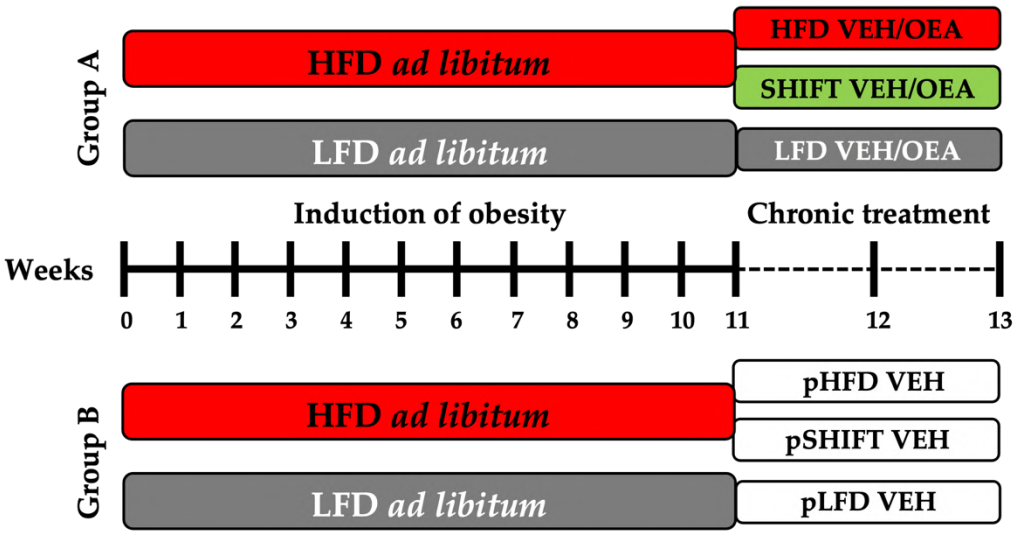

Fig. 2.2: Time course of the experiment, the diets and the treatments the animals were exposed to

- **Terminal experiment**

On the day of the terminal experiment, all food was removed from the cages 1h prior to dark onset. All the animals were administered with either VEH or OEA (10 mg/kg, i.p.) 10 minutes before the dark phase, and then had again free access to food. One hour after the administration, a first group of animals was deeply anesthetized with pentobarbital sodium (80 mg/kg; 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 48h at 4°C), and then snap frozen in dry-ice-cold 2-methylbutane (-60°C), to be stored at -80°C until processed for immunohistochemical analyses. Moreover, one hour after the administration a second group of animals was euthanized by CO2 overdose and the livers immediately collected, snap frozen in 2-metylbutane (-60°C) and stored at -80°C until processed for hepatic metabolic and oxidative stress analyses.

3.4. Immunohistochemistry and immunofluorescence experiments and semiquantitative densitometric analyses

For immunohistochemistry and immunofluorescence experiments, brains collected from LF and HF rats treated with both VEH and OEA (10 mg/kg i.p.) were sliced into 20 μm serial coronal sections by using a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA); these coronal slices were mounted on positively charged slides (Super Frost Plus, Menzel, Germany).

For GFAP immunofluorescence experiment, we adapted the protocol already published in previous papers [49,50]; it was performed in the

HYPO, HIPPO and DG of the HIPPO, brain areas strongly involved in the neuroinflammatory process related to obesity. For Immunofluorescence experiments for VIM and ZO-1, we also adapted the protocols already published in previous papers [51,52]; these immunofluorescent experiments were performed in the AP in the nucleus of the solitary tract (NST) and in the arcuate nucleus (ARC) of the HYPO, brain areas strongly involved in the regulation of eating behavior and in the pro-satiety effect of OEA [53]. Moreover, the ARC is in close contact with the ME; particularly, the AP and the ME, termed circumventricular organs (CVOs), are characterized by extensive fenestrated vasculature and lack of the usual BBB and result strongly affected in the neuroinflammatory process related to obesity [5,10].

Briefly, brain slices, containing the desired brain areas, underwent an initial antigen retrieval procedure by submerging selected slides in a sodium citrate buffer (10 mM pH 6,0) heated at 95°C for 5 min, as previous reported in a paper published by my research group [53]. Sections were rinsed with PBS (pH 7,4) and incubated for 1h in a blocking solution contained 0,3% Triton X-100 (Sigma–Aldrich) and either 2,5% of Normal Goat Serum (NGS; Jackson Immunoresearch) for VIM and ZO-1 or 2% of Normal Donkey Serum (NDS; Jackson Immunoresearch) for GFAP. Sections were then incubated, overnight at 4°C, with the blocking solution containing the primary antibody (mouse anti-GFAP, 1:250 dilution, Sigma-Aldrich; chicken anti-VIM, 1:1000 dilution, Sigma-Aldrich; rabbit anti-ZO-1, 1:500 dilution, Thermo Fisher). During the second day, after additional washes, sections were incubated, 2h at room temperature in dark conditions, with the blocking solution containing the secondary antibody (for GFAP: 488 Alexa fluor donkey anti-mouse, 1:300 dilution, Invitrogen; for VIM: 488 Alexa fluor goat anti-chicken, 1:1000 dilution, Invitrogen; for ZO-1: 594

Alexa fluor goat anti-rabbit, 1:300 dilution, Invitrogen). After additional washes sections were incubated, 20 min at room temperature in dark conditions, with Hoechst (1:5000 dilution; Sigma–Aldrich), used to detect cell nuclei. After final washes, slides were cover-slipped with Fluoromount (Sigma-Aldrich).

DCX immunohistochemistry experiment was performed in the DG of the HIPPO, an adult neurogenic site strongly involved in the neurogenesis process [24], adapting a protocol already published in previous paper [54]. Briefly, brain slices, containing the desired brain areas, underwent an initial antigen retrieval procedure by submerging selected slides in a sodium citrate buffer (10 mM pH 6,0) heated at 95°C for 5 min as previous reported in a paper published by my research group [53]. Sections were rinsed with PBS (pH 7,4) and washed for 20 min with a solution containing PBS + 0,1% Triton X-100 (Sigma–Aldrich) + 20% methanol (Merk) + 1,5% hydrogen peroxide (Merk). After additional washes sections were incubated for 1h in a blocking solution contained 0.3% Triton X-100 (Sigma–Aldrich) and 10% of Normal Donkey Serum (NDS; Jackson Immunoresearch. Sections were then incubated, overnight at 4°C, with solution containing the primary antibody (mouse anti-DCX, 1:400 dilution, Santa Cruz Biothecnology) and 0,3% Triton X-100 (Sigma–Aldrich). During the second day, after additional washes, sections were incubated, 2h at room temperature, with the secondary antibody (biotinylated donkey anti-mouse, 1:500 dilution, Jackson Immunoresearch) in PBS + 0,3% Triton X-100 (Sigma–Aldrich). After incubation for 1 h with the ABC Kit (Vectastain® ABC kit, Peroxidase; Vector Laboratories), sections were stained by incubation in DAB substrate kit (Vector Laboratories) chromogen solution. The slides were then rinsed with TBS (pH 7,3), dehydrated in graded alcohol, immersed in xylene and cover-slipped with Eukitt (Sigma–Aldrich).

The slices were then 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 both epifluorescent (GFAP, VIM and ZO-1) and light field (DCX) conditions and the rat brain atlas by Paxinos and Watson [55] was used as reference for the localization of the brain areas of interest.

GFAP, VIM and ZO-1 immunostainings were measured semi-quantitatively as optical density (OD) by using the Scion Image J software and considering, for background normalization, the averaged OD either of non-immunoreactive regions or of white matter structures within the same brain slice. DCX-positive neurons were manually counted using the Scion Image J software. For these analyses the investigator was blind to experimental groups and measurements were obtained in at least five consecutive tissue sections per animal containing the desired structure.

3.5. Assays of hepatic antioxidant enzymatic activities and oxidative stress parameters

By different assays, we evaluated in the liver of VEH-treated LFD rats, HF rats treated with both VEH and OEA (10 mg/kg i.p.) and pair-HF rats, the activities of key enzymes involved in the balance of the redox state, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase and oxidative stress parameters such as H_2O_2 and malondialdehyde (MDA). Particularly, the glutathione peroxidase (GPx) assay was performed following the instructions reported in Cayman's GPx assay kit (#703102, Cayman Chemical, Ann Arbor, MI, USA). The activity was measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione produced upon reduction of hydroperoxide by GPx was recycled to its reduced state by GR and nicotinamide adenine dinucleotide

phosphate. The change in absorbance at 340 nm was monitored for 3 min. A blank with all ingredients except for the sample was also included in the procedure. Specific activity was calculated as U/mg protein.

The activity of SOD was assayed by SOD assay Kit (#19160, Sigma-Aldrich), which utilized a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. SOD activity was calculated using the equation obtained from the linear regression of a standard curve built in the same experimental conditions.

Catalase activity was assayed by a catalase assay kit (#219265, Sigma-Aldrich) that uses a reaction between the catalase present in the sample and hydrogen peroxide (H_2O_2) to produce water and oxygen. The unconverted H2O2 reacts with a probe to generate a product that can be measured colorimetrically at 540 nm.

For oxidative stress parameters, H_2O_2 was measured by Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (#A22188, Thermo Fisher Scientific). The oxidation product of 1:1 stoichiometric reaction between Amplex® Red reagent and H_2O_2 was spectrophotometrically measured at 571 nm.

Finally, for the MDA assay, liver tissues were homogenized in the lysis buffer. MDA levels were measured by Lipid Peroxidation MDA assay kit according to manufacturer's instructions (#MAK085, Sigma-Aldrich).

3.6. Oil Red-O staining of lipid droplets and quantification

Fresh snap-frozen liver samples of VEH-treated LFD rats, HF rats treated with both VEH and OEA (10 mg/kg i.p.) and pair-HF rats were sliced on a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA) in 20-μm-thick serial sections, were mounted on positively charged slides

(Super Frost Plus, Menzel, Germany) and stored at −80 °C until processed for Oil Red-O staining. On the day of the experiment, following the protocol used in a paper already published [56], liver sections were fixed in 4% paraformaldehyde in PBS (pH 7,4) for 5 min at room temperature; after several washes in distilled water sections were stained with Oil Red-O solution at 5% in isopropyl alcohol for 1h. Sections were then rinsed with distilled water, washed with 10% isopropanol, and counterstained with hematoxylin for 30s. After final washes, slides were cover-slipped with Fluoromount (Sigma-Aldrich).

Sections 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 light field condition at 40× magnification.

Lipid droplet average size was determined as reported in [56] through the Scion Image J software. For the analyses the investigator was blind to experimental groups and measures were obtained by analyzing at least 50 sections for each group in three independent experiments.

3.7. Western blot analysis

A first western blot experiment was conducted only in HF rats treated with both VEH and OEA (10 mg/kg i.p.) to evaluate the expression of:

- key enzymes involved in different steps of fatty acid and triacylglycerol (TAG) syntheses: diacylglycerol acyltransferase (DGAT)-1, 2, stearoyl-CoA desaturase (SCD)-1, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [57,58]
- proteins involved in lipid uptake such as the cluster of differentiation (CD)36 [59].

- the peroxisome proliferator-activated receptor (PPAR)-γ as regulator of several proteins associated with fatty acid synthesis and TAG storage [60]
- the sterol regulatory element-binding protein (SREBP)-1 that represents the master transcription factor regulating lipogenic enzyme expression in the liver [61].

Livers from VEH and OEA rats were suspended in a medium containing 250 mM sucrose, 1,0 mM Tris-HCl (pH 7,4), 0,5 mM EGTA. In the same medium, the liver was gently homogenized with a Potter-Elvehjem homogenizer.

Proteins were extracted from the whole liver homogenate using RIPA lysis buffer (15 mM Tris-HCl, 165 mM-NaCl, 0,5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS), with a protease inhibitor cocktail (1:1000; Sigma-Aldrich, St. Louis, MI, USA) and 1 mM- PMSF (phenyl-methane-sulfonyl fluoride solution). Total protein levels of the lysate were determined using the Bradford method (Bio-Rad Laboratories). After boiling for 5 min, proteins were loaded and separated by SDS-polyacrylamide gel. The samples were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and blocked at room temperature for 1h using 5% (*w*/*v*) non-fat milk in TBS-Tris buffer (Tris-buffered saline (TBS) plus 0.5% (*v*/*v*) Tween-20, TTBS). The membranes were incubated overnight at 4 °C with primary antibodies against ACC (Cell Signaling #3676, Rabbit 1:1000), FAS (Cell Signaling #3180, Rabbit 1:1000), DGAT1 (Novus Biologicals #NB110-41487, Rabbit 1:1000), DGAT2 (Novus Biologicals #NBP1-71701, Mouse 1:1000), PPAR-γ (Santa Cruz #sc-7273, Mouse 1:500), SREBP-1 (Santa Cruz #sc-365513, Mouse 1:500), CD36 (Santa Cruz #sc-7309, Mouse 1:1000), and SCD1 (Santa Cruz #sc-58420, Mouse 1:1000). β-actin antibody (Cell

Signaling #8457, Rabbit 1:1000), was used to determine loading fairness. After washing with TTBS, the blots were incubated with peroxidase-conjugated secondary antibodies (#A3687 and #A3682, Sigma-Aldrich) at 1:10.000 dilutions at room temperature for 1–2h. The blots were then washed thoroughly in TTBS. Western blotting analyses were performed using Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK). Densitometric analysis of the immunoblots was performed using Image Lab^{TM} Version 6.0.1 2017 (Bio-Rad Laboratories, Inc., Segrate (MI)-Italy) software.

A second western blot experiment was conducted in VEH-treated LFD rats, HF rats treated with both VEH and OEA (10 mg/kg i.p.) and pair-HF rats to evaluate the expression of:

- key enzymes involved in fatty acid synthesis and transport: SCD1, ACC FAS [57,58] and low-density lipoprotein receptor (LDLR)
- proteins directly or indirectly involved in the fatty acid oxidation: carnitine palmitoyl-transferase (CPT)-1 and PPAR-α receptor [60]
- transcription factors involved in both oxidative stress response and regulation of lipid metabolism: nuclear respiratory factor (Nrf)-1,2 [62–64] and PPAR-γ receptor as an Nrf-2-downstream transcriptional factor in the regulation of lipid metabolism [65–67]
- signals that coordinate the unfold protein response (UPR) involved in the readjustment of ER folding capacity to restore protein homeostasis: X-box-binding protein (XBP)-1, activating transcription factor (ATF)-6 and binding-immunoglobulin protein aka GRP-78 (BIP) [68–73].

As reported above, livers from VEH-treated LFD rats, HF rats treated with both VEH and OEA (10 mg/kg i.p.) and pair-HF rats were suspended in a

medium containing 250 mM sucrose, 1,0 mM Tris-HCl (pH 7,4), 0,5 mM EGTA. In the same medium, the liver was gently homogenized with a Potter-Elvehjem homogenizer.

Proteins were extracted from the whole liver homogenate using RIPA lysis buffer (15 mM Tris-HCl, 165 mM-NaCl, 0,5% Na-deoxycholate, 1% Triton X-100 and 0.1% SDS), with a protease inhibitor cocktail (1:1000; Sigma-Aldrich, St. Louis, MI, USA) and 1 mM- PMSF (phenyl-methane-sulfonyl fluoride solution). Total protein levels of the lysate were determined using the Bradford method (Bio-Rad Laboratories). After boiling for 5 min, proteins were loaded and separated by SDS-polyacrylamide gel. The samples were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and blocked at room temperature for 1h using 5% (*w*/*v*) non-fat milk in TBS-Tris buffer (Tris-buffered saline (TBS) plus 0.5% (*v*/*v*) Tween-20, TTBS). The membranes were incubated overnight at 4 ˚C with primary antibodies against LDLR (#PAB8804, Rabbit 1:1000, Abnova, Milan, Italy), CPT-1 (#12252S, Rabbit 1:500, Cell Signaling), PPAR-α (#sc-9000, Rabbit 1:1000, Santa Cruz), Nrf1 (#sc-28379, Mouse 1:1000, Santa Cruz), Nrf2 (#sc-365949, Mouse 1:1000, Santa Cruz), XBP1 (#sc-7160, Rabbit 1:1000, Santa Cruz), ATF6 (#sc-166659, Mouse 1:1000, Santa Cruz), and BIP (#3177, Rabbit 1:1000, Cell Signaling). β-actin antibody (Cell Signaling #8457, Rabbit 1:1000), was used to determine loading fairness. After washing with TTBS, the blots were incubated with peroxidase-conjugated secondary antibodies (#A3687 and #A3682, Sigma-Aldrich) at 1:10.000 dilutions at room temperature for 1–2h. The blots were then washed thoroughly in TTBS. Western blotting analyses were performed using Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Little Chalfont, UK). Densitometric analysis of the immunoblots was

performed using Image Lab^{TM} Version 6.0.1 2017 (Bio-Rad Laboratories, Inc., Segrate (MI)-Italy) software.

3.8. Statistical analysis

All data were expressed as mean ± SEM. Regarding the behavioral experiment, the data obtained from the daily monitoring of the BWG and FI 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 (IBM SPSS, version 22, IBM Analytics). Data obtained from immunohistochemical, immunofluorescence and Oil Red-O staining results were statistically analyzed by two-way ANOVA with "diet" (LF and HF) and "treatment" (VEH, OEA and pair feeding only for Oil Red-O staining) as the two factors. Tukey's test was used as a *post hoc* test to perform multiple comparisons (IBM SPSS, version 22, IBM Analytics). In the first western blot experiment, the comparison between the two groups (HF rats treated with both VEH and OEA) was analyzed by Student's t-test (GraphPad Software, San Diego, CA, USA). Data obtained from second western blot experiment and from the assays of hepatic antioxidant enzymatic activities and oxidative stress parameters, were statistically analyzed by one-way ANOVA setting "treatment" (VEH, OEA, pair-feeding) as fixed variable and the Tuckey test was used as *post hoc* to perform multiple comparisons (GraphPad Software, San Diego, CA, USA). Moreover because of the difference in the number of brain slices and liver samples 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, as reported in a paper published by my research group [53].

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

4. Results

4.1. Effects of chronic Oleoylethanolamide treatment on food intake and body weight gain

To evaluate the hypophagic effect of OEA administration (10 mg/kg, i.p.), the **FI (kcal/kg)** was monitored throughout the two weeks of chronic treatment. Particularly, in LFD animals, the two-way ANOVA for repeated measures analysis showed a significant effect of the time (F_{time}= 2275,787; df= 1/35; °°°p<0,001), a significant effect of the treatment ($F_{treatment}$= 26,165; df= 1/35; °°°p<0,001), and a significant effect of the 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 FI from the third day of treatment (LFDV vs LFDO: °p<0,05), and until the end of the experiment (°°p<0,01 on the fourth day and °°°p<0,001 from the fifth to the last day) (Fig. 2.3-A).

Further the FI intake of HFD animals was analyzed. The results obtained with the two-way ANOVA for repeated measures analysis showed a significant effect of the time (F_{time}= 1938,974; df= 1/28; °°°p<0,001), a significant effect of the treatment ($F_{treatment}$= 8,585; df= 1/28; °°p<0,01) and a significant effect of the 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 FI in HFD animals from the eighth day of treatment (HFDV vs HFDO: °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. 2.3-B).

Lastly, the FI of the SHIFT animals was analyzed. The two-way ANOVA for repeated measures analysis showed a significant effect of the time (F_{time}= 869,836; df= 1/26; °°°p<0,001), a significant effect of the treatment ($F_{treatment}$= 14,760; df= 1/26; °°°p<0,001), and a significant effect of the 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 treatment significantly decreases the FI of SHIFT animals from day 6 (°p<0,05) 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. 2.3-C).

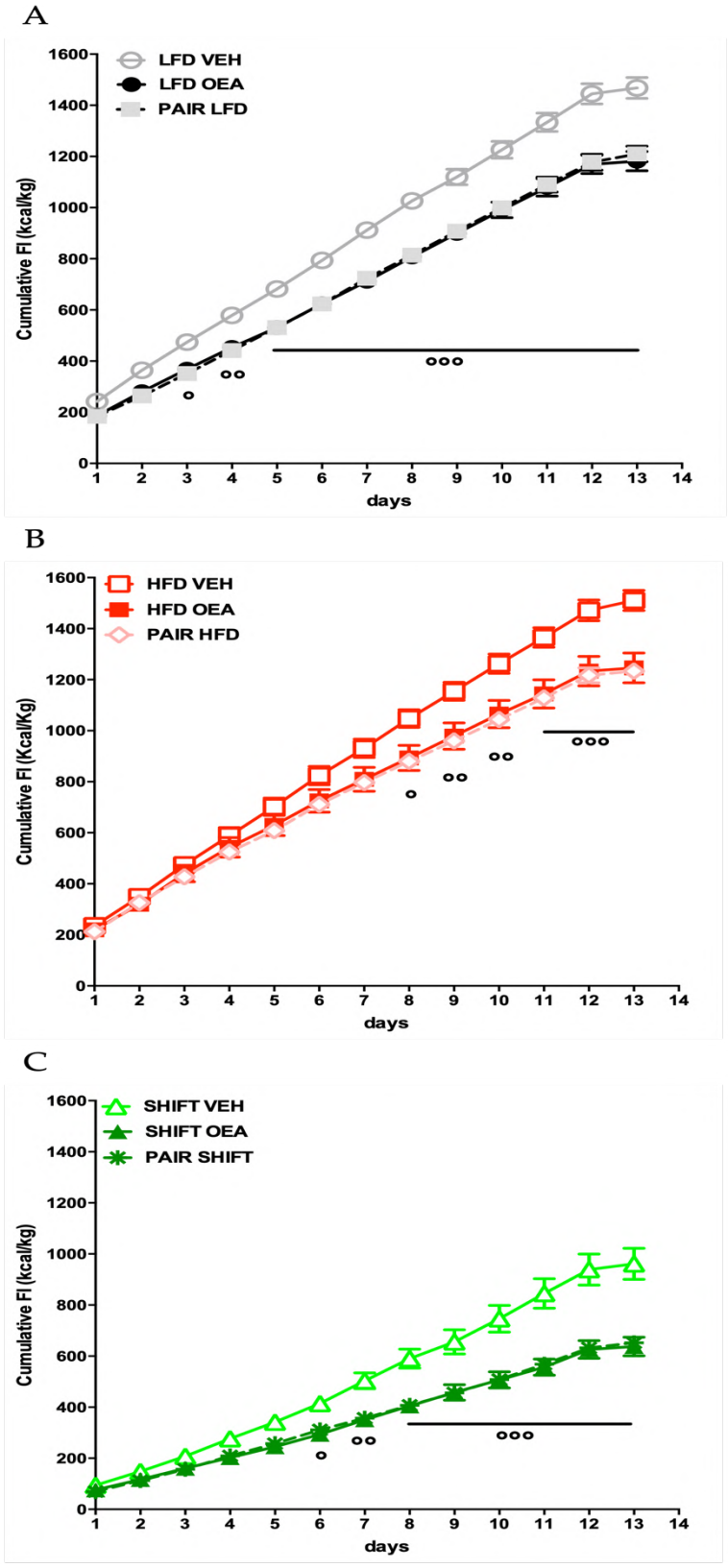

Fig 2.3: Time course of the caloric intake of animals exposed to LFD (A), HFD (B) or SHIFT (C) diet and treated with either VEH or OEA (10 mg/kg, i.p.) for fourteen days. Data are expressed as mean ±SEM. °p<0,05, °°p<0,01; °°°p<0,001 vs VEH-treated controls.

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 the time (F_{time}= 2,479; df= 1/35; *p<0,05), a significant effect of the treatment ($F_{treatment}$= 23,393; df= 1/35; ***p<0,001), and a significant effect of the 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 fourth day of treatment, chronic OEA administration significantly decreases the BW of LFD animals (LFDV vs LFDO: °°p<0,01 on the fourth day; °°°p<0.001 from the fifth day to the end of the treatment) (Fig. 2.4-A).

In the same way, the results obtained with the two-way ANOVA for repeated measures showed, in HFD animals, a significant effect of the time (F_{time}= 12,719; df= 1/28; ***p<0,001), a significant effect of the treatment ($F_{treatment}$= 8,397; df= 1/28; **p<0,01) and a significant effect of the 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 treatment significantly decreases the BW of HFD-fed animals from the sixth day of chronic administration to the end of the treatment (HFDV vs HFDO: °°p<0,01 on the sixth day; °°°p<0,001 for the remaining days) (Fig. 2.4-B).

The chronic treatment with OEA (10 mg/kg, i.p.), in addition, decreases the BW gain in SHIFT animals. In fact, the results of the two-way ANOVA for repeated measures showed a significant effect of the time (F_{time}= 125,040; df= 1/26; ***p<0,001) and a significant effect of the interaction between time and treatment ($F_{interaction}$= 6,550; df= 1/26; ***p<0,001), but no significant effect of the treatment ($F_{treatment}$= 2,126; df= 1/26; p=0,141). Moreover, the Bonferroni post hoc test for multiple comparisons showed that OEA treatment

decreases BW in SHIFT animals from the day eleventh to the end of the treatment (SHIFTV vs SHIFTO: °°p<0,01 on day eleventh; °°°p<0,001 for the remaining days) (Fig. 2.4-C).

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, i.p.) induces, in the LFD group, a significant decrease of BW compared to pLFD animals from the day seventh to the end of the treatment (LFDO vs pLFD: *p<0,05 on day 7 and 11; ***p<0.001 from day 8 to day 11 and from day 12 to the end of the treatment) (Fig. 2.4-A).

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 (HFDO vs pHFD: **p<0,01 on days 9 and 10; ***p<0,001 from the eleventh day) (Fig. 2.4-B).

Finally, the Bonferroni post hoc test for multiple comparisons showed that, in SHIFT animals, OEA induces a significant decrease of BW gain on days 12 and 13 compared to pSHIFT animals (SHIFTO vs pSHIFT= *p<0,05) (Fig. 2.4-C).

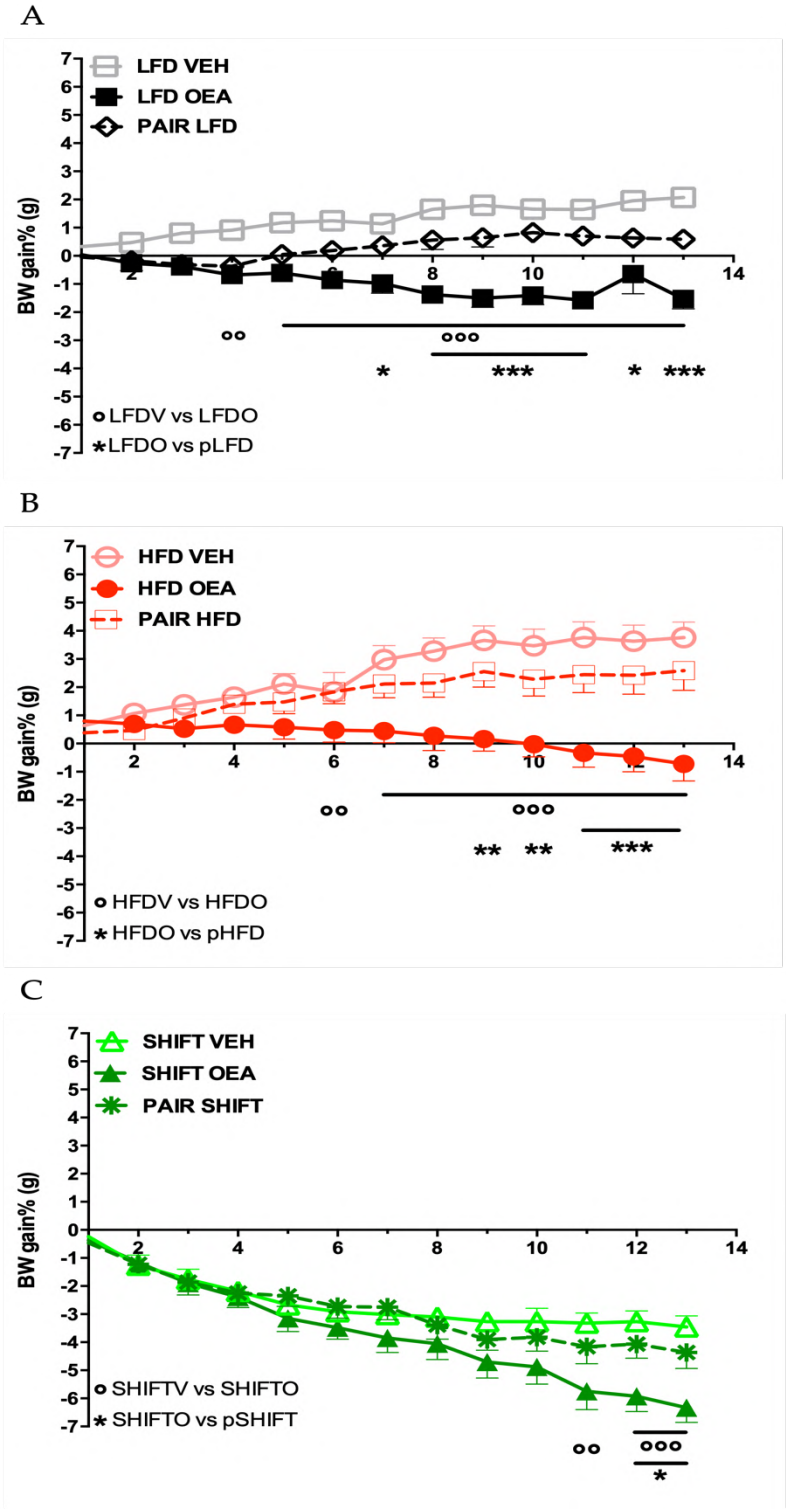

Fig. 2.4: Time course of the body weight gain of animals exposed to LFD (A), HFD (B) or SHIFT (C) diet and treated with either VEH or OEA (10 mg/kg, i.p.) for fourteen days. Data are expressed as mean± SEM. °°p<0,01; °°°p<0,001 vs VEH-treated controls; *p< 0,05, **p<0,01, ***p<0,001 vs pair-feeding animals in the same diet group.

4.2. Effects of chronic Oleoylethanolamide treatment on neuroinflammation

Concerning the levels of GFAP expression in the HYPO, HIPPO and DG of DIO rat model (Fig. 2.5-A), the results obtained by two-way ANOVA showed a significant effect of the diet in the HYPO (F_{diet}= 13,693, df= 1/11, p<0,01), HIPPO (F_{diet}= 4,976, df= 1/11, p<0,05), and DG (F_{diet}= 13,301, df= 1/11, p<0,01), a significant effect of the treatment in the HIPPO ($F_{treatment}$= 13,516, df= 1/11, p<0,01), but no significant effect of the treatment in both the HYPO ($F_{treatment}$= 2,631, df= 1/11, p=0,13) and the DG ($F_{treatment}$= 3,559, df= 1/11, p=0,08) and no significant effect of the interaction between the two factors in the HYPO ($F_{interaction}$= 4,729, df= 1/11, p=0,05), HIPPO ($F_{interaction}$= 2,345, df= 1/11, p=0,153) and DG ($F_{interaction}$= 1,533, df= 1/11, p=0,24).

The results obtained from post-hoc Tuckey test, in the HYPO, HIPPO and DG, showed a significant increase of the levels of GFAP expression in HF rats treated with VEH compared to VEH-treated LF rats (HYPO and DG: p<0,01; HIPPO: p<0,01) and a significant decrease of the levels of GFAP expression in HF rats treated with OEA compared to the rats belonging to the same diet group but treated with VEH (HYPO and HIPPO: p<0,01; DG: p<0,05) (Fig. 2.5-B).

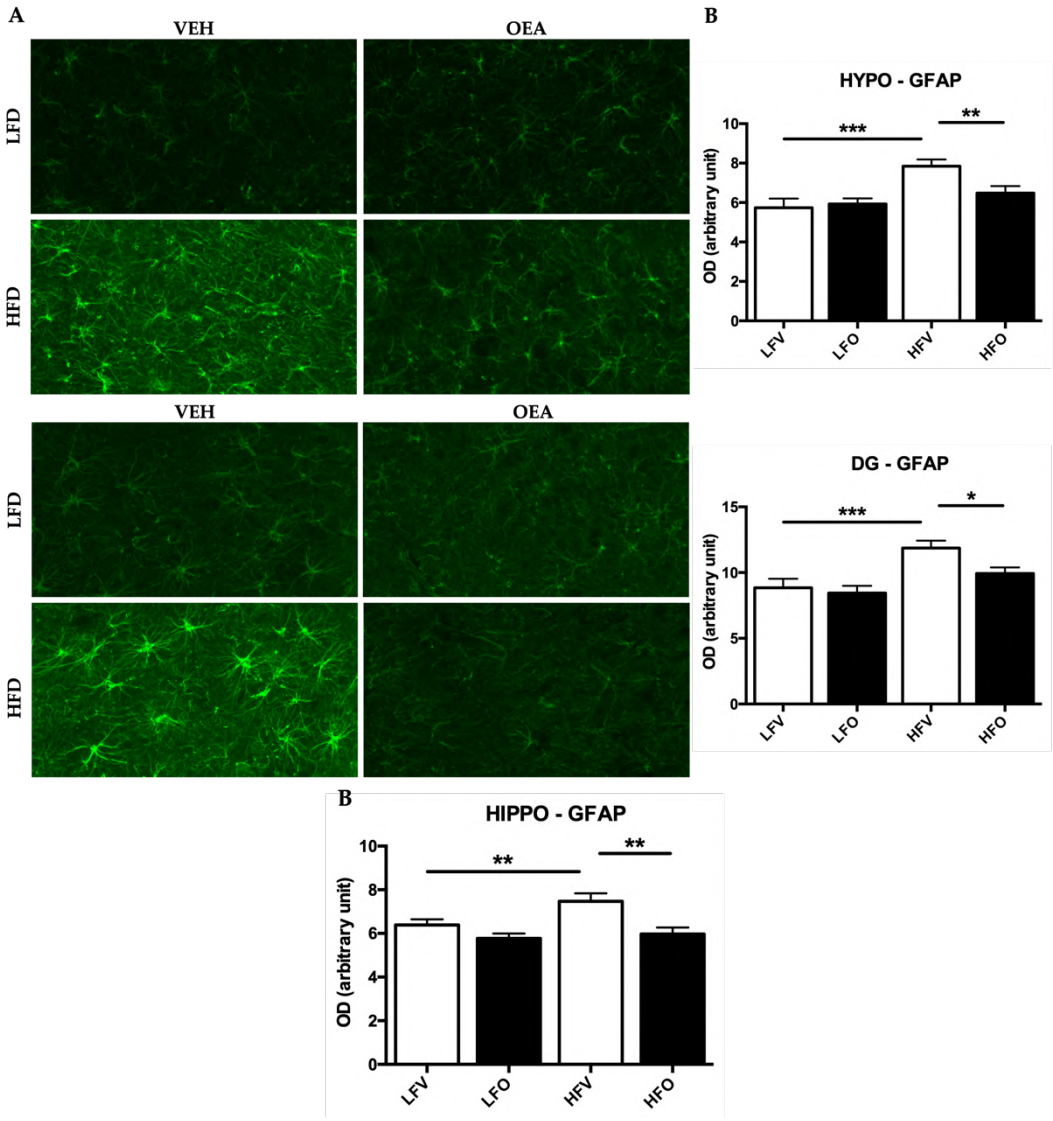

Fig. 2.5: Effects of chronic OEA treatment on the expression of GFAP in the HYPO, HIPPO and DG of DIO rat model. (A) Representative photomicrographs (x20 magnification) of fluorescent GFAP immunostaining within the HYPO and DG; representative images of fluorescent GFAP immunostaining within the HIPPO are absent as it represents the sum of the results obtained in its sub-regions. (B) Semiquantitative densitometric analysis of the expression of GFAP within the HYPO, HIPPO and DG. Data are expressed as mean ± SEM. *p<0,05 **p<0,01 ***p<0,001

4.3. Effects of chronic Oleoylethanolamide treatment on neurogenesis

Concerning DCX-positive neurons in the DG of DIO rat model (Fig 2.6 A), the results obtained by two-way ANOVA showed a significant effect of the treatment ($F_{treatment}$= 71,558, df= 1/11, p=0,13) and no significant effect of both the diet (F_{diet}= 4,088, df= 1/11, p=0,06) and the interaction between the two factors ($F_{interaction}$= 2,965, df= 1/11, p=0,11).

The results obtained from post-hoc Tuckey test, showed a significant increase of DCX-positive neurons in both LF and HF rats treated with OEA compared to the respective group of rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig 2.6-B).

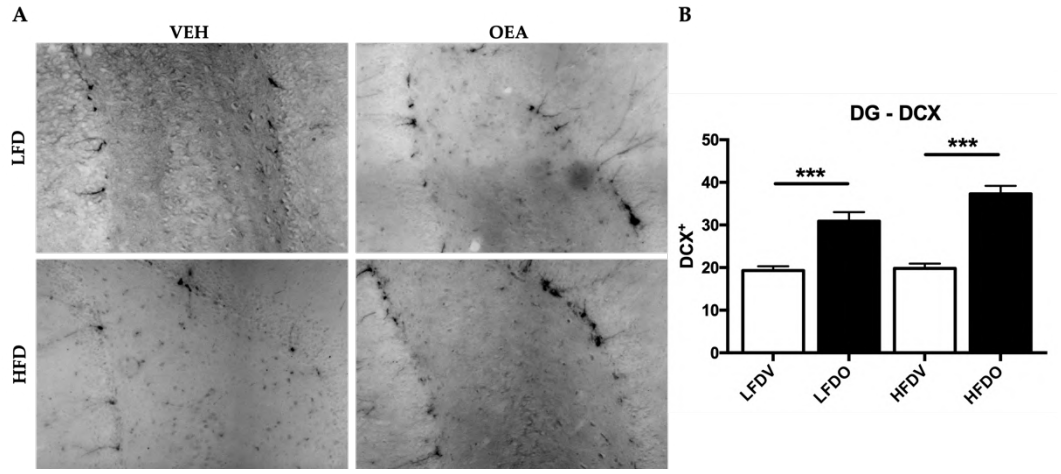

Fig. 2.6: Effects of chronic OEA treatment on DCX-positive neurons in the DG of DIO rat model. (A) Representative photomicrographs (x10 magnification) of DCX-immuno-positive neurons within the DG; (B) Analysis of manually counted DCX-positive neurons within the DG. Data are expressed as mean ± SEM. ***p<0,001

4.4. Effects of chronic Oleoylethanolamide treatment on the expression of Blood Brain Barrier proteins

Concerning the levels of VIM expression in the ARC of DIO rat model (2.7-A), the results obtained by two-way ANOVA showed no significant effect of the diet (F_{diet}= 4,430, df= 1/11, p=0,06), no significant effect of the treatment ($F_{treatment}$= 0,299, df= 1/11, p=0,59) and no significant effect of the interaction between the two factors ($F_{interaction}$= 0,842, df= 1/11, p=0,38).

No significantly variation were obtained in the results from post-hoc Tuckey test (Fig 2.7-B).

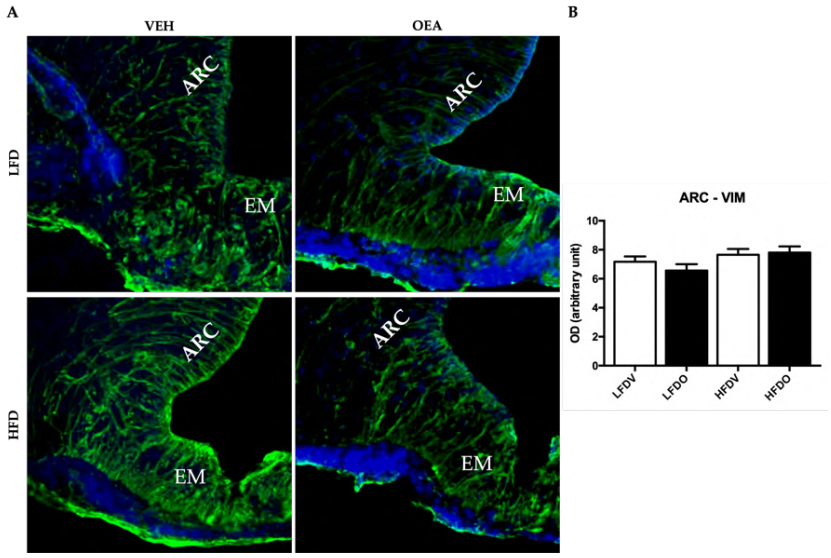

Fig. 2.7: Effects of chronic OEA treatment on the expression of VIM in the ARC of DIO rat model. (A) Representative photomicrographs (x20 magnification) of fluorescent VIM immunostaining within the ARC; (B) Semiquantitative densitometric analysis of the expression of VIM within the ARC. Data are expressed as mean ± SEM.

The results obtained by two-way ANOVA, concerning the levels of VIM expression in the AP of DIO rat model (Fig. 2.8-A), showed a significant effect of both the diet (F_{diet}= 6,490, df= 1/11, p<0,05) and the treatment ($F_{treatment}$= 4,936, df= 1/11, p<0,05) but no significant effect of the interaction between the two factors ($F_{interaction}$= 2,433, df= 1/11, p=0,14). The results

obtained from post-hoc Tuckey test, showed a significant increase of the levels of VIM expression in LF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH ($p<0,05$) (Fig 2.8-B).

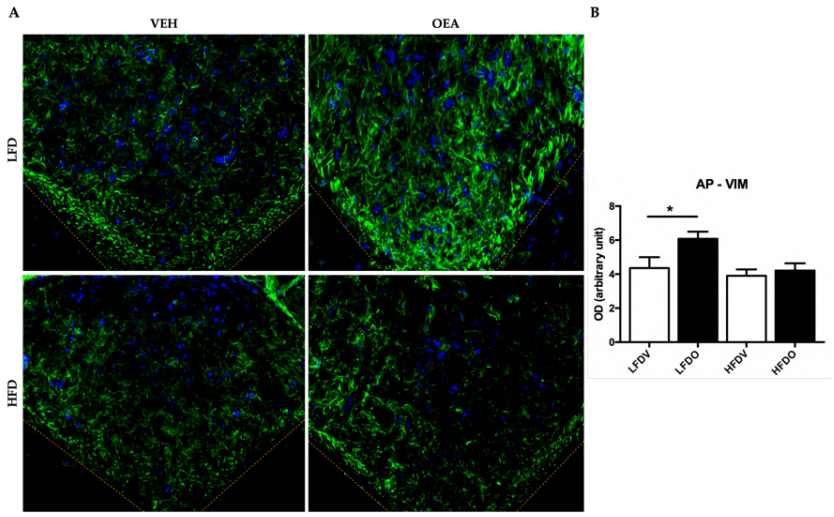

Fig. 2.8: Effects of chronic OEA treatment on the expression of VIM in the AP of DIO rat model. (A) Representative photomicrographs (x20 magnification) of fluorescent VIM immunostaining within the AP; (B) Semiquantitative densitometric analysis of the expression of VIM within the AP. Data are expressed as mean ± SEM. *$p<0,05$

Concerning the levels of VIM expression in the NST and its sub-nuclei (SOLC, SOLDM, SOLM, SOLVL) of DIO rat model (Fig. 2.9-B), the results obtained by two-way ANOVA showed:

- in the NST, no significant effect of the diet (F_{diet}= 2,129, df= 1/11, p=0,17), no significant effect of the treatment ($F_{treatment}$= 3,434, df= 1/11, p=0,09) and no significant effect of the interaction between the two factors ($F_{interaction}$= 3,331, df= 1/11, p=0,09)
- in the SOLC, no significant effect of the diet (F_{diet}= 4,796, df= 1/11, p=0,05), no significant effect of the treatment ($F_{treatment}$= 0,535, df= 1/11, p=0,47) and no significant effect of the interaction between the two factors ($F_{interaction}$= 2,356, df= 1/11, p=0,15)

- in the SOLDM, a significant effect of the interaction between the two factors ($F_{interaction}$= 5,934, df= 1/11, p<0,05) but no significant effect of the diet (F_{diet}= 0,079, df= 1/11, p=0,78) and no significant effect of the treatment ($F_{treatment}$= 0,172, df= 1/11, p=0,68)
- in the SOLM, no significant effect of the diet (F_{diet}= 1,131, df= 1/11, p=0,31), no significant effect of the treatment ($F_{treatment}$= 1,488, df= 1/11, p=0,248) and no significant effect of the interaction between the two factors ($F_{interaction}$= 1,265, df= 1/11, p=0,284)
- in the SOLVL, a significant effect of both the diet (F_{diet}= 8,577, df= 1/11, p<0,05) and the treatment ($F_{treatment}$= 11,558, df= 1/11, p<0,01) but no significant effect of the interaction between the two factors ($F_{interaction}$= 2,978, df= 1/11, p=0,11)

The results obtained from post-hoc Tuckey test, showed, in the SOLVL, a significant increase of the levels of VIM expression in LF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,01) (Fig 2.9-F), a significant decrease of the levels of VIM expression in the SOLC of HF rats treated with VEH compared to VEH-treated LF rats (p<0,05) (Fig. 2.9-C) and a decrease of the levels of VIM expression in the NST of HF rats treated with VEH compared to VEH-treated LF rats (p<0,05) (Fig. 2.9-G); moreover, the results obtained showed a significant increase of the levels of VIM expression in both SOLDM and NST of HF rats treated with OEA compared to VEH-treated HF rats (p<0,05) (Fig. 2.9-D,G) and an increase, although not statistically significant, of the levels of VIM expression in both SOLC and SOLM of HF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p=0,09) (Fig. 2.9-C,E).

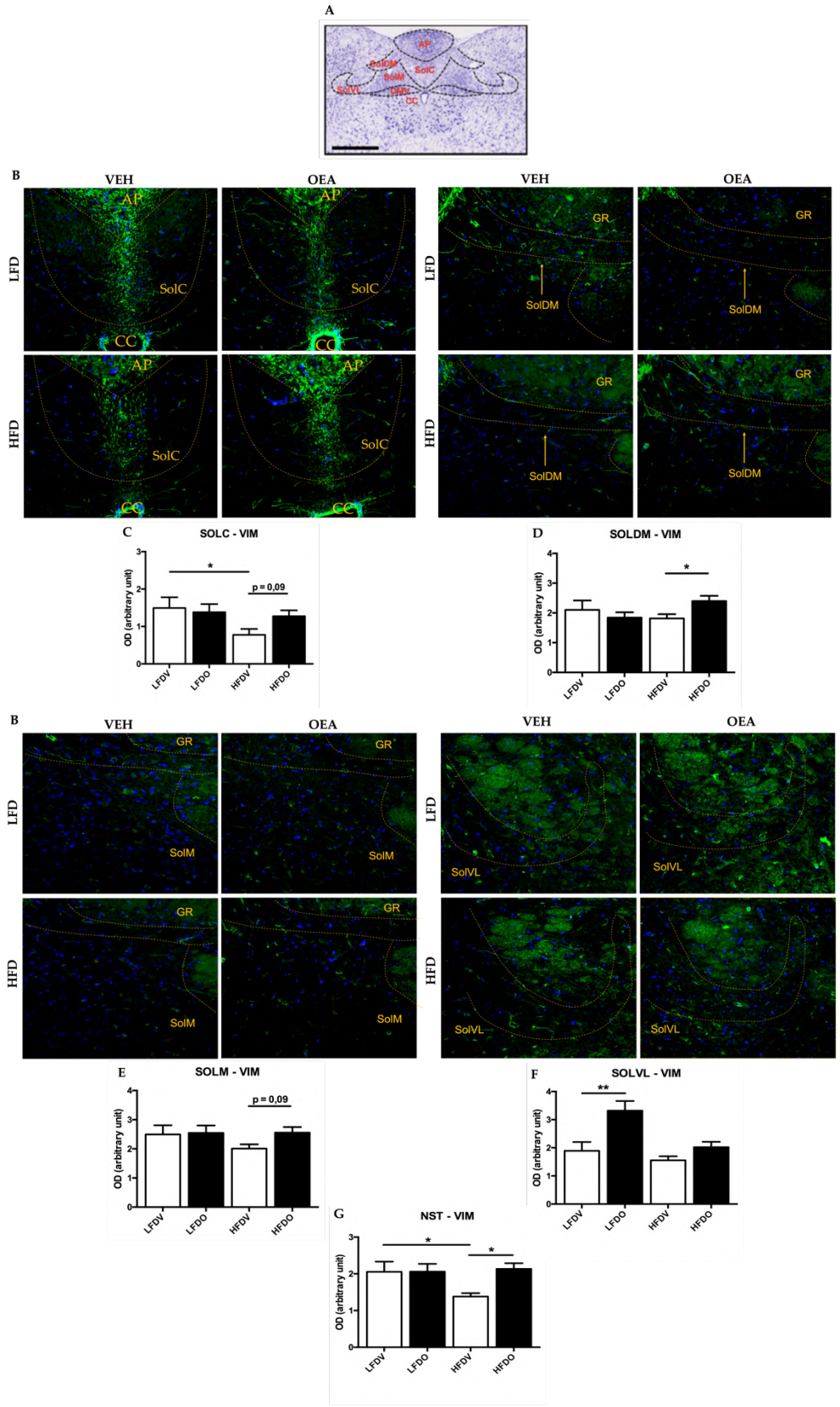

Fig. 2.9: Effects of chronic OEA treatment on the expression of VIM in the NST and its sub-nuclei of DIO rat model. (A) Representative photomicrographs showing Nissl staining of coronal brainstem sections; (B) Representative photomicrographs (x20 magnification) of fluorescent VIM

immunostaining within the NST and its sub-nuclei; representative images of fluorescent VIM immunostaining within the NST are absent as it represents the sum of the results obtained in its sub-nuclei. Semiquantitative densitometric analysis of the expression of VIM within the SOLC (C), SOLDM (D), SOLM (E), SOLVL (F) and NST (G). Data are expressed as mean ± SEM. *p<0,05 **p<0,01.

Concerning the levels of ZO-1 expression in the ARC of DIO rat model (Fig. 2.10-A), the results obtained by two-way ANOVA showed a significant effect of the interaction between the two factors ($F_{interaction}$= 6,218, df= 1/11, p<0,05) but no significant effect of both the diet (F_{diet}= 0,204, df= 1/11, p=0,66) and the treatment ($F_{treatment}$= 0,536, df= 1/11, p=0,47).

The results obtained from post-hoc Tuckey test, showed a significant increase of the levels of ZO-1 expression in HF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,05) (Fig 2.10-B).

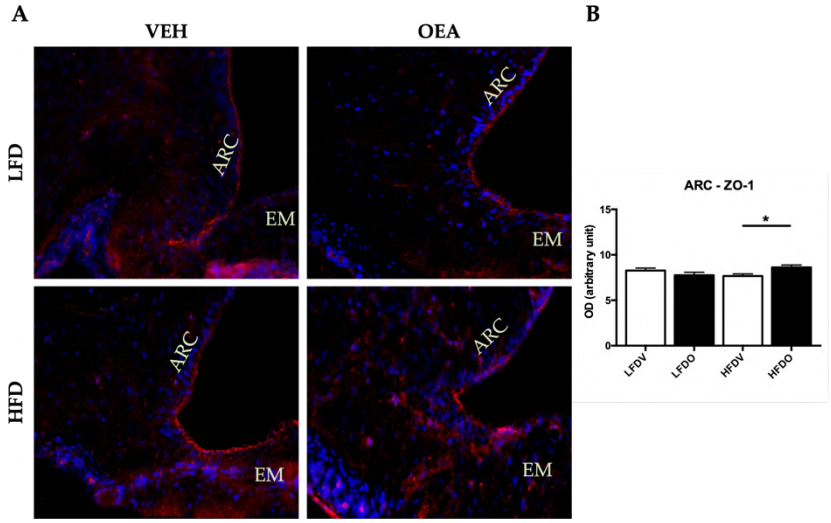

Fig. 2.10: Effects of chronic OEA treatment on the expression of ZO-1 in the ARC of DIO rat model. (A) Representative photomicrographs (x20 magnification) of fluorescent ZO-1 immunostaining within the ARC; (B) Semiquantitative densitometric analysis of the expression of ZO-1 within the ARC. Data are expressed as mean ±SEM. *p<0,05

The results obtained by two-way ANOVA, concerning the levels of ZO-1 expression in the AP of DIO rat model (Fig. 2.11-A), showed a significant

effect of the diet (F_{diet}= 6,116, df= 1/11, p<0,05) but no significant effect of both the treatment ($F_{treatment}$= 4,099, df= 1/11, p=0,07) and the interaction between the two factors ($F_{interaction}$= 1,376, df= 1/11, p=0,26). The results from post-hoc Tuckey test, showed a significantly increase of the levels of ZO-1 expression in HF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,05) (Fig 2.11-B).

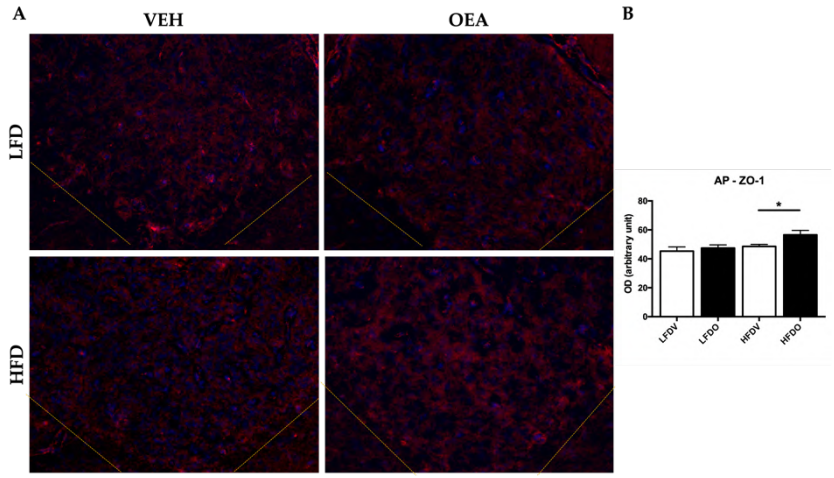

Fig. 2.11: Effects of chronic OEA treatment on the expression of ZO-1 in the AP of DIO rat model. (A) Representative photomicrographs (x20 magnification) of fluorescent ZO-1 immunostaining within the AP; (B) Semiquantitative densitometric analysis of the expression of ZO-1 within the AP. Data are expressed as mean ±SEM. *p<0,05

Concerning the levels of ZO-1 expression in the NST and its sub-nuclei (SOLC, SOLDM, SOLM, SOLVL) of DIO rat model (Fig 2.12-B), the results by two-way ANOVA showed:

- in the NST, no significant effect of the diet (F_{diet}= 1,049, df= 1/11, p=0,32), no significant effect of the treatment ($F_{treatment}$= 2,793, df= 1/11, p=0,12) and no significant effect of the interaction between the two factors ($F_{interaction}$= 0,079, df= 1/11, p=0,41)
- in the SOLC, no significant effect of the diet (F_{diet}= 1,873, df= 1/11, p=0,19), no significant effect of the treatment ($F_{treatment}$= 4,278, df= 1/11, p=0,06) and

no significant effect of the interaction between the two factors ($F_{interaction}$= 0,026, df= 1/11, p=0,87)

- in the SOLDM, no significant effect of the diet (F_{diet}= 0,175, df= 1/11, p=0,68), no significant effect of the treatment ($F_{treatment}$= 1,920, df= 1/11, p=0,19) and no significant effect of the interaction between the two factors ($F_{interaction}$= 1,414, df= 1/11, p=0,25)
- in the SOLM, no significant effect of the diet (F_{diet}= 1,115, df= 1/11, p=0,31), no significant effect of the treatment ($F_{treatment}$= 3,485, df= 1/11, p=0,08) and no significant effect of the interaction between the two factors ($F_{interaction}$= 0,207, df= 1/11, p=0,65)
- in the SOLVL, no significant effect of the diet (F_{diet}= 0,001, df= 1/11, p=0,97), no significant effect of the treatment ($F_{treatment}$= 3,522, df= 1/11, p=0,08) and no significant effect of the interaction between the two factors ($F_{interaction}$= 1,488, df= 1/11, p=0,24)

The results obtained from post-hoc Tuckey test, showed, in the SOLVL, a significant increase of the levels of ZO-1 expression in HF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH ($p<0,05$) (Fig 2.12-F) and an increase, although not statistically significant, of the levels of ZO-1 expression in the SOLDM and NST of HF rats treated with OEA compared to VEH-treated HF rats (SOLDM: p=0,06; NST: p=0,08) (Fig. 2.12-D,G). No significant variations were obtained in the levels of ZO-1 expression in both SOLC and SOLM (Fig. 2.12-C,E).

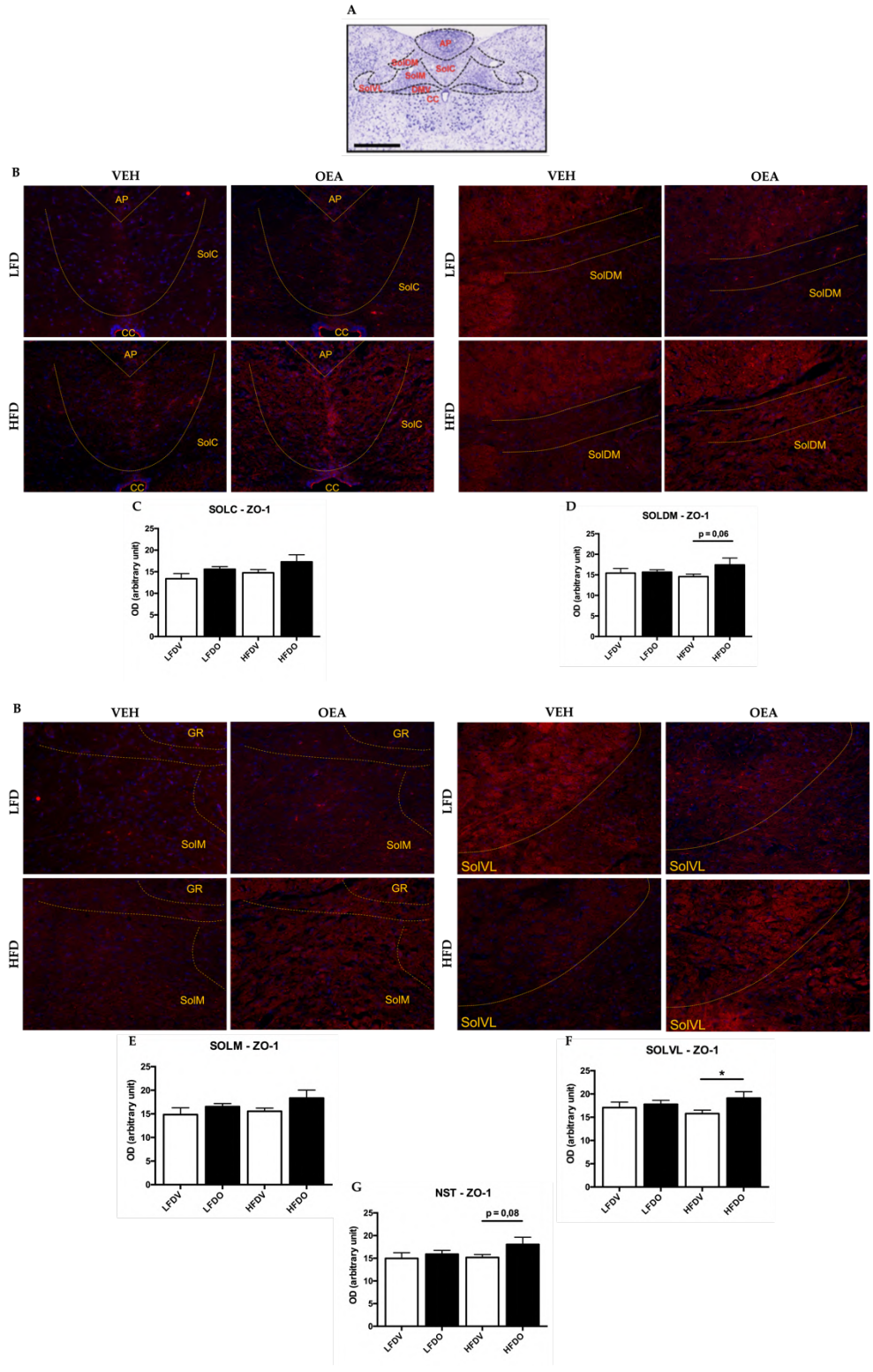

Fig. 2.12: Effects of chronic OEA treatment on the expression of ZO-1 in the NST and its sub-nuclei of DIO rat model. (A) Representative photomicrographs showing Nissl staining of coronal brainstem sections; (B) Representative photomicrographs (x20 magnification) of fluorescent ZO-1 immunostaining within the NST and its sub-nuclei; representative images of fluorescent ZO-1

immunostaining within the NST are absent as it represents the sum of the results obtained in its sub-nuclei. Semiquantitative densitometric analysis of the expression of ZO-1 within the SOLC (C), SOLDM (D), SOLM (E), SOLVL (F) and NST (G). Data are expressed as mean ± SEM. *p<0,05

4.5. Effects of chronic Oleoylethanolamide treatment on the expression of enzymes and proteins involved in lipid metabolism

The results obtained by Student's t-test, concerning the first set of western blot experiments for the expression of enzymes and proteins involved in lipid metabolism (Fig. 2.13-A and Fig 2.14-A), revealed in OEA-treated rats with respect to VEH-treated rats, a significant decreased expression of hepatic DGAT1 (p<0,01) and DGAT2 (p<0,05) (Fig. 2.13-B), SCD1 (p<0,05; Fig. 2.13-C), ACC (p<0,01; Fig. 2.13-D), FAS (p<0,01; Fig. 2.13-E) and CD36 (p<0,01; Fig. 2.13-F). Moreover, the results obtained showed a significant decreased protein level of both PPAR-γ (p<0,01; Fig. 2.14-B) and SREBP-1 (p<0,05; Fig. 2.14-C) in the liver of OEA rats with respect to VEH rats.

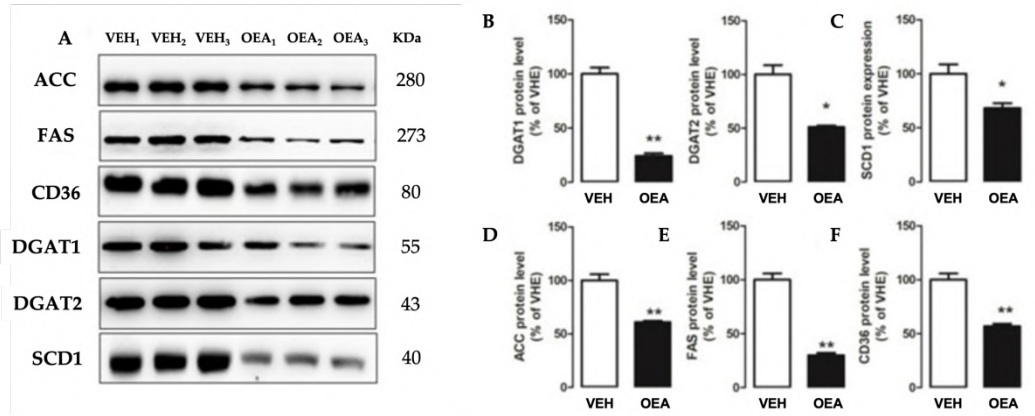

Fig. 2.13: Hepatic fatty acid and triacylglycerol synthesis enzymes. (A) Proteins extracted from hepatic VEH and OEA samples were subjected to SDS-page electrophoresis. Membranes were analyzed by immunoblotting using specific antibodies for acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS), CD36, diacylglycerol acyltransferase (DGAT)-1, DGAT2, and stearoyl-CoA desaturase 1 (SCD1). Each blot was normalized to the proper specific β-actin. (B-F) Blot signals were

quantified by densitometric analysis and reported as % of the VEH. Data are expressed as mean ± SEM. *p<0,05 **p<0,01

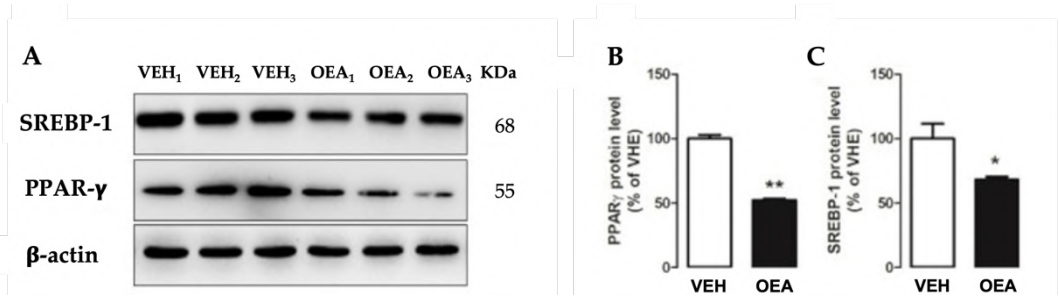

Fig. 2.14: Hepatic PPAR-γ and SREBP-1 protein expression. (A) Hepatic proteins from VEH- and OEA-treated rats were subjected to SDS-page electrophoresis. Membranes were then subjected to immunoblotting using specific antibodies for sterol regulatory binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor γ (PPAR-γ). (B, C) Signals were quantified by densitometric analysis and expressed as % of the VEH. Data are expressed as mean ± SEM. *p<0,05 **p<0,01

The results obtained by one-way ANOVA, concerning the expression of enzymes and proteins involved in lipid metabolism showed a significant effect of the treatment for: ACC ($F_{treatment}$= 183,3, df= 1/9, p<0,001), FAS ($F_{treatment}$= 116,6, df= 1/9, p<0,001), CPT1 ($F_{treatment}$= 13,78, df= 1/10, p<0,01), PPAR-α ($F_{treatment}$= 12,68, df= 1/8, p<0,01) LDLR ($F_{treatment}$= 69,06, df= 1/10, p<0,001) and SCD1 ($F_{treatment}$= 45,29, df= 1/10, p<0,001).

The results from second western blot experiment (Fig. 2.15-A), obtained from post hoc Tuckey test, showed that the hepatic expression of both ACC and FAS in the HF rats treated with VEH was significantly lower compared to VEH-treated LF rats (p<0,001; Fig. 2.15-B, C). Moreover, a significantly reduced expression in LDLR, involved in the transport of lipoproteins into the liver, after HFD feeding, was also detected (p<0,001; Fig. 2.15-E). When compared to the HV group, HO rats showed significantly decreased expression of ACC (p<0,01; Fig. 2.15-B), FAS (p<0,01; Fig. 2.15-C) and LDLR (p<0,05; Fig. 2.15-E). To note that both ACC and FAS expression were lower in HO than in HP (vs HV p<0,05), thus indicating that these effects could be

specifically attributed to OEA and not to its anorexigenic effect. Moreover, OEA administration to HFD-fed rats significantly increased expression of both CPT-1 (Fig. 2.15-D) and PPAR-α (Fig. 2.15-F). Finally, HF rats treated with OEA showed a significant decrease in the level of SCD1 compared to both HV ($p<0,01$; Fig. 2.15-G) and HP ($p<0,001$; Fig. 2.15-G) groups of rats.

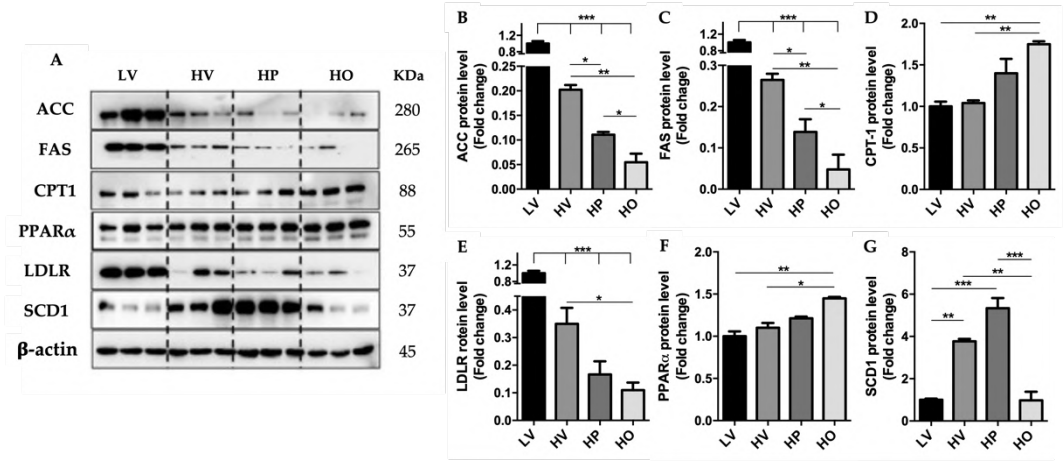

Fig. 2.15: (A) Representative immunoblots for acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), carnitine palmitoyltransferase-1 (CPT-1), peroxisome proliferator-activated receptor α (PPAR-α), low-density lipoprotein receptor (LDLR), and stearoyl-CoA desaturase 1 (SCD1); β-actin was used as a loading control. (B-G) Quantification of protein expression. Values in the histogram represent the mean ±SEM and are expressed as fold change relative to LV. *p<0,05 **p<0,01 ***p<0,001

4.6. Effects of chronic Oleoylethanolamide treatment on hepatic histopathological changes

The results obtained by two-way ANOVA, concerning the histological analyses of liver sections conducted through the O-red Oil staining protocol, showed a significant effect of both the diet (F_{diet}= 213,824, df= 1/11, $p<0,001$) and of the treatment ($F_{treatment}$= 66,439, df= 1/11, $p<0,05$); the interaction between the two factors was not considered.

The results obtained from post-hoc Tuckey test, showed a significant increase of the mean particle size in HF rats treated with VEH compared to VEH-treated LF rats ($p<0,001$; Fig. 2.16-B) and a significant decrease of the

mean particle size in both the HO and HP groups of rats, as compared to the HV group (p<0,001; Fig. 2.16-B); moreover, animals treated with OEA showed a further significant decrease in the mean particle size, compared to HP rats (p<0,05; Fig. 2.16-B), thus suggesting that the fat accumulation of HFD-fed rats can be successfully decreased by OEA treatment, and this effect might not be the mere consequence of the reduced caloric intake induced by OEA. Moreover, it has been shown that HFD per se caused an increase in the amount of lipid stored as lipid droplets (HV vs. all the other groups, Fig. 2.16-A) and that such increase was abolished in both the HO and HP groups of rats, as compared to the HV group (Fig. 2.16-A).

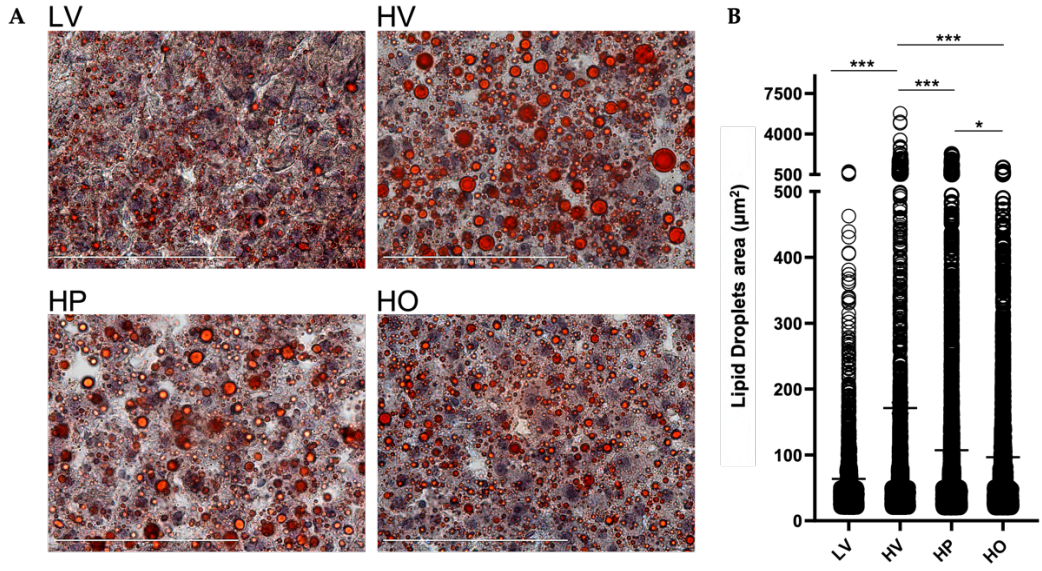

Fig. 2.16: (A) Representative microscopic observations of the Oil red-O-stained lipid droplets and relative quantification (B) from low-fat + VEH (LV) (n = 5), high fat + VEH (HV), pair feeding (HP) and high fat + OEA (HO) rats. Scale bar = 50 µm. Data are expressed as mean ±SEM. *p<0,05 ***p<0,001.

4.7. Effects of chronic Oleoylethanolamide treatment on hepatic oxidative and endoplasmic reticulum stress

- *OEA improved hepatic oxidative stress*

The results obtained by one-way ANOVA, concerning selected parameters associated with oxidative stress showed a significant effect of the treatment for: H_2O_2 ($F_{treatment}$= 7,856, df= 1/18, p<0,05), MDA ($F_{treatment}$= 21,50, df= 1/9, p<0,01), catalase ($F_{treatment}$= 13,40, df= 1/10, p<0,01), GPx ($F_{treatment}$= 5,955, df= 1/10, p<0,05) and SOD ($F_{treatment}$= 36,28, df= 1/10, p<0,001).

The results obtained from post hoc Tuckey test, showed a significant increase of H_2O_2 levels in HP rats compared to VEH-treated LF rats (p<0,01) and a significant decrease of H_2O_2 levels in HF rats treated with OEA compared to both HV and HP rats (HO vs HV: p<0,01; HO vs HP: p<0,001) (Fig. 2.17-A); moreover, the results obtained, showed a significant increase of MDA levels in HF rats treated with VEH compared to VEH-treated LF rats (p<0,05) and a significant decrease of MDA levels in HF rats treated with OEA compared to LV, HV and HP rats groups (HO vs LV: p<0,05; HO vs HV: p<0,001; HO vs HP: p<0,001) (Fig. 2.17-A).

The results obtained from post hoc Tuckey test showed a significant decrease of catalase activity in HF rats treated with VEH compared to VEH-treated LF rats (p<0,01) and a significant increase of catalase activity in HF rats treated with OEA compared to rats belonging to the same diet group but treated with VEH (p<0,01) (Fig. 2.17-B).

Moreover, the results obtained showed a significant decrease of GPx activity in both HV and HP rats compared to VEH-treated LF rats (p<0,05) and an increase, although not statistically significant, of GPx activity in HF rats treated with OEA (Fig. 2.17-B).

Finally, the results obtained showed a significant decrease of SOD activity in both HV and HP rats compared to VEH-treated LF rats ($p<0,001$) and a significant increase in HF rats treated with OEA compared to both HV and HP rats ($p<0,01$) (Fig. 2.17-B).

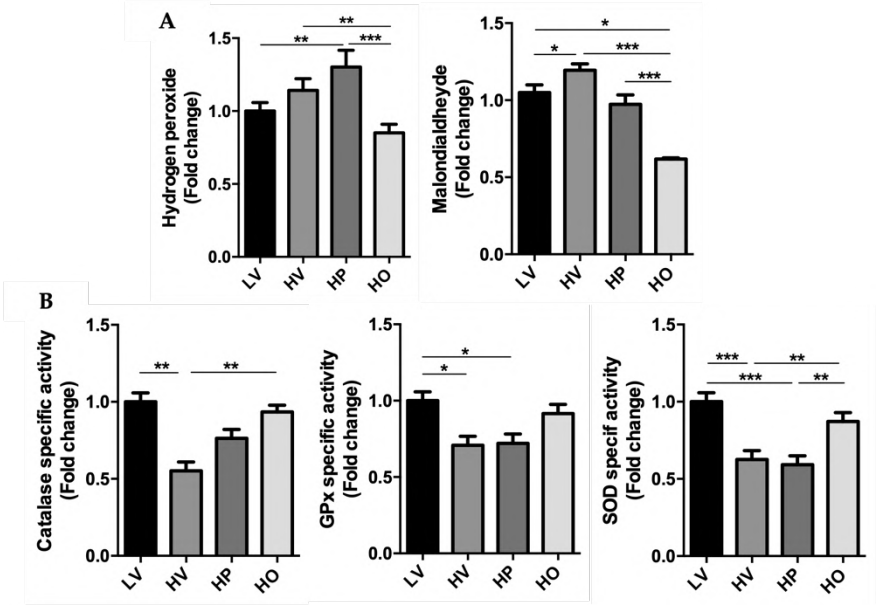

Fig. 2.17: (A) Hydrogen peroxide and malondialdehyde measurements were done as reported in Materials and Methods. Values, expressed in fold change of LV, are the mean ± SEM of five different samples. (B) Catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD), specific activities were assayed in liver homogenates as reported in Materials and Methods. Values, expressed in fold change of LV, are the mean ± SEM of five different samples. mean ± SEM of five different samples. *p<0,05 **p<0,01 ***p<0,001.

- *OEA reduced the ER stress*

The results obtained by one-way ANOVA, concerning proteins involved in UPR signaling and in the control of protein folding during ER stress, showed a significant effect of the treatment for BIP ($F_{treatment}$= 7,518, df= 1/27, $p<0,05$), but no significant effect of the treatment for both XBP1 ($F_{treatment}$= 3,489, df= 1/19, p=0,07) and ATF6 ($F_{treatment}$= 4,471, df= 1/13, p=0,05).

The results from second western blot experiment (Fig. 2.18-A), obtained from post hoc Tuckey test, showed a significant increase of ATF6 and BIP

expressions in HV rats compared to VEH-treated LF rats (p<0,05) and a significant increase of XBP1 expression in HP rats compared to VEH-treated LF rats (p<0,05); moreover, the results obtained showed a significant decrease of BIP expression in HF rats treated with OEA compared to both HV (p<0,05) and HP (p<0,01) rats and a significant decrease of XBP1 expression in HF rats treated with OEA compared to rats belonging to the same diet group but treated with VEH (p<0,05) (Fig. 2.18-B).

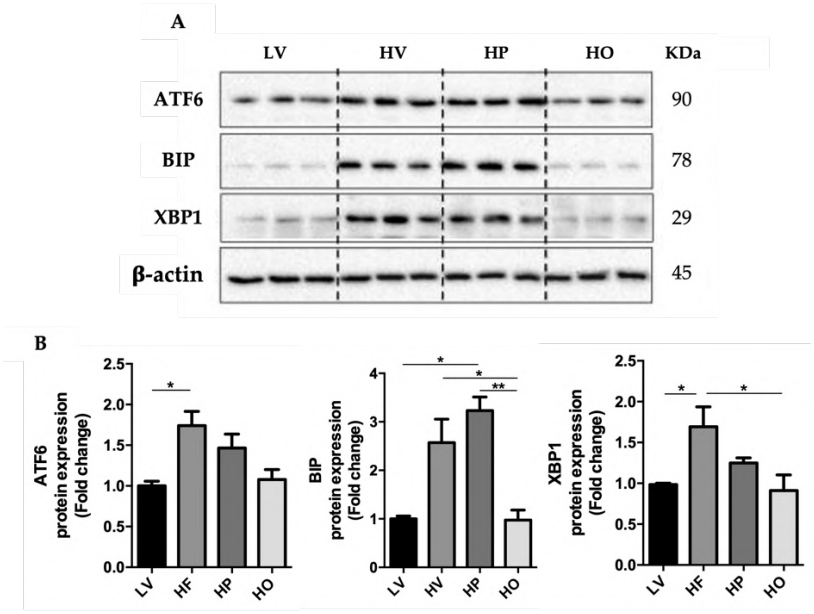

Fig. 2.18: (A) Representative immunoblots for Activating Transcription Factor 6 (ATF6), binding-immunoglobulin protein aka GRP-78 (BIP), and X-box-Binding Protein-1 (XBP1); β-actin was used as a loading control. (B) Quantification of protein expression. Values in the histogram were expressed as fold change relative to LV, the data represent the mean ± SEM. *p<0,05 **p<0,01

- *OEA differentially regulated the expression of the transcription factors Nrf1 and Nrf2*

The results obtained by one-way ANOVA, concerning transcriptions factors of metabolic pathways modulate by oxidative stress, showed a significant effect of the treatment for: Nrf1 ($F_{treatment}$= 19,68, df= 1/10, p<0,01),

Nrf2 ($F_{treatment}$= 17,30, df= 1/10, p<0,01) and PPAR-γ ($F_{treatment}$= 62,00, df= 1/10, p<0,001).

The results from second western blot experiment (Fig. 2.19-A), obtained from post hoc Tuckey test, showed a significant decrease of Nrf1 expression in HP rats compared to both LV (p<0,01) and HV (p<0,05) rats and a significant increase in HF rats treated with OEA compared to both HV (p<0,05) and HP (p<0,001) rats (Fig. 2.19-B).

Moreover, the results obtained showed a significant decrease of Nrf2 expression in HF rats treated with OEA compared to LV (p<0,05), HV (p<0,01) and HP (p<0,01) rats (Fig. 2.19-B).

Finally, the results obtained showed a significant increase of PPAR-γ expression in both HV and HP rats compared to VEH-treated LF rats (p<0,001) and a significant decrease of PPAR-γ expression in HF rats treated with OEA compared to both HV and HP rats (p<0,001) (Fig. 2.19-B).

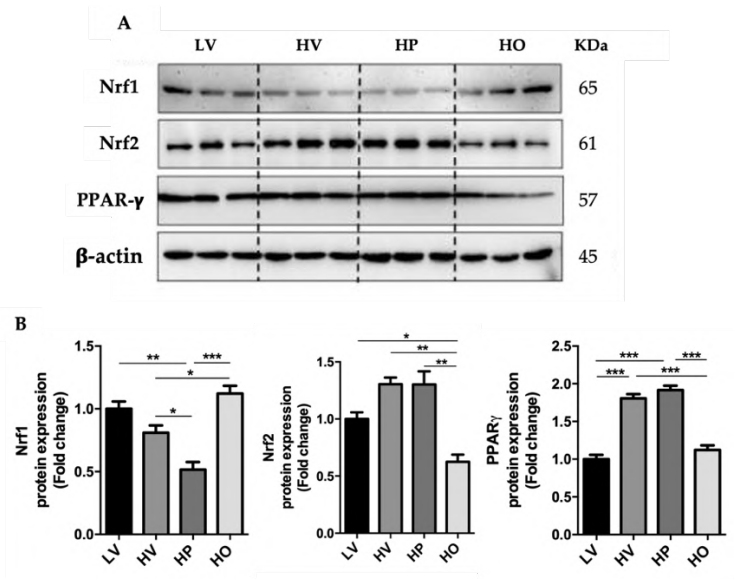

Fig. 2.19: (A) Representative immunoblots for nuclear factor erythroid-derived 2-related factor 1 (Nrf1), Nrf2 and peroxisome proliferator-activated receptor γ (PPAR-γ); β-actin was used as a loading control. (B) Quantification of protein expression reported as fold change relative to the control. Values in the histogram were expressed as fold change relative to the LV. Data are expressed as mean ± SEM. *p<0,05 **p<0,01 ***p<0,001

5. Discussion

In the present study, we reported that a two-week treatment with OEA, to rats exposed to an HFD, exert anti-obesity effects and ameliorates both peripheral and central alterations related to obesity, including neuroinflammation markers, modifications in the expression of BBB proteins, fatty liver diseases and oxidative stress phenomena.

The rational of this study derives from several studies that have been demonstrated that far from being limited to weight gain, obesity is characterized by chronic, unresolved tissue inflammation associated with a cluster of central and peripheral alterations [3,11–14].

Our behavioral data showed an effect of OEA treatment on both food intake and body weight gain in all the diet groups studied, although with some peculiarities. Indeed, in obese animals, the anorexigenic effect of OEA appears to be delayed compared to both control (LFD) animals and animals that underwent, from the first day of treatment, a shift (SHIFT animals) from the HFD to LFD ad libitum, to mimic dieting. Particularly we observed a reduction of food intake, in obese animals, after eight days of chronic treatment with OEA. According to the literature [74], the exposure to HFD dampen the synthesis and mobilization of OEA; therefore, we hypothesized that such disruption of the OEA signaling pathway could cause the delayed anorexigenic effect of OEA observed in obese animals.

Moreover, the data obtained in the present work showed the hypophagic effect of OEA also in SHIFT animals in which such effect is observed earlier compared to animals with an obese phenotype. According to the literature [75,76] the change of the diet induces per se a reduction in the caloric intake; therefore, we hypothesized that the shift of the diet might, in part, restore the sensitivity to the biological action of OEA, and the imbalance between

mediators regulating energy homeostasis that is disrupted upon the exposure to HF [77,78].

We further analyzed the BW gain of the animals in all diet groups. Our data demonstrate that OEA decreases BW gain in all diet groups compared to VEH-treated animals, as expected. In this regard, pair feeding groups were introduced to investigate whether the effects of OEA on BW are only due to the reduction of the food consumed or to additional effects on the metabolism. Indeed, this group was treated only with the VEH, and received the average of the amount of food consumed by the OEA-treated animals of the matching diet group.

We found that the OEA-induced weight loss, as for the FI, has a different onset depending on the diet group. In fact, in animals with an obese phenotype, 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 [79], that leads to a reduced responsivity to its anorexigenic effect [80]. Regarding the SHIFT animals, the OEA-induced reduction of BW, seems to be delayed compared to the onset of the effect on the FI. It could be hypothesized that OEA's effect is dampened by the effect that the shift of the diet already has on weight loss.

Interestingly, we also found that the BW of OEA-treated animals was significantly lower than that of pair-feeding animals in all diet groups. 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. Indeed, several studies, in keeping with our results, have demonstrated that OEA affects the metabolism by stimulating fatty acid β-oxidation [48], by inhibiting lipogenesis and lipoprotein secretion in

hepatocytes [81] and by improving HFD-induced liver steatosis in rats [48] and humans [82,83].

Regarding central alterations related to obesity, the results obtained from GFAP immunofluorescence analyses revealed an increased expression of this astrocytic marker in the HYPO, HIPPO and DG of animals with an obese phenotype suggesting that the prolonged exposure to the HFD induced, in these animals, a neuroinflammatory response. In keeping with our results, the study published in 2005 [84] by De Souza and colleagues was the first to demonstrate that long-term feeding of a lard based HFD to rats both increased hypothalamic activation of different inflammatory pathways and impaired insulin and leptin signaling. Interestingly, it has also been demonstrated that HYPO inflammation appears to precede low-grade chronic systemic inflammation in obesity as the diet itself could affect inflammatory biomarkers related to neuroinflammation [85]. Indeed, Thaler et al. reported that, in rodent models, markers of hypothalamic inflammation are elevated within 24 hours of HFD exposure, while in peripheral tissues, inflammation process develops over weeks to months of HFD feeding; moreover, although inflammation and gliosis are initially transient in the HYPO, suggesting an effective neuroprotective response, they return and become established with continued HFD exposure [86]. However, the neuroinflammation derived from obesity is not restricted to the HYPO; particularly, other than the HYPO, the HIPPO is a best-studied CNS structure in this context. Indeed, numerous experimental studies, using animal models of HFD-induced obesity, have revealed modifications in the hippocampal structure and function [32,86–91].

Interestingly, in our results we showed that the chronic OEA treatment, in the animals with obese phenotype, completely reverted the increased expression of GFAP to the level observed in control groups, supporting the

potential beneficial role of OEA in modulating neuroinflammatory responses related to obesity.

Moreover, we hypothesized that alterations in the integrity and functionality of the BBB could play a crucial role in the neuroinflammatory responses observed in the animals with an obese phenotype. Indeed, low-grade inflammation characteristic of obesity can lead to neuroinflammation through several mechanisms, including the disruption of the BBB [14].

The BBB is a regulatory interface between the CNS and the peripheral circulation. It is a complex three-dimensional structure composed of specialized endothelial cells that are held together by tight junctions (TJs) and are reinforced by pericytes, astrocytic end-feet and extracellular matrix [9]. The BBB is present throughout the CNS except for the CVOs, located around the third and fourth cerebral ventricles, such as the AP and the ME [5]. CVOs are characterized by extensive fenestrated vasculature and by lack of TJs complexes, the hallmark of the CNS barriers; therefore, CVOs are in persistent contact with signaling molecules circulating in the bloodstream [92]. However, CVOs also possess a blood-cerebrospinal fluid (CSF) barrier, at the ventricular wall, composed of tanycyte that are highly specialized ependymal cells which present well-organized TJs [93]. Therefore, the displacement of barrier properties from the vascular to the ventricular side allows the diffusion of blood-borne molecules into the parenchyma of the CVOs while tanycyte TJs control their diffusion into the CSF, thus maintaining brain homeostasis [93].

In the context of obesity-induced neuroinflammation, different cellular types, involved in the functionality of both BBB and CVOs, result to be affected [6–10]; particularly VIM and ZO-1 represents good markers for barrier integrity loss. VIM is an intermediary filament protein of the cytoskeleton, expressed in both brain vessels and tanycytes, also involved

in the regulation of the low-density lipoprotein-derived cholesterol transport [94]; ZO-1 is a key cytoplasmatic component of the tight junctions which hold together both endothelial cells of the BBB and tanycyte of the CVOs, limiting exchanges with the periphery [95–97].

The results obtained from VIM immunofluorescence analyses showed no significant effect of OEA treatment in the ARC (located adjacent to the ME) and in the AP of animals with an obese phenotype. We speculated that these results could be due to both the distinctive anatomy and localization of the CVOs in the brain and the specific function of VIM. Indeed CVOs, which are in persistent contact with signaling molecules circulating in the bloodstream, are particularly and rapidly responsive to the presence of high amounts of fatty acids present in the HFD. Therefore, at tanycyte level, VIM, which is crucially involved in the regulation of the low-density lipoprotein-derived cholesterol transport, could be irreversibly down-regulated by the prolonged presence of high amounts of fatty acids observed in HFD-fed animals, making OEA treatment ineffective. This hypothesis might be supported by both the increased VIM expression we observed in the AP of control animals treated with OEA (not exposed to the HFD) and the effect of OEA treatment we observed in the NST and its subnuclei, brain areas characterized by the presence of the BBB (unlike the CVOs). Indeed, in the SOLC and in the NST of animals with an obese phenotype we first observed a reduction of VIM expression. In keeping with our result, it has been demonstrated that in a mouse model of DIO, the exposure for 2 months to HFD induce the downregulation of many proteins of the BBB, including cytoskeletal proteins, such as VIM [9]. Interestingly, OEA treatment, in animals with an obese phenotype, induced an increase of VIM expression in both NST and SOLDM and an increasing trend in the other subnuclei,

suggesting, at this level, an effect of OEA treatment on VIM regulation and consequently a potential effect on the fluidity of the BBB.

Concerning ZO-1, we showed that OEA treatment, in animals with an obese phenotype, induced an increase in the expression of this TJs marker in the ARC, AP and SOLVL and an increasing trend in NST and its other subnuclei. It has been demonstrated that the expression of these TJs is downregulated in animals exposed to prolonged HFD [6,9]; therefore, our results supported a role of OEA treatment in maintaining the stability and functions of the TJs, suggesting a potential effect of OEA on the integrity of both BBB and CVOs, to limit exchanges with the periphery and to maintain CNS homeostasis.

Moreover, in an obesity context, also neurons result to be affected. Indeed, neurogenesis, a process involving the proliferation, migration, differentiation, survival, and integration of new neurons into existing circuitry [24], result dysregulated during metabolic diseases, such as obesity and T2DM [18–23]. Many researchers have reported that neurogenesis in the hippocampal DG is decreased in rodents with a chronic intake of obesogenic diets, including a HFD [25–30,98–105] For example, mice administered a HFD show impaired neurogenesis and decreased brain-derived neurotrophic factor, as well as increased lipid peroxidation [18].

Our results showed that in the DG of animals with obese phenotype the chronic OEA treatment increased the number of neurons positive to DCX, a marker of differentiation of new neurons into existing circuitry, strictly involved in brain plasticity [24]. This increased number of DCX-positive neurons was observed, interestingly, also in control rats treated with OEA suggesting that, at this level, the potential neuroprotective effect of OEA, in stimulating neurogenesis processes, is independent of the diet condition.

As further step of our study, in collaboration with Prof.ssa Anna Maria Giudetti of University of Salento, we have recently published two articles on the effects of the chronic OEA treatment on hepatic alterations related to obesity.

In our first study, conducted only in animals with an obese phenotype, we observed that OEA-induced a significant decrease of PPAR-γ receptor expression and of its downstream target protein CD36. PPAR-γ and CD36 mRNA expression are up-regulated in high-fat diet-induced liver steatosis in mice [106]. CD36 expression has been associated with insulin resistance in humans with type 2 diabetes [107,108] and increased hepatic *Cd36* gene expression was reported to increase fatty uptake, TAG accumulation and fatty liver [109,110]. We hypothesized that the hepatic reduction of PPAR-γ expression was linked to a reduction in TAG content and this observation is in keeping with previous studies demonstrating that a reduced PPAR-γ expression in the liver is associated with hepatic lipogenesis and TAG content [111].

We also investigate the impact of chronic administration of OEA on the expression of enzymes involved in TAG and fatty acid synthesis. Particularly, the final step in TAG synthesis is catalyzed by diacylglycerol acyltransferase (DGAT) isoforms, DGAT1 and DGAT2 [112] with distinct protein sequences and different biochemical, cellular, and physiological functions [113]. PPAR-γ is involved in the regulation of both DGAT1 and DGAT2 enzyme expression, and interestingly knockdown of hepatic PPAR-γ was reported to reduce hepatic lipid accumulation and both DGAT1 and DGAT2 expressions [111,112,114,115]. We found that DGAT1 and DGAT2 expression decreased in the livers of OEA-treated rats. OEA treatment significantly also decreased the hepatic level of stearoyl-CoA desaturase (SCD1), a member of the fatty acid desaturase family that catalyzes the

conversion of stearoyl-CoA to oleoyl-CoA, a major substrate for TAG synthesis [58].

Moreover, sterol regulatory element-binding protein (SREBP)-1 is a transcription factor that activates the expression of most genes required for hepatic lipogenesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and SCD1 [116,117]. Increased levels of SREBP-1 mRNA were demonstrated in livers of several mouse models characterized by insulin resistance and increased rates of hepatic lipogenesis [117].

Li et al. reported that OEA inhibits hepatic de novo lipogenesis throughout a SREBP-1-mediated inhibition of SCD1, and ACC mRNA expression [48]. According to that study, we found that OEA decreases the expression of SREBP-1. Moreover, we observed that OEA decreases ACC, FAS, and SCD1 protein expression, thus suggesting a possible link between the decreased level of ACC, FAS, and SCD1 and the down-regulation of SREBP-1.

Taken together, our results propose a dual role played by OEA in the reduction of hepatic TAG levels: by recruiting the SREBP-1 system OEA might regulate key proteins involved in fatty acid synthesis, whereas by affecting PPAR-γ, it might influence fatty acid uptake, binding, and transport.

Our second study was carried out on animals with obese phenotype treated with both VEH and OEA (10 mg/kg i.p.) (HV, HO) and on the corresponding control and pair-feeding groups (LV and HP).

The results obtained from the histological analyses of liver sections conducted through the O-red oil staining protocol showed that chronic OEA treatment completely abolished, in animals with an obese phenotype and in corresponding pair-feeding groups, the increased amount of lipid stored as lipid droplets caused by prolonged exposure to HFD. Interestingly, animals with an obese phenotype treated with OEA showed

a further significant decrease in the mean particle size, compared to HP rats, thus suggesting that the fat accumulation of HFD-fed rats can be successfully decreased by OEA treatment, and this effect might not be the mere consequence of the reduced caloric intake induced by OEA.

Moreover, in the present study, we confirm that OEA treatment stimulates β-oxidation and, in addition, exerts inhibitory effects on the expressions of key enzymes involved in fatty acid synthesis and transport, such as FAS, ACC, SCD1, and low-density lipoprotein receptor (LDLR).

After OEA treatment we found reduced oxidative stress in the animals with an obese phenotype, as detected by a decreased level of hydrogen peroxide (H_2O_2) and activation of key enzymes involved in the balance of the redox state, such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx). Considering the role played by SOD and catalase in the protection of cells against oxidative damage [118,119] the increased activity of these enzymes following OEA treatment suggests a decreased hepatic oxidative stress in OEA-treated rats. The decreased hepatic oxidative state of OEA-treated rats ameliorated the oxidative damage as suggested by the reduction in the hepatic levels of malondialdehyde (MDA), which represents the most prevalent biomarkers of protein and lipid peroxidation during liver injury [120,121].

Interestingly, all the antioxidant effects observed in the liver of the animals with an obese phenotype treated with OEA, were significantly different from those of the HP group, thus indicating that OEA effects on the selected parameters might be specifically attributed to its direct pharmacological actions, rather than to its anorexigenic effect.

The ER plays an important role in the post-translational modifications and folding of proteins synthesized in the ribosomes [62]. One of the most abundant ER-resident proteins that increase the efficiency of protein folding

of the nascent polypeptide is the Hsp70-type chaperone, BIP. Aberrant folding processes may lead to the accumulation of misfolded proteins, also known as ER stress. The unfolded protein response (UPR), a cell-signaling system that readjusts ER folding capacity to restore protein homeostasis, involves three signaling arms coordinated by IRE1-XBP1, PERK-eIF2a-ATF4, and ATF6 [119].

Liver fat causes the emergence of many complications such as ER stress [66]. ER stress markers are increased in patients with obesity-associated metabolic syndrome [67]. Both in vitro and in vivo studies have reported that ER stress, and the activation of the UPR signaling, play a critical role in the regulation of hepatic lipid metabolism [122]. Thus, the UPR pathway can activate lipogenic enzymes such as ACC2 and SCD1 and induce hepatic steatosis [123,124].

In our study, we showed that UPR activation, induced by HFD, was significantly reduced by OEA, as suggested by the lower expression of XBP1, ATF6 and BIP in HO vs both HF and HP groups of rats. To our knowledge, this is the first report showing a reduction of ER stress after OEA administration in a rat model of NAFLD. We can speculate that the OEA-induced antisteatotic effect might relieve the pressure induced by lipid accumulation, thus ameliorating ER stress.

We found that OEA regulates the protein expression of Nrf1 and Nrf2 in opposite manners, i.e., increasing Nrf1 and decreasing Nrf2, as compared to both HP and HV groups. Both observations are in accordance with the different roles recently attributed to these proteins. Nrf1 and Nrf2 are members a family of proteins that play a crucial role in NAFLD and related oxidative stress response [63]. Moreover, these transcription factors are involved in the regulation of lipid metabolism [62,64]. It has been demonstrated that Nrf1 knockout mice accumulate lipids in the liver and

exhibit a phenotype that mimics human non-alcoholic steatohepatitis. The somatic inactivation of Nrf1 expression leads to NAFLD and sensitizes hepatocytes to oxidative stress-induced cell toxicity and injury [125]. The lower expression of Nrf1 we observed in the liver of rats with an obese phenotype, as compared to control rats, is coherent with these data reported in the literature. On the other hand, OEA-induced increase of Nrf1 expression might represent a novel mechanism by which OEA can regulate hepatic lipid metabolism in HFD-fed rats.

Nrf1, a protein largely localized in the cytoplasm, where it is anchored to the ER membrane [118], has been reported to regulate the proteasome system to ensure that misfolded proteins do not accumulate in the cell [126]. Although the role of Nrf1 in regulating the transcription of antioxidant genes is not yet fully understood, based on our results, we hypothesize a relationship between antioxidant enzyme activity and Nrf1 expression. This aspect will be elucidated in our future studies. However, considering the relationship between ER and Nrf1, with our data a possible post-translational control of antioxidant enzymes cannot be excluded.

Nrf2 is a transcription factor that plays a critical role in inflammation and antioxidant responses by regulating antioxidant and anti-inflammatory genes [120]. Indeed, Nrf2 is activated by ROS, inflammatory cytokines, and ER stresses [120]. Moreover, a role in the regulation of lipid metabolism and the occurrence of liver steatosis, has been also reported [62].

In our experimental model of diet-induced obesity, we found that Nrf2 expression was increased upon HFD feeding, probably due to the established high oxidative stress, and was decreased after OEA treatment.

As mentioned above, PPAR-γ is a regulator of lipid metabolism in hepatocytes; changes in the expression of this protein have been associated with non-alcoholic fatty liver diseases through the induction of lipogenic

factors [67]. Here, we found that HFD increased PPAR-γ expression and induced liver steatosis, both restored by OEA administration. Considering the role of PPAR-γ as a positive modulator of de novo lipogenic enzymes, the decreased expression we measured in the liver of OEA-treated animals is well-correlated with the lower expression of ACC and FAS we measured in HO vs. HV rats. Based on recent findings reporting an Nrf2-mediated regulation of PPAR-γ expression in the modulation of hepatic lipid metabolism [127], and the coordinate modulation of these factors we measured, the relationship between Nrf2 and PPAR-γ in the liver of HFD-fed rats awaits further investigations. In summary, our data demonstrated that OEA can reduce fatty liver and ameliorate parameters of oxidative stress and ER stress in the liver of HDF-fed rats.

Taken together, our data demonstrated that OEA exert an anti-obesity effect that is accompanied centrally by a reduction of neuroinflammatory response and an increase of neuroplasticity and peripherally by an amelioration of fatty liver and oxidative and ER stress parameters. Therefore, our study further supports the potential use of OEA for the treatment of obesity and for both central and peripheral obesity-related alteration.

6. References

1. Kopelman, P.G. Obesity as a Medical Problem. *Nature* 2000.

2. Yach, D.; Stuckler, D.; Brownell, K.D. Epidemiologic and Economic Consequences of the Global Epidemics of Obesity and Diabetes. *Nature Medicine* 2006, *12*.

3. Ying, W.; Fu, W.; Lee, Y.S.; Olefsky, J.M. The Role of Macrophages in Obesity-Associated Islet Inflammation and β-Cell Abnormalities. *Nature Reviews Endocrinology* 2020.

4. Chow, B.W.; Gu, C. The Molecular Constituents of the Blood-Brain Barrier. *Trends in Neurosciences* 2015, *38*.

5. Al-Kaabi, M.; Hussam, F.; Al-Marsoummi, S.; Al-Anbaki, A.; Al-Salihi, A.; Al-Aubaidy, H. Expression of ZO1, Vimentin, Pan-Cadherin and AGTR1 in Tanycyte-like Cells of the Sulcus Medianus Organum. *Biochemical and Biophysical Research Communications* **2018**, *502*, doi:10.1016/j.bbrc.2018.05.151.

6. Nerurkar, P. v.; Johns, L.M.; Buesa, L.M.; Kipyakwai, G.; Volper, E.; Sato, R.; Shah, P.; Feher, D.; Williams, P.G.; Nerurkar, V.R. Momordica Charantia (Bitter Melon) Attenuates High-Fat Diet-Associated Oxidative Stress and Neuroinflammation. *Journal of Neuroinflammation* **2011**, *8*, doi:10.1186/1742-2094-8-64.

7. Elahy, M.; Lam, V.; Pallebage-Gamarallage, M.M.; Giles, C.; Mamo, J.C.L.; Takechi, R. Nicotine Attenuates Disruption of Blood-Brain Barrier Induced by Saturated-Fat Feeding in Wild-Type Mice. *Nicotine and Tobacco Research* **2015**, *17*, doi:10.1093/ntr/ntv044.

8. Takechi, R.; Pallebage-Gamarallage, M.M.; Lam, V.; Giles, C.; Mamo, J.C. Nutraceutical Agents with Anti-Inflammatory Properties Prevent Dietary Saturated-Fat Induced Disturbances in Blood-Brain Barrier

Function in Wild-Type Mice. *Journal of Neuroinflammation* **2013**, *10*, doi:10.1186/1742-2094-10-73.

9. Ouyang, S.; Hsuchou, H.; Kastin, A.J.; Wang, Y.; Yu, C.; Pan, W. Diet-Induced Obesity Suppresses Expression of Many Proteins at the Blood-Brain Barrier. *Journal of Cerebral Blood Flow and Metabolism* **2014**, *34*, doi:10.1038/jcbfm.2013.166.

10. Severi, I.; Fosca, M.; Colleluori, G.; Marini, F.; Imperatori, L.; Senzacqua, M.; di Vincenzo, A.; Barbatelli, G.; Fiori, F.; Rau, J. v.; et al. High-Fat Diet Impairs Mouse Median Eminence: A Study by Transmission and Scanning Electron Microscopy Coupled with Raman Spectroscopy. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22158049.

11. Boulangé, C.L.; Neves, A.L.; Chilloux, J.; Nicholson, J.K.; Dumas, M.E. Impact of the Gut Microbiota on Inflammation, Obesity, and Metabolic Disease. *Genome Medicine* 2016.

12. Klöting, N.; Blüher, M. Adipocyte Dysfunction, Inflammation and Metabolic Syndrome. *Reviews in Endocrine and Metabolic Disorders* 2014.

13. Guillemot-Legris, O.; Muccioli, G.G. Obesity-Induced Neuroinflammation: Beyond the Hypothalamus. *Trends in Neurosciences* 2017, *40*.

14. Novo, A.M.; Batista, S. Multiple Sclerosis: Implications of Obesity in Neuroinflammation. In *Advances in Neurobiology*; 2017; Vol. 19.

15. Jha, M.K.; Lee, W.H.; Suk, K. Functional Polarization of Neuroglia: Implications in Neuroinflammation and Neurological Disorders. *Biochemical Pharmacology* 2016, *103*.

16. Colombo, E.; Farina, C. Astrocytes: Key Regulators of Neuroinflammation. *Trends in Immunology* 2016, *37*.

17. Linnerbauer, M.; Wheeler, M.A.; Quintana, F.J. Astrocyte Crosstalk in CNS Inflammation. *Neuron* 2020, *108*.

18. Park, H.R.; Park, M.; Choi, J.; Park, K.Y.; Chung, H.Y.; Lee, J. A High-Fat Diet Impairs Neurogenesis: Involvement of Lipid Peroxidation and Brain-Derived Neurotrophic Factor. *Neuroscience Letters* **2010**, *482*, doi:10.1016/j.neulet.2010.07.046.

19. Ho, N.; Sommers, M.S.; Lucki, I. Effects of Diabetes on Hippocampal Neurogenesis: Links to Cognition and Depression. *Neuroscience and Biobehavioral Reviews* 2013, *37*.

20. Nam, S.M.; Kim, J.W.; Yoo, D.Y.; Jung, H.Y.; Choi, J.H.; Hwang, I.K.; Seong, J.K.; Yoon, Y.S. Reduction of Adult Hippocampal Neurogenesis Is Amplified by Aluminum Exposure in a Model of Type 2 Diabetes. *Journal of Veterinary Science* **2016**, *17*, doi:10.4142/jvs.2016.17.1.13.

21. Nam, S.M.; Kim, J.W.; Kwon, H.J.; Yoo, D.Y.; Jung, H.Y.; Kim, D.W.; Hwang, I.K.; Seong, J.K.; Yoon, Y.S. Differential Effects of Low- and High-Dose Zinc Supplementation on Synaptic Plasticity and Neurogenesis in the Hippocampus of Control and High-Fat Diet-Fed Mice. *Neurochemical Research* **2017**, *42*, doi:10.1007/s11064-017-2353-2.

22. Tang, S.S.; Ren, Y.; Ren, X.Q.; Cao, J.R.; Hong, H.; Ji, H.; Hu, Q.H. ERα and/or ERβ Activation Ameliorates Cognitive Impairment, Neurogenesis and Apoptosis in Type 2 Diabetes Mellitus Mice. *Experimental Neurology* **2019**, *311*, doi:10.1016/j.expneurol.2018.09.002.

23. Tanokashira, D.; Kurata, E.; Fukuokaya, W.; Kawabe, K.; Kashiwada, M.; Takeuchi, H.; Nakazato, M.; Taguchi, A. Metformin Treatment Ameliorates Diabetes-Associated Decline in Hippocampal Neurogenesis and Memory via Phosphorylation of Insulin Receptor Substrate 1. *FEBS Open Bio* **2018**, *8*, doi:10.1002/2211-5463.12436.

24. Christie, B.R.; Cameron, H.A. Neurogenesis in the Adult Hippocampus. *Hippocampus* **2006**, *16*, doi:10.1002/hipo.20151.

25. Boitard, C.; Etchamendy, N.; Sauvant, J.; Aubert, A.; Tronel, S.; Marighetto, A.; Layé, S.; Ferreira, G. Juvenile, but Not Adult Exposure to High-Fat Diet Impairs Relational Memory and Hippocampal Neurogenesis in Mice. *Hippocampus* **2012**, *22*, doi:10.1002/hipo.22032.

26. Hwang, I.K.; Kim, I.Y.; Kim, D.W.; Yoo, K.Y.; Kim, Y.N.; Yi, S.S.; Won, M.H.; Lee, I.S.; Yoon, Y.S.; Seong, J.K. Strain-Specific Differences in Cell Proliferation and Differentiation in the Dentate Gyrus of C57BL/6N and C3H/HeN Mice Fed a High Fat Diet. *Brain Research* **2008**, *1241*, doi:10.1016/j.brainres.2008.08.024.

27. Pathak, N.M.; Pathak, V.; Lynch, A.M.; Irwin, N.; Gault, V.A.; Flatt, P.R. Stable Oxyntomodulin Analogues Exert Positive Effects on Hippocampal Neurogenesis and Gene Expression as Well as Improving Glucose Homeostasis in High Fat Fed Mice. *Molecular and Cellular Endocrinology* **2015**, *412*, doi:10.1016/j.mce.2015.05.035.

28. Vinuesa, A.; Pomilio, C.; Menafra, M.; Bonaventura, M.M.; Garay, L.; Mercogliano, M.F.; Schillaci, R.; Lux Lantos, V.; Brites, F.; Beauquis, J.; et al. Juvenile Exposure to a High Fat Diet Promotes Behavioral and Limbic Alterations in the Absence of Obesity. *Psychoneuroendocrinology* **2016**, *72*, doi:10.1016/j.psyneuen.2016.06.004.

29. Yoo, D.Y.; Kim, W.; Nam, S.M.; Yoo, K.Y.; Lee, C.H.; Choi, J.H.; Won, M.H.; Hwang, I.K.; Yoon, Y.S. Reduced Cell Proliferation and Neuroblast Differentiation in the Dentate Gyrus of High Fat Diet-Fed Mice Are Ameliorated by Metformin and Glimepiride Treatment. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-011-0566-3.

30. Klein, C.; Jonas, W.; Iggena, D.; Empl, L.; Rivalan, M.; Wiedmer, P.; Spranger, J.; Hellweg, R.; Winter, Y.; Steiner, B. Exercise Prevents High-Fat Diet-Induced Impairment of Flexible Memory Expression in the Water Maze and Modulates Adult Hippocampal Neurogenesis in Mice.

Neurobiology of Learning and Memory **2016**, *131*, doi:10.1016/j.nlm.2016.03.002.

31. Kriegstein, A.; Alvarez-Buylla, A. The Glial Nature of Embryonic and Adult Neural Stem Cells. *Annual Review of Neuroscience* 2009, *32*.

32. Lu, J.; Wu, D. mei; Zheng, Y. lin; Hu, B.; Cheng, W.; Zhang, Z. feng; Shan, Q. Ursolic Acid Improves High Fat Diet-Induced Cognitive Impairments by Blocking Endoplasmic Reticulum Stress and IκB Kinase β/Nuclear Factor-KB-Mediated Inflammatory Pathways in Mice. *Brain, Behavior, and Immunity* **2011**, *25*, doi:10.1016/j.bbi.2011.06.009.

33. Beilharz, J.E.; Maniam, J.; Morris, M.J. Short-Term Exposure to a Diet High in Fat and Sugar, or Liquid Sugar, Selectively Impairs Hippocampal-Dependent Memory, with Differential Impacts on Inflammation. *Behavioural Brain Research* **2016**, *306*, doi:10.1016/j.bbr.2016.03.018.

34. Guillemot-Legris, O.; Masquelier, J.; Everard, A.; Cani, P.D.; Alhouayek, M.; Muccioli, G.G. High-Fat Diet Feeding Differentially Affects the Development of Inflammation in the Central Nervous System. *Journal of Neuroinflammation* **2016**, *13*, doi:10.1186/s12974-016-0666-8.

35. Tapia-González, S.; García-Segura, L.M.; Tena-Sempere, M.; Frago, L.M.; Castellano, J.M.; Fuente-Martín, E.; García-Cáceres, C.; Argente, J.; Chowen, J.A. Activation of Microglia in Specific Hypothalamic Nuclei and the Cerebellum of Adult Rats Exposed to Neonatal Overnutrition. *Journal of Neuroendocrinology* **2011**, *23*, doi:10.1111/j.1365-2826.2011.02113.x.

36. Kim, C.H.; Younossi, Z.M. Nonalcoholic Fatty Liver Disease: A Manifestation of the Metabolic Syndrome. *Cleveland Clinic Journal of Medicine* 2008, *75*.

37. Wang, X.; Rao, H.; Liu, F.; Wei, L.; Li, H.; Wu, C. Recent Advances in Adipose Tissue Dysfunction and Its Role in the Pathogenesis of Non-Alcoholic Fatty Liver Disease. *Cells* 2021, *10*.

38. Kartha, G.K.; Moshal, K.S.; Sen, U.; Joshua, I.G.; Tyagi, N.; Steed, M.M.; Tyagi, S.C. Renal Mitochondrial Damage and Protein Modification in Type-2 Diabetes. *Acta Diabetologica* **2008**, *45*, doi:10.1007/s00592-008-0025-z.

39. Flessa, C.M.; Kyrou, I.; Nasiri-Ansari, N.; Kaltsas, G.; Papavassiliou, A.G.; Kassi, E.; Randeva, H.S. Endoplasmic Reticulum Stress and Autophagy in the Pathogenesis of Non-Alcoholic Fatty Liver Disease (NAFLD): Current Evidence and Perspectives. *Current Obesity Reports* 2021, *10*.

40. Lim, S.; Kim, J.W.; Targher, G. Links between Metabolic Syndrome and Metabolic Dysfunction-Associated Fatty Liver Disease. *Trends in Endocrinology and Metabolism* 2021, *32*.

41. di Meo, S.; Reed, T.T.; Venditti, P.; Victor, V.M. Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxidative Medicine and Cellular Longevity* 2016, *2016*.

42. Romano, A.; Tempesta, B.; Provensi, G.; Passani, M.B.; Gaetani, S. Central Mechanisms Mediating the Hypophagic Effects of Oleoylethanolamide and N-Acylphosphatidylethanolamines: Different Lipid Signals? *Frontiers in Pharmacology* 2015.

43. Gaetani, S.; Romano, A.; Provensi, G.; Ricca, V.; Lutz, T.; Passani, M.B. Eating Disorders: From Bench to Bedside and Back. *Journal of Neurochemistry* 2016.

44. Romano, A.; Micioni Di Bonaventura, M.V.; Gallelli, C.A.; Koczwara, J.B.; Smeets, D.; Giusepponi, M.E.; de Ceglia, M.; Friuli, M.; Micioni Di Bonaventura, E.; Scuderi, C.; et al. Oleoylethanolamide Decreases Frustration Stress-Induced Binge-like Eating in Female Rats: A Novel Potential Treatment for Binge Eating Disorder. *Neuropsychopharmacology* **2020**, doi:10.1038/s41386-020-0686-z.

45. Rodríguez De Fonseca, F.; Navarro, M.; Gómez, R.; Escuredo, L.; Nava, F.; Fu, J.; Murillo-Rodríguez, E.; Giuffrida, A.; Loverme, J.; Gaetani, S.; et al. An Anorexic Lipid Mediator Regulated by Feeding. *Nature* **2001**, doi:10.1038/35102582.

46. Fu, J.; Oveisi, F.; Gaetani, S.; Lin, E.; Piomelli, D. Oleoylethanolamide, an Endogenous PPAR-α Agonist, Lowers Body Weight and Hyperlipidemia in Obese Rats. *Neuropharmacology* **2005**, doi:10.1016/j.neuropharm.2005.02.013.

47. Serrano, A.; del Arco, I.; Javier Pavón, F.; Macías, M.; Perez-Valero, V.; Rodríguez de Fonseca, F. The Cannabinoid CB1 Receptor Antagonist SR141716A (Rimonabant) Enhances the Metabolic Benefits of Long-Term Treatment with Oleoylethanolamide in Zucker Rats. *Neuropharmacology* **2008**, doi:10.1016/j.neuropharm.2007.03.007.

48. Li, L.; Li, L.; Chen, L.; Lin, X.; Xu, Y.; Ren, J.; Fu, J.; Qiu, Y. Effect of Oleoylethanolamide on Diet-Induced Nonalcoholic Fatty Liver in Rats. *Journal of Pharmacological Sciences* **2015**, doi:10.1016/j.jphs.2014.12.001.

49. Buckman, L.B.; Thompson, M.M.; Lippert, R.N.; Blackwell, T.S.; Yull, F.E.; Ellacott, K.L.J. Evidence for a Novel Functional Role of Astrocytes in the Acute Homeostatic Response to High-Fat Diet Intake in Mice. *Molecular Metabolism* **2015**, *4*, doi:10.1016/j.molmet.2014.10.001.

50. Buckman, L.B.; Thompson, M.M.; Moreno, H.N.; Ellacott, K.L.J. Regional Astrogliosis in the Mouse Hypothalamus in Response to Obesity. *Journal of Comparative Neurology* **2013**, *521*, doi:10.1002/cne.23233.

51. Parkash, J.; Messina, A.; Langlet, F.; Cimino, I.; Loyens, A.; Mazur, D.; Gallet, S.; Balland, E.; Malone, S.A.; Pralong, F.; et al. Semaphorin7A Regulates Neuroglial Plasticity in the Adult Hypothalamic Median Eminence. *Nature Communications* **2015**, *6*, doi:10.1038/ncomms7385.

52. Jiang, H.; Gallet, S.; Klemm, P.; Scholl, P.; Folz-Donahue, K.; Altmüller, J.; Alber, J.; Heilinger, C.; Kukat, C.; Loyens, A.; et al. MCH Neurons Regulate Permeability of the Median Eminence Barrier. *Neuron* **2020**, *107*, doi:10.1016/j.neuron.2020.04.020.

53. Romano, A.; Gallelli, C.A.; Koczwara, J.B.; Braegger, F.E.; Vitalone, A.; Falchi, M.; Micioni Di Bonaventura, M.V.; Cifani, C.; Cassano, T.; Lutz, T.A.; et al. Role of the Area Postrema in the Hypophagic Effects of Oleoylethanolamide. *Pharmacological Research* **2017**, doi:10.1016/j.phrs.2017.05.017.

54. Hülskötter, K.; Lühder, F.; Flügel, A.; Herder, V.; Baumgärtner, W. Tamoxifen Application Is Associated with Transiently Increased Loss of Hippocampal Neurons Following Virus Infection. *International Journal of Molecular Sciences* **2021**, *22*, doi:10.3390/ijms22168486.

55. Paxinos, G.; Watson, C. The Rat Brain in Stereotaxic Coordinates Seventh Edition. *Elsevier Academic Press* **2014**, *170*.

56. Giudetti, A.M.; Guerra, F.; Longo, S.; Beli, R.; Romano, R.; Manganelli, F.; Nolano, M.; Mangini, V.; Santoro, L.; Bucci, C. An Altered Lipid Metabolism Characterizes Charcot-Marie-Tooth Type 2B Peripheral Neuropathy. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **2020**, doi:10.1016/j.bbalip.2020.158805.

57. Giudetti, A.M.; de Domenico, S.; Ragusa, A.; Lunetti, P.; Gaballo, A.; Franck, J.; Simeone, P.; Nicolardi, G.; de Nuccio, F.; Santino, A.; et al. A Specific Lipid Metabolic Profile Is Associated with the Epithelial Mesenchymal Transition Program. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **2019**, doi:10.1016/j.bbalip.2018.12.011.

58. Flowers, M.T.; Ntambi, J.M. Role of Stearoyl-Coenzyme A Desaturase in Regulating Lipid Metabolism. *Current Opinion in Lipidology* 2008.

59. Coburn, C.T.; Hajri, T.; Ibrahimi, A.; Abumrad, N.A. Role of CD36 in Membrane Transport and Utilization of Long-Chain Fatty Acids by Different Tissues. In Proceedings of the Journal of Molecular Neuroscience; 2001.

60. Desvergne, B.; Wahli, W. Peroxisome Proliferator-Activated Receptors: Nuclear Control of Metabolism. *Endocrine Reviews* 1999.

61. Jump, D.B.; Tripathy, S.; Depner, C.M. Fatty Acid-Regulated Transcription Factors in the Liver. *Annual Review of Nutrition* **2013**, doi:10.1146/annurev-nutr-071812-161139.

62. Huang, J.; Tabbi-Anneni, I.; Gunda, V.; Wang, L. Transcription Factor Nrf2 Regulates SHP and Lipogenic Gene Expression in Hepatic Lipid Metabolism. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **2010**, *299*, doi:10.1152/ajpgi.00322.2010.

63. Ohtsuji, M.; Katsuoka, F.; Kobayashi, A.; Aburatani, H.; Hayes, J.D.; Yamamoto, M. Nrf1 and Nrf2 Play Distinct Roles in Activation of Antioxidant Response Element-Dependent Genes. *Journal of Biological Chemistry* **2008**, *283*, doi:10.1074/jbc.M804597200.

64. Hirotsu, Y.; Hataya, N.; Katsuoka, F.; Yamamoto, M. NF-E2-Related Factor 1 (Nrf1) Serves as a Novel Regulator of Hepatic Lipid Metabolism through Regulation of the Lipin1 and PGC-1 β Genes . *Molecular and Cellular Biology* **2012**, *32*, doi:10.1128/mcb.06706-11.

65. Romano, A.; Friuli, M.; del Coco, L.; Longo, S.; Vergara, D.; del Boccio, P.; Valentinuzzi, S.; Cicalini, I.; Fanizzi, F.P.; Gaetani, S.; et al. Chronic Oleoylethanolamide Treatment Decreases Hepatic Triacylglycerol Level in Rat Liver by a Pparγ/Srebp-Mediated Suppression of Fatty Acid and Triacylglycerol Synthesis. *Nutrients* **2021**, *13*, doi:10.3390/nu13020394.

66. Li, L.; Fu, J.; Liu, D.; Sun, J.; Hou, Y.; Chen, C.; Shao, J.; Wang, L.; Wang, X.; Zhao, R.; et al. Hepatocyte-Specific Nrf2 Deficiency Mitigates

High-Fat Diet-Induced Hepatic Steatosis: Involvement of Reduced PPARγ Expression. *Redox Biology* **2020**, *30*, doi:10.1016/j.redox.2019.101412.

67. Souza-Mello, V. Peroxisome Proliferator-Activated Receptors as Targets to Treat Non-Alcoholic Fatty Liver Disease. *World Journal of Hepatology* 2015, *7*.

68. Damiano, F.; Tocci, R.; Gnoni, G.V.; Siculella, L. Expression of Citrate Carrier Gene Is Activated by ER Stress Effectors XBP1 and ATF6α, Binding to an UPRE in Its Promoter. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* **2015**, *1849*, doi:10.1016/j.bbagrm.2014.10.004.

69. Li, J.; Huang, J.; Li, J.S.; Chen, H.; Huang, K.; Zheng, L. Accumulation of Endoplasmic Reticulum Stress and Lipogenesis in the Liver through Generational Effects of High Fat Diets. *Journal of Hepatology* **2012**, *56*, doi:10.1016/j.jhep.2011.10.018.

70. Sharma, N.K.; Das, S.K.; Mondal, A.K.; Hackney, O.G.; Chu, W.S.; Kern, P.A.; Rasouli, N.; Spencer, H.J.; Yao-Borengasser, A.; Elbein, S.C. Endoplasmic Reticulum Stress Markers Are Associated with Obesity in Nondiabetic Subjects. *Journal of Clinical Endocrinology and Metabolism* **2008**, *93*, doi:10.1210/jc.2008-1001.

71. Liu, J.; Jin, X.; Yu, C.H.; Chen, S.H.; Li, W.P.; Li, Y.M. Endoplasmic Reticulum Stress Involved in the Course of Lipogenesis in Fatty Acids-Induced Hepatic Steatosis. *Journal of Gastroenterology and Hepatology (Australia)* **2010**, *25*, doi:10.1111/j.1440-1746.2009.06086.x.

72. Haque, E.; Rezaul Karim, M.; Teeli, A.S.; Śmiech, M.; Leszczynski, P.; Winiarczyk, D.; Parvanov, E.D.; Atanasov, A.G.; Taniguchi, H. Molecular Mechanisms Underlying Hepatocellular Carcinoma Induction by Aberrant Nrf2 Activation-Mediated Transcription Networks: Interaction of Nrf2-Keap1 Controls the Fate of Hepatocarcinogenesis. *International Journal of Molecular Sciences* 2020, *21*.

73. Steffen, J.; Seeger, M.; Koch, A.; Krüger, E. Proteasomal Degradation Is Transcriptionally Controlled by TCF11 via an ERAD-Dependent Feedback Loop. *Molecular Cell* **2010**, *40*, doi:10.1016/j.molcel.2010.09.012.

74. Igarashi, M.; DiPatrizio, N. v.; Narayanaswami, V.; Piomelli, D. Feeding-Induced Oleoylethanolamide Mobilization Is Disrupted in the Gut of Diet-Induced Obese Rodents. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **2015**, *1851*, doi:10.1016/j.bbalip.2015.05.006.

75. Everard, A.; Cani, P.D. Diabetes, Obesity and Gut Microbiota. *Best Practice and Research: Clinical Gastroenterology* 2013, *27*.

76. Berthoud, H.R.; Lenard, N.R.; Shin, A.C. Food Reward, Hyperphagia, and Obesity. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 2011, *300*.

77. Hariri, N.; Thibault, L. High-Fat Diet-Induced Obesity in Animal Models. *Nutrition Research Reviews* **2010**, *23*, doi:10.1017/S0954422410000168.

78. Woods, S.C.; D'Alessio, D.A.; Tso, P.; Rushing, P.A.; Clegg, D.J.; Benoit, S.C.; Gotoh, K.; Liu, M.; Seeley, R.J. Consumption of a High-Fat Diet Alters the Homeostatic Regulation of Energy Balance. *Physiology and Behavior* **2004**, *83*, doi:10.1016/j.physbeh.2004.07.026.

79. Diep, T.A.; Madsen, A.N.; Holst, B.; Kristiansen, M.M.; Wellner, N.; Hansen, S.H.; Hansen, H.S. Dietary Fat Decreases Intestinal Levels of the Anorectic Lipids through a Fat Sensor. *The FASEB Journal* **2011**, *25*, doi:10.1096/fj.10-166595.

80. Sihag, J.; Jones, P.J.H. Oleoylethanolamide: The Role of a Bioactive Lipid Amide in Modulating Eating Behaviour. *Obesity Reviews* 2018.

81. Pan, X.; Schwartz, G.J.; Hussain, M.M. Oleoylethanolamide Differentially Regulates Glycerolipid Synthesis and Lipoprotein Secretion in Intestine and Liver. *Journal of Lipid Research* **2018**, doi:10.1194/jlr.M089250.

82. Tutunchi, H.; Naeini, F.; Saghafi-Asl, M.; Farrin, N.; Monshikarimi, A.; Ostadrahimi, A. Effects of Oleoylethanolamide Supplementation on Atherogenic Indices and Hematological Parameters in Patients with Nonalcoholic Fatty Liver Disease: A Clinical Trial. *Health Promotion Perspectives* **2020**, *10*, doi:10.34172/hpp.2020.56.

83. Tutunchi, H.; Ostadrahimi, A.; Saghafi-Asl, M.; Hosseinzadeh-Attar, M.J.; Shakeri, A.; Asghari-Jafarabadi, M.; Roshanravan, N.; Farrin, N.; Naemi, M.; Hasankhani, M. Oleoylethanolamide Supplementation in Obese Patients Newly Diagnosed with Non-Alcoholic Fatty Liver Disease: Effects on Metabolic Parameters, Anthropometric Indices, and Expression of PPAR-α, UCP1, and UCP2 Genes. *Pharmacological Research* **2020**, *156*, doi:10.1016/j.phrs.2020.104770.

84. de Souza, C.T.; Araujo, E.P.; Bordin, S.; Ashimine, R.; Zollner, R.L.; Boschero, A.C.; Saad, M.J.A.; Velloso, L.A. Consumption of a Fat-Rich Diet Activates a Proinflammatory Response and Induces Insulin Resistance in the Hypothalamus. *Endocrinology* **2005**, *146*, doi:10.1210/en.2004-1520.

85. Marques, C.G.; dos Santos Quaresma, M.V.L.; Nakamoto, F.P.; Magalhães, A.C.O.; Lucin, G.A.; Thomatieli-Santos, R.V. Does Modern Lifestyle Favor Neuroimmunometabolic Changes? A Path to Obesity. *Frontiers in Nutrition* 2021, *8*.

86. Dutheil, S.; Ota, K.T.; Wohleb, E.S.; Rasmussen, K.; Duman, R.S. High-Fat Diet Induced Anxiety and Anhedonia: Impact on Brain Homeostasis and Inflammation. *Neuropsychopharmacology* **2016**, *41*, doi:10.1038/npp.2015.357.

87. Kang, E.B.; Koo, J.H.; Jang, Y.C.; Yang, C.H.; Lee, Y.; Cosio-Lima, L.M.; Cho, J.Y. Neuroprotective Effects of Endurance Exercise Against High-Fat Diet-Induced Hippocampal Neuroinflammation. *Journal of Neuroendocrinology* **2016**, *28*, doi:10.1111/jne.12385.

88. Miao, Y.; Ren, J.; Jiang, L.; Liu, J.; Jiang, B.; Zhang, X. α-Lipoic Acid Attenuates Obesity-Associated Hippocampal Neuroinflammation and Increases the Levels of Brain-Derived Neurotrophic Factor in Ovariectomized Rats Fed a High-Fat Diet. *International Journal of Molecular Medicine* **2013**, *32*, doi:10.3892/ijmm.2013.1482.

89. Cai, M.; Wang, H.; Li, J. jing; Zhang, Y.L.; Xin, L.; Li, F.; Lou, S. jie The Signaling Mechanisms of Hippocampal Endoplasmic Reticulum Stress Affecting Neuronal Plasticity-Related Protein Levels in High Fat Diet-Induced Obese Rats and the Regulation of Aerobic Exercise. *Brain, Behavior, and Immunity* **2016**, *57*, doi:10.1016/j.bbi.2016.05.010.

90. Tucsek, Z.; Toth, P.; Sosnowska, D.; Gautam, T.; Mitschelen, M.; Koller, A.; Szalai, G.; Sonntag, W.E.; Ungvari, Z.; Csiszar, A. Obesity in Aging Exacerbates Blood-Brain Barrier Disruption, Neuroinflammation, and Oxidative Stress in the Mouse Hippocampus: Effects on Expression of Genes Involved in Beta-Amyloid Generation and Alzheimer's Disease. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* **2014**, *69*, doi:10.1093/gerona/glt177.

91. Jeon, B.T.; Jeong, E.A.; Shin, H.J.; Lee, Y.; Lee, D.H.; Kim, H.J.; Kang, S.S.; Cho, G.J.; Choi, W.S.; Roh, G.S. Resveratrol Attenuates Obesity-Associated Peripheral and Central Inflammation and Improves Memory Deficit in Mice Fed a High-Fat Diet. *Diabetes* **2012**, *61*, doi:10.2337/db11-1498.

92. Collins, P. The Sulcus Medianus Organ in the Fourth Cerebral Ventricle of the Rabbit: A Previously Undescribed Circumventricular Organ. *Journal of Anatomy* **1989**, *162*.

93. Langlet, F.; Mullier, A.; Bouret, S.G.; Prevot, V.; Dehouck, B. Tanycyte-like Cells Form a Blood-Cerebrospinal Fluid Barrier in the

Circumventricular Organs of the Mouse Brain. *Journal of Comparative Neurology* **2013**, *521*, doi:10.1002/cne.23355.

94. Sarria, A.J.; Panini, S.R.; Evans, R.M. A Functional Role for Vimentin Intermediate Filaments in the Metabolism of Lipoprotein-Derived Cholesterol in Human SW-13 Cells. *Journal of Biological Chemistry* **1992**, *267*, doi:10.1016/s0021-9258(18)41797-8.

95. Reese, T.S.; Karnovsky, M.J. Fine Structural Localization of a Blood-Brain Barrier to Exogenous Peroxidase. *The Journal of cell biology* **1967**, *34*, doi:10.1083/jcb.34.1.207.

96. Brightman, M.W.; Reese, T.S. Junctions between Intimately Apposed Cell Membranes in the Vertebrate Brain. *The Journal of cell biology* **1969**, *40*, doi:10.1083/jcb.40.3.648.

97. Westergaard, E.; Brightman, M.W. Transport of Proteins across Normal Cerebral Arterioles. *Journal of Comparative Neurology* **1973**, *152*, doi:10.1002/cne.901520103.

98. Rivera, P.; Pérez-Martín, M.; Pavón, F.J.; Serrano, A.; Crespillo, A.; Cifuentes, M.; López-Ávalos, M.D.; Grondona, J.M.; Vida, M.; Fernández-Llebrez, P.; et al. Pharmacological Administration of the Isoflavone Daidzein Enhances Cell Proliferation and Reduces High Fat Diet-Induced Apoptosis and Gliosis in the Rat Hippocampus. *PLoS ONE* **2013**, *8*, doi:10.1371/journal.pone.0064750.

99. Gault, V.A.; Lennox, R.; Flatt, P.R. Sitagliptin, a Dipeptidyl Peptidase-4 Inhibitor, Improves Recognition Memory, Oxidative Stress and Hippocampal Neurogenesis and Upregulates Key Genes Involved in Cognitive Decline. *Diabetes, Obesity and Metabolism* **2015**, *17*, doi:10.1111/dom.12432.

100. Yoo, D.Y.; Choi, J.H.; Kim, W.; Yoo, K.Y.; Lee, C.H.; Yoon, Y.S.; Won, M.H.; Hwang, I.K. Effects of Melissa Officinalis L. (Lemon Balm) Extract on

Neurogenesis Associated with Serum Corticosterone and GABA in the Mouse Dentate Gyrus. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-010-0312-2.

101. Yoo, D.Y.; Kim, W.; Kim, D.W.; Yoo, K.Y.; Chung, J.Y.; Youn, H.Y.; Yoon, Y.S.; Choi, S.Y.; Won, M.H.; Hwang, I.K. Pyridoxine Enhances Cell Proliferation and Neuroblast Differentiation by Upregulating the GABaergic System in the Mouse Dentate Gyrus. *Neurochemical Research* **2011**, *36*, doi:10.1007/s11064-010-0385-y.

102. Yoo, D.Y.; Nam, S.M.; Kim, W.; Lee, C.H.; Won, M.H.; Hwang, I.K.; Yoon, Y.S. N-Acetylserotonin Increases Cell Proliferation and Differentiating Neuroblasts with Tertiary Dendrites through Upregulation of Brain-Derived Neurotrophic Factor in the Mouse Dentate Gyrus. *Journal of Veterinary Medical Science* **2011**, *73*, doi:10.1292/jvms.11-0123.

103. Yoo, D.Y.; Kim, W.; Kim, D.W.; Nam, S.M.; Jung, H.Y.; Kim, J.W.; Lee, C.H.; Choi, J.H.; Won, M.H.; Yoon, Y.S.; et al. Cell Proliferation and Neuroblast Differentiation in the Dentate Gyrus of High-Fat Diet-Fed Mice Are Increased after Rosiglitazone Treatment. *Journal of Veterinary Science* **2014**, *15*, doi:10.4142/jvs.2014.15.1.27.

104. Yoo, D.Y.; Kim, W.; Yoo, K.Y.; Nam, S.M.; Chung, J.Y.; Yoon, Y.S.; Won, M.H.; Hwang, I.K. Effects of Pyridoxine on a High-Fat Diet-Induced Reduction of Cell Proliferation and Neuroblast Differentiation Depend on Cyclic Adenosine Monophosphate Response Element Binding Protein in the Mouse Dentate Gyrus. *Journal of Neuroscience Research* **2012**, *90*, doi:10.1002/jnr.23035.

105. Hamilton, A.; Patterson, S.; Porter, D.; Gault, V.A.; Holscher, C. Novel GLP-1 Mimetics Developed to Treat Type 2 Diabetes Promote Progenitor Cell Proliferation in the Brain. *Journal of Neuroscience Research* **2011**, *89*, doi:10.1002/jnr.22565.

106. Bonen, A.; Parolin, M.L.; Steinberg, G.R.; Calles-Escandon, J.; Tandon, N.N.; Glatz, J.F.C.; Luiken, J.J.F.P.; Heigenhauser, George.J.F.; Dyck, D.J. Triacylglycerol Accumulation in Human Obesity and Type 2 Diabetes Is Associated with Increased Rates of Skeletal Muscle Fatty Acid Transport and Increased Sarcolemmal FAT/CD36. *The FASEB Journal* **2004**, doi:10.1096/fj.03-1065fje.

107. Su, X.; Abumrad, N.A. Cellular Fatty Acid Uptake: A Pathway under Construction. *Trends in Endocrinology and Metabolism* 2009.

108. Koonen, D.P.Y.; Jacobs, R.L.; Febbraio, M.; Young, M.E.; Soltys, C.L.M.; Ong, H.; Vance, D.E.; Dyck, J.R.B. Increased Hepatic CD36 Expression Contributes to Dyslipidemia Associated with Diet-Induced Obesity. *Diabetes* **2007**, doi:10.2337/db07-0907.

109. Zhou, J.; Febbraio, M.; Wada, T.; Zhai, Y.; Kuruba, R.; He, J.; Lee, J.H.; Khadem, S.; Ren, S.; Li, S.; et al. Hepatic Fatty Acid Transporter Cd36 Is a Common Target of LXR, PXR, and PPARγ in Promoting Steatosis. *Gastroenterology* **2008**, doi:10.1053/j.gastro.2007.11.037.

110. Hui, S.T.; Parks, B.W.; Org, E.; Norheim, F.; Che, N.; Pan, C.; Castellani, L.W.; Charugundla, S.; Dirks, D.L.; Psychogios, N.; et al. The Genetic Architecture of NAFLD among Inbred Strains of Mice. *eLife* **2015**, doi:10.7554/eLife.05607.

111. Miyazaki, M.; Flowers, M.T.; Sampath, H.; Chu, K.; Otzelberger, C.; Liu, X.; Ntambi, J.M. Hepatic Stearoyl-CoA Desaturase-1 Deficiency Protects Mice from Carbohydrate-Induced Adiposity and Hepatic Steatosis. *Cell Metabolism* **2007**, doi:10.1016/j.cmet.2007.10.014.

112. Zhang, W.; Sun, Q.; Zhong, W.; Sun, X.; Zhou, Z. Hepatic Peroxisome Proliferator-Activated Receptor Gamma Signaling Contributes to Alcohol-Induced Hepatic Steatosis and Inflammation in Mice. *Alcoholism: Clinical and Experimental Research* **2016**, doi:10.1111/acer.13049.

113. Bonzom, P.M.A.; Nicolaou, A.; Zloh, M.; Baldeo, W.; Gibbons, W.A. NMR Lipid Profile of Agaricus Bisporus. *Phytochemistry* **1999**, doi:10.1016/S0031-9422(98)00703-1.

114. Boelsterli, U.A.; Bedoucha, M. Toxicological Consequences of Altered Peroxisome Proliferator-Activated Receptor γ (PPARγ) Expression in the Liver: Insights from Models of Obesity and Type 2 Diabetes. *Biochemical Pharmacology* 2002.

115. Bensinger, S.J.; Tontonoz, P. Integration of Metabolism and Inflammation by Lipid-Activated Nuclear Receptors. *Nature* 2008.

116. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: Activators of the Complete Program of Cholesterol and Fatty Acid Synthesis in the Liver. *Journal of Clinical Investigation* **2002**, doi:10.1172/jci15593.

117. Liang, G.; Yang, J.; Horton, J.D.; Hammer, R.E.; Goldstein, J.L.; Brown, M.S. Diminished Hepatic Response to Fasting/Refeeding and Liver X Receptor Agonists in Mice with Selective Deficiency of Sterol Regulatory Element-Binding Protein-1c. *Journal of Biological Chemistry* **2002**.

118. Wang, W.; Chan, J.Y. Nrf1 Is Targeted to the Endoplasmic Reticulum Membrane by an N-Terminal Transmembrane Domain: Inhibition of Nuclear Translocation and Transacting Function. *Journal of Biological Chemistry* **2006**, *281*, doi:10.1074/jbc.M602802200.

119. Zhang, Y.; Crouch, D.H.; Yamamoto, M.; Hayes, J.D. Negative Regulation of the Nrf1 Transcription Factor by Its N-Terminal Domain Is Independent of Keap1: Nrf1, but Not Nrf2, Is Targeted to the Endoplasmic Reticulum. *Biochemical Journal* **2006**, *399*, doi:10.1042/BJ20060725.

120. Ma, Q. Role of Nrf2 in Oxidative Stress and Toxicity. *Annual Review of Pharmacology and Toxicology* 2013, *53*.

121. Taguchi, K.; Motohashi, H.; Yamamoto, M. Molecular Mechanisms of the Keap1-Nrf2 Pathway in Stress Response and Cancer Evolution. *Genes to Cells* 2011, *16*.

122. Pi, J.; Leung, L.; Xue, P.; Wang, W.; Hou, Y.; Liu, D.; Yehuda-Shnaidman, E.; Lee, C.; Lau, J.; Kurtz, T.W.; et al. Deficiency in the Nuclear Factor E2-Related Factor-2 Transcription Factor Results in Impaired Adipogenesis and Protects against Diet-Induced Obesity. *Journal of Biological Chemistry* **2010**, *285*, doi:10.1074/jbc.M109.093955.

123. Lee, A.H.; Scapa, E.F.; Cohen, D.E.; Glimcher, L.H. Regulation of Hepatic Lipogenesis by the Transcription Factor XBP1. *Science* **2008**, *320*, doi:10.1126/science.1158042.

124. Basseri, S.; Austin, R.C. Endoplasmic Reticulum Stress and Lipid Metabolism: Mechanisms and Therapeutic Potential. *Biochemistry Research International* 2012.

125. Parola, M.; Novo, E. Nrf1 Gene Expression in the Liver: A Single Gene Linking Oxidative Stress to NAFLD, NASH and Hepatic Tumours. *Journal of Hepatology* **2005**, *43*, doi:10.1016/j.jhep.2005.09.008.

126. Hamazaki, J.; Murata, S. Er-Resident Transcription Factor NRF1 Regulates Proteasome Expression and Beyond. *International Journal of Molecular Sciences* 2020, *21*.

127. Skat-Rørdam, J.; Højland Ipsen, D.; Lykkesfeldt, J.; Tveden-Nyborg, P. A Role of Peroxisome Proliferator-Activated Receptor γ in Non-Alcoholic Fatty Liver Disease. *Basic and Clinical Pharmacology and Toxicology* 2019, *124*.

Chapter 3:

Deranged central oxytocinergic signaling in pre-clinical models of obesity and hedonic feeding: effects of increased oleoylethanolamide tone

1. Abstract

The neuropeptide oxytocin (OXY), by binding its receptor (oxytocin receptor, OXYR), is implicated in a variety of processes, including the modulation of both eating behavior and metabolism, at the crossroad between homeostatic and non-homeostatic systems. Concerning the regulation of eating behavior, great attention has been dedicated to the naturally occurring bioactive lipid oleoylethanolamide (OEA), which belongs to the family of N-acyl-ethanolamides (NAEs), for its pro-satiety effect that is strictly associated to the activation of selected brain areas, which actively participate in the control of energy homeostasis and feeding behavior. Moreover, it has been demonstrated that the anorexigenic effect of OEA is associated to the activation of the central oxytocinergic system, thus suggesting an interplay between OEA and OXY signaling in the regulation of eating behavior.

Therefore, the aim of the present chapter was to evaluate whether rats with different aberrant food intake behavior showed deranged oxytocinergic signaling, by measuring both OXY and OXY-R expression in specific brain regions; furthermore, we evaluated whether a direct or indirect pharmacological manipulation of the endogenous OEA tone might affect these potential alterations.

2. Introduction

Oxytocin (OXY) is a nine-amino acid neuropeptide hormone, mainly produced in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus (HYPO) [1,2].

In addition to its extensively studied influence on social behavior and reproductive function, it is now well established the key role of central oxytocinergic signaling in the modulation of eating behavior [3]. Particularly, in the homeostatic control of eating, the oxytocinergic system has been reported to have a complex downstream signaling pathway, which comprises the involvement of a variety of different neuropeptides and hormones [2]. However, in addition to acting in brain regions, such as the HYPO and the brainstem (which includes the area postrema and the nucleus of the solitary tract; AP and NST) that regulate "homeostatic" or energy need-based aspects of food intake, OXY acts within the brain reward circuitry to suppress consumption and the motivated response to palatable food [3]. Therefore, OXY integrates homeostatic signals derived from the HYPO with hedonic signals arising from the mesolimbic system (which includes caudate putamen and nucleus accumbens; CPU and NAc) with inputs from superordinate decision-making centers, such as the medial prefrontal cortex (mPFC), to coordinate a harmonized response on eating behavior [2].

Concerning the regulation of eating behavior, great attention has been dedicated to a class of endogenous lipid molecules, the N-acyl-ethanolamides (NAEs) to which belong the endocannabinoid anandamide (AEA) and other endocannabinoid-like compounds (structurally like endocannabinoids but which do not bind the endocannabinoid receptors) such as palmitoylthanolamide, oleoylethanolamide (OEA),

stearoylethanolamide and linoleoylethanolamide. Particularly, OEA, by recruiting PPAR-α receptors, reduces food intake and body weight gain [4–6] in both lean and obese rodents and is, also, able to restore a physiologic sensitivity to the rewarding properties of fat in a mouse model of diet-induce obesity (DIO) [7]. Moreover, evidence accumulated over the years revealed, in the pro-satiety effect evoked by OEA, the involvement of selected brain areas, which actively participate in the control of energy homeostasis and feeding behavior, such as PVN and SON in HYPO and AP and NST in brainstem [4,8–10]. It has also been demonstrated a necessary role of different neuronal circuits, including oxytocinergic system, in mediating OEA action as appetite suppressor [11]. Thus, the peripheral administration of OEA induces the release of OXY from the PVN, SON and neurohypophysis and the central administration of the selective OXY-R antagonist, L-368,899, completely abolishes OEA-induced satiety, supporting the pivotal role played by this neuropeptide in mediating OEA's effects [8,10]. Moreover, the multitude of hormonal and neurotransmitter functions of OXY are mediated by the specific oxytocin receptor (OXY-R), a member of the G-protein coupled receptor family, widely expressed in all the brain nuclei activated by OEA [1,2].

Therefore, the aim of the present chapter was to evaluate whether rats with different aberrant food intake behavior show deranged oxytocinergic signaling, by measuring both OXY and OXY-R expression in specific brain regions; furthermore, we evaluated whether a direct or indirect pharmacological manipulation of the endogenous OEA tone might affect these potential alterations.

We used three different preclinical model of aberrant eating pattern:

1. a rat model of DIO, 2. a rat model of binge-like eating, in which the combination of dieting and stress is a common trigger for the onset of the

aberrant food intake, and a 3. a rat model of DIO based on hedonic overfeeding induced by the exposure to a high palatable food (HPF, cafeteria diet) for 40 days and subsequently to an abstinence period from this HPF.

The animal model and the experimental paradigms were described in detail in the next paragraphs.

The impact of the stimulation of the endogenous OEA tone on the central oxytocinergic system (OXY and OXY-R expression levels) was assessed pharmacologically either directly by peripherally administering OEA (10 mg/kg i.p.) to bingeing and to DIO rats or indirectly by administering the fatty acid amide hydrolase (FAAH, the enzyme involved in the catabolism of the acylethanolamides) inhibitor, PF-3845 (10 mg/kg subcutaneously), to rats that underwent an abstinence period from the cafeteria diet.

3. Materials and methods

3.1.Animals and diets

- **Binge eating model**

Female Sprague Dawley rats (Charles River, Italy), weighing 200–225 g at the beginning of the experiments, were group housed under a 12 hours (h) light/dark cycle (lights on at 8:00 A.M.), at constant temperature (20–22°C) and humidity (45–55%) and with access to food and water *ad libitum* for 2 weeks before the experiments. According to the dietary schedule, the rats were given standard food pellets (4RF18; Mucedola; 2.6 kcal/g) or HPF (3.63 kcal/g) consisting in a paste prepared by mixing Nutella (Ferrero®) chocolate cream (5.33 kcal/g; 56, 31, and 7% from carbohydrate, fat, and protein, respectively), ground food pellets (4RF18), and water in the following w/w/w percent ratio: 52% Nutella, 33% food pellets, and 15% water.

The procedure for binge eating induction was performed according to previous studies published by my research group [12–14]and in collaboration with Prof. Carlo Cifani of the University of Camerino. Briefly, two groups of female rats were housed individually in metal cages (30 x 30 x 30 cm) and exposed (or not exposed) for 24 days to three 8-day cycles of intermittent food restriction (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle), during which they were given access to HPF for 2 h during the light cycle between 10:00 A.M. and 12:00 A.M. (2 h after the onset of the light cycle) on days 5–6 and 13–14 of the first two cycles (total of four exposures) [13]. On the test day (day 25), at 10 A.M., half of the animals in each group was subjected to a 15 min frustration stress, consisting of the exposure to HPF placed out of reach [12]. During this 15

min period, the rats could smell and see the HPF and repeatedly attempted to reach it. The second half of animals in each group was not exposed to the stress manipulation, therefore we will refer to dietary restricted (R) vs non restricted (NR) rats and exposed to stress (S) vs non exposed to stress (NS) rats. After 15 min stress exposure, the HPF was placed inside the cage for all rats. In accordance with previous studies published by my research group [12–14], binge eating behavior occurred in R+S rats as demonstrated by the immediate and persistent consumption of a larger amount of HPF within the first 15 min access, with respect to the other groups. Vaginal smears were collected at the end of experiments to exclude from the results rats in the estrous phase [14].

All experiments were carried out in accordance with the European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals.

The procedure of binge eating induction is depicted in Fig. 3.1.

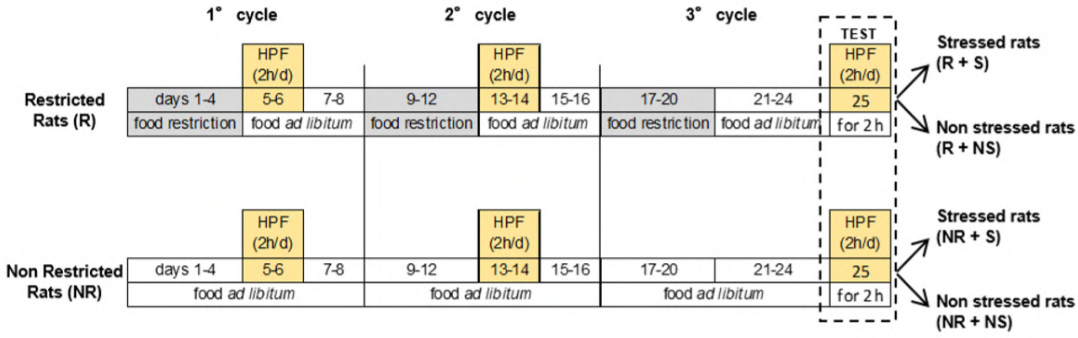

Fig. 3.1: Procedure of binge eating induction.

- **DIO model**

This preclinical model is a useful tool to study the obesity state, sharing several common features with human conditions. Indeed, the availability of

high-fat diet (HFD) and the resulting overconsumption represents the etiology of obesity in modern societies [15].

This model has already been described in detail in the chapter 2.

- **Cafeteria model**

This model was developed in accordance with previous model described by Johnson and Kenny [16].

Male Wistar rats (Charles River, Italy) were used in this study, weighing 300–350 g at the beginning of the experiments. Rats were group-housed under a 12h light/dark cycle, at constant temperature (20 –22°C) and humidity (45–55%) and with access to food and water *ad libitum* for the entire period before starting the experiments. All experiments were performed in accordance with the European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals.

During the phase 1 of the experiment (from day 1 to day 40) animals were randomly subdivided into two different groups:

- Control group rats (CHOW; n=14) with *ad libitum* access to standard food pellets (4RF18; Mucedola; 2.6 kcal/g);
- Cafeteria group rats (CAF; n=12) with *ad libitum* access to both standard food and Cafeteria diet consisting in a mixture of various foods available for human consumption such as: mortadella (3.2 kcal/g), cookies (Macine, Mulino Biaco; 4.8 kcal/g) muffin (Mr Day; 4.5 kcal/g), cheese chips (Fonzies; 5.3 kcal/g), cheese (4.3 kcal/g), sippets (San Carlo; 5.5 kcal/g) and lard (9 kcal/g). Each food was individually weighed before being made available to the rats. The caloric intake from the various foods was calculated based on the nutritional information provided by the manufacturer.

Animals followed the eating schedule described until the end (day 40) of phase 1 of the experiment. Body intake and food intake (expressed as mean kcal/kg ingested ± S.E.M.) were measured every day.

The detailed timeline of the experiment is depicted in Fig. 3.3.

3.2.Drugs and treatments

- **Binge eating model**

Experiment 1: effect of OEA on stress-induced binge-eating

The first set of rats ($N = 144$) was divided into 16 groups ($N = 9$ per group) in a 2 (history of intermittent food restriction: yes (R), no (NR) rats) × 2 (stress during testing: yes (S), no (NS) rats) × 4 (OEA dose: 0, 2,5, 5, and 10 mg/kg) factorial design, to evaluate the behavioral effects of OEA during the test day. To this aim, OEA or VEH (saline/PEG/Tween80, 90/5/5 v/v/v) were acutely administered intraperitoneally (i.p.) 1h before the access to HPF; rats were exposed (or not exposed) to the 15-min frustration stress, and once they had access to the HPF, the intake was measured at the following time points (15, 30, 60, and 120 min).

The experimental paradigm is depicted in Fig. 3.2.

Experiment 2: effect of OEA on oxytocin receptor expression

A second experiment was conducted focusing on the NR+S and R+S rat's groups acutely treated with either VEH or OEA at the highest dose of 10 mg/kg i.p, based on the observation made in experiment 1. The rationale of the choice of these groups was based on the observation that intermittent caloric restriction was the predisposing condition that allowed stress to act as a trigger (R + S), whereas the ad libitum feeding condition represented the baseline control, in which stress was ineffective (NR + S), thus also providing the control for the stress effect. In this experiment we evaluated

whether the interaction between dietary regimen and stress exposure was accompanied by alteration of OXY-R immunoreactivity in selected brain regions, and whether OEA treatment might affect this endpoint. Therefore, 30 rats were subjected to the same procedure used in experiment 1 but, on the test day, were allowed to consume the HPF only for 1 hour (Fig. 3.2). At the end of this procedure all rats were sacrificed, their brains immediately collected, snap frozen in 2-metylbutane (-60°C) and stored at -80°C until processed for immunohistochemical analyses.

The experimental paradigm is depicted in Fig. 3.2

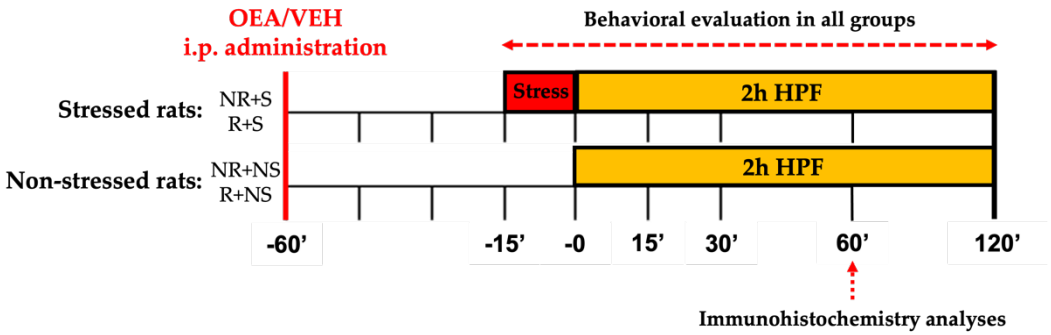

Fig 3.2: Timeline of treatments and frustration stress procedure.

- **DIO model**

After the induction of the obese phenotype, 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 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 (saline/PEG/Tween80, 90/5/5 v/v/v, 2 ml/kg) or OEA (10 mg/kg, 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, pSHIFT and pLFD). 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.

On the day of the terminal experiment, all food was removed from the cages 1h prior to dark onset. All the animals were administered with either VEH or OEA (10 mg/kg, i.p.) 10 minutes before the dark phase, and then had again free access to food. One hour after the administration, a first group of animals was deeply anesthetized with pentobarbital sodium (80 mg/kg; 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 48h at 4°C), and then snap frozen in dry-ice-cold 2-methylbutane (-60°C), to be stored at -80°C until processed for immunohistochemical analyses.

The detailed timeline of the experiment is depicted in Fig. 2.2 of chapter 2.

- **Cafeteria model**

Starting from day 41, CAF rats underwent an abstinence period of 28 days (phase 2) from Cafeteria diet; in particular, CAF rats had *ad libitum* access only to standard food pellets (4RF18; Mucedola; 2.6 kcal/g) until the end of the experiment (day 68); CHOW rats were maintained with *ad libitum* standard food until the end of the experiment. During phase 2 of the experiment CAF rats were chronically treated every other day with PF-3845 (10 mg/kg s.c.; CAF PF group n=5) or with VEH (ethanol/tween80/saline, 5/5/90 v/v/v; CAF VEH group n=7); identically, also animals from CHOW group were treated either with PF-3845 (10 mg/kg s.c.; CHOW PF group n=6) or with VEH (CHOW VEH group n=8).

On the day of terminal experiment the animals were euthanized by CO2 overdose. Brains were extracted and immediately snap-frozen in 2-methyl butane (-60°C) and stored at -80°C until processed for immunohistochemical analyses.

Body intake and food intake (expressed as mean kcal/kg ingested ± S.E.M.) were measured every day, in both phase 1 and phase 2 of the experiment.

The detailed timeline of both phase 1 (40 days) and phase 2 (28 days) of the experiment is depicted in Fig. 3.3.

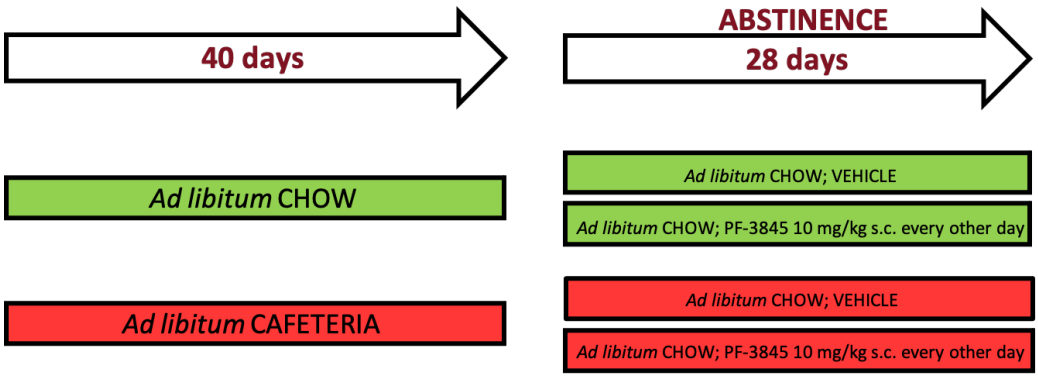

Fig. 3.3: Diagram of the timeline of the experiment with the diets and the treatments to which animals were exposed; subcutaneously (s.c.)

3.3. Immunohistochemistry experiments and densitometric analysis

Brains were collected from: NR+S and R+S rats' groups acutely treated with both VEH and OEA (10 mg/kg i.p.) for binge eating model; LF, HF and SHIFT rats' groups chronically treated with both VEH and OEA (10 mg/kg i.p.) for DIO models; CHOW rats and rats abstinent from the cafeteria diet chronically treated with both VEH and PF-3845 (10 mg/kg s.c.) for cafeteria model.

Each brain was sliced into 20 μm serial coronal sections by using a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA). The coronal slices were mounted on positively charged slides (Super Frost Plus, Menzel, Germany) and stored either at -20°C (binge eating and DIO models) or at -80° (cafeteria model) until further analyzed.

For OXY-R immunohistochemistry experiments, we adapted the protocol already published in a previous paper [17]; these experiments were performed in different brain areas including the mPFC that participates to the mechanisms regulating eating on the bases of a conditioned response arising from previous eating-acquired experiences (superordinate decision-making area), the CPU and NAc involved in the non-homeostatic regulation of food intake as part of the reward system. Moreover, only in DIO rats, OXY-R immunohistochemistry was also performed in the PVN, the HYPO, the AP, the NST and its sub-nuclei (SolC, SolDM, SolM and SolVL), all brain areas crucially involved in the hypophagic effect of OEA and, according to the literature, all brain areas presenting the OXY-R.

Unlike binge eating and cafeteria models, brain slices of DIO rats, containing the desired brain areas, underwent an initial antigen retrieval procedure by submerging selected slides in a sodium citrate buffer (10 mM

pH 6,0) heated at 95°C for 5 min, as previous reported in a paper published by my research group [18]. The subsequent steps of the protocol used were the same for all the slices derived from the brains of the three animal models studied. Briefly, sections were rinsed with PBS (pH 7,4) and washed for 20 min with a solution containing PBS + 0,1% Triton X-100 (Sigma–Aldrich) + 20% methanol (Merk) + 1,5% hydrogen peroxide (Merk). After additional washes sections were incubated for 1h in a blocking solution contained 0,05% Triton X-100 (Sigma–Aldrich) and 10% of bovine serum albumin (BSA; SERVA Electrophoresis GmbH) with 2 drops of avidin for binge eating and DIO models and with 2% of Normal Donkey Serum (NDS; Jackson Immunoresearch) for cafeteria model. Sections were then incubated, overnight at 4°C, with a solution containing the primary antibody (rabbit anti-OXY-R, 1:500 dilution, Alomone Labs) and 0,2% Triton X-100 (Sigma–Aldrich) for binge eating and DIO models and blocking solution for cafeteria model.

During the second day, after additional washes, sections were incubated, for 2h at room temperature, with the secondary antibody (biotinylated donkey anti-rabbit, 1:500 dilution, Jackson Immunoresearch) in PBS + 0,3% Triton X-100 (Sigma–Aldrich). After incubation for 1h with the ABC Kit (Vectastain® ABC kit, Peroxidase; Vector Laboratories), sections were stained by incubation in DAB substrate kit (Vector Laboratories) chromogen solution. The slides were then rinsed with TBS (pH 7,3), dehydrated in graded alcohol, immersed in xylene and cover-slipped with Eukitt (Sigma–Aldrich).

Moreover, in DIO rats also OXY immunohistochemistry was performed in the PVN, one of the key brain areas implicated in the oxytocinergic signaling, following the protocol reported in papers already published by my research group [10,18,19].

Briefly, brain slices, containing the desired brain areas, underwent an initial antigen retrieval procedure by submerging selected slides in a sodium citrate buffer (10 mM pH 6,0) heated at 95°C for 5 min, as previous reported in a paper published by my research group [18]. Sections were rinsed with PB (pH 7,4) and washed for 20 min with a solution containing PB + 0.1% Triton X-100 (Sigma–Aldrich) + 20% methanol (Merk) + 1,5% hydrogen peroxide (Merk). After additional washes sections were incubated for 1h in a blocking solution contained PB + 0,1% Triton X-100 (Sigma–Aldrich) + 2% NDS (Jackson Immunoresearch). Sections were then incubated, overnight at 4°C, with the blocking solution containing the primary antibody (mouse anti-OXY, 1:1000 dilution, Millipore). During the second day, after additional washes, sections were incubated, for 1h at room temperature, with the secondary antibody (biotinylated donkey anti-mouse, 1:500 dilution, Jackson Immunoresearch) in PB + 0,1% Triton X-100 (Sigma–Aldrich). After incubation for 1h with the ABC Kit (Vectastain® ABC kit, Peroxidase; Vector Laboratories), sections were stained by incubation in DAB substrate kit (Vector Laboratories) chromogen solution. The slides were then rinsed with TBS (pH 7,3), dehydrated in graded alcohol, immersed in xylene and cover-slipped with Eukitt (Sigma–Aldrich).

The sliced were then 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 light field using either a 4× (mPFC, CPU, NAc, PVN and HYPO) or 10× (AP, NST and its sub-nuclei) objectives and the rat brain atlas by Paxinos and Watson [20] was used as reference for the localization of the brain areas of interest.

OXY-R and OXY DAB-immunostaining were measured semi-quantitatively as optical density (OD) by using the Scion Image J software and considering, for background normalization, the averaged OD either of

non-immunoreactive regions or of white matter structures within the same brain slice. For this analysis the investigator was blind to experimental groups and measurements were obtained in at least five consecutive tissue sections per animal containing the desired structure.

3.4.Statistical analysis

All data were expressed as mean ± SEM. For binge eating model feeding data showed in Fig. 3.4-A were statistically analyzed by three-way ANOVA for repeated measures, which included the intermittent food restriction (R or NR) and the frustration stress during testing (S or NS) as the between-subjects factors, with sessions time (0-15, 15-30, 30-60, 60-120 min) as the within-subject factor. Moreover, feeding data showed in Fig. 3.4-B, were statistically analyzed by two-way ANOVA with intermittent food restriction and stress as the two factors. Finally, feeding data showed in Fig. 3.5 A-D were statistically analyzed by one-way ANOVA with treatment as between-subject factor. Bonferroni's test for multiple comparisons (Systat Software 10.0) was used for post hoc analyses of all feeding data.

For cafeteria model the data obtained from the daily monitoring of the body weight gain and food intake in phase one were analyzed by a two-way ANOVA for repeated measures, setting "diet" (CHOW, CAF) and "time" as fixed variables, and the Bonferroni's test was used as post-hoc analysis for multiple comparisons. Similarly, data from phase two were analyzed with a two-way ANOVA for repeated measures (within the same treatment group), setting "diet" (CHOW, CAF) and "time" as fixed variables and with a Two-way ANOVA for repeated measures (within the same diet group), setting "treatment" (VEH, PF-3845) and "time" as fixed variables; the Bonferroni's test was used as post-hoc analysis for multiple comparisons.

Results obtained from immunohistochemistry were statistically analyzed by two-way ANOVA with two different fixed variables for each animal model: "restriction" (NR and R for binge eating model) and "treatment" (S + VEH and S + OEA) for binge eating model, "diet" (LFD, HFD and SHIFT) and "treatment" (VEH and OEA) for DIO model and "diet" (CHOW and CAF) and "treatment" (VEH and OEA) for cafeteria model. Tukey's test was used as a post hoc test to perform multiple comparisons (IBM SPSS, version 22, IBM Analytics). Moreover because of the difference in the number of slices examined and the high degree of freedom, the error degrees of freedom were kept constant at a value based on the actual number of animals per group used in each experiment [18].

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

4. Results

4.1. Behavioral results

- **Binge eating model**

The combination of caloric restriction and stress exposure induced BED

The statistical analyses of palatable food intakes during the test day demonstrated a significant interaction among the three factors (food restriction x stress x sessions time) ($F_{interaction} = 6,902$, df = 3/78, $p<0,001$). Post hoc tests revealed a significant ($p<0,001$) increase in HPF consumption at the 0-15 min time point in rats with a history of food restriction and exposed to frustration stress (R + S), as compared to the other groups, while no change in palatable food consumption was observed during the other sessions time of the test (15–30; 30-60; 60-120 min) among all groups (Fig. 3.4-A). Two-way ANOVA of the 120 min cumulative palatable food intake showed a two-way interaction (food restriction x stress) ($F_{interaction} = 4,460$, df

= 1/26, p<0,05) and the post hoc analyses (p<0,001) revealed that R + S rats were the only group showing increased HPF intake with respect to the other groups (Fig. 3.4-B). These results, in agreement with our previous studies [12–14] demonstrated that stress exposure was able to trigger a binge-like behavior in R + S rats, which consumed a large amount of HPF within a short period of time, while it had no effect on HPF intake in rats that did not have history of food restriction.

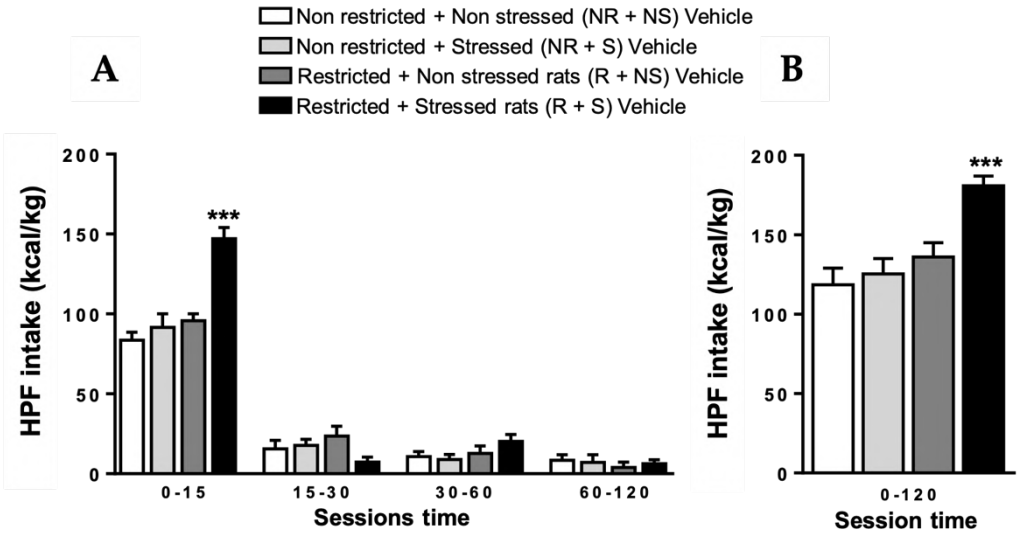

Fig. 3.4: Palatable food intake (kcal/kg) at different sessions time (0-15, 15-30, 30-60, 60-120 min) during testing (A) and total 120 min palatable food intake (B) in the VEH-injected rats in experiment 1. Data are expressed as mean ±SEM ***p<0,001, different from the other three groups.

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

We found that acute treatment with OEA, systemically administered to rats 1h before giving access to HPF (Fig. 3.2), selectively prevented binge-like eating of R + S rats (Fig. 3.5-A), without altering feeding behavior in the other experimental groups (Fig. 3.5 B-D). Particularly, OEA decreased frustration stress-induced HPF overconsumption in a dose- and time-dependent manner, with the strongest and long-lasting effect observed at the dosage of 10 mg/kg i.p. (Fig. 3.5A). The intermediate dose of OEA (5 mg/kg i.p.) was effective only at the 15-min time point, while the lowest dose of OEA was ineffective. The results obtained from ANOVA showed a significant effect of treatment in the session time 0–15 min ($F_{treatment} = 29{,}763$, df = 3/27, $p<0{,}001$) and in 0–120 min ($F_{treatment} = 5{,}758$, df = 3/27, $p<0{,}01$). Significant differences among groups evaluated by the post hoc analyses are indicated in Fig 3.5-A

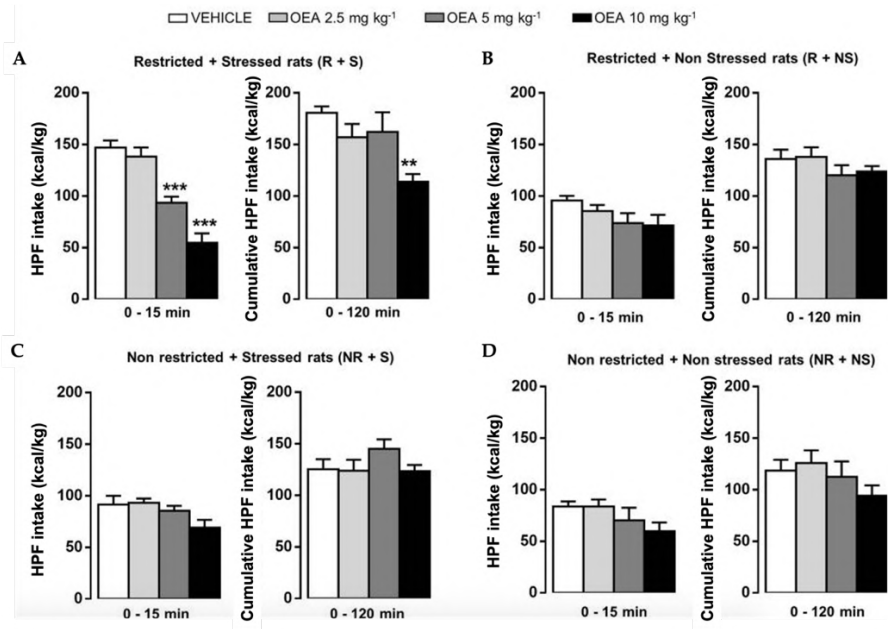

Fig. 3.5: HPF intake (kcal/kg) during the first 15 min (left) and the total 120 min (right) test session after vehicle (VEH) or 3 different doses of OEA (2,5, 5 and 10 mg/kg i.p.) administration to R+S (A, restricted–stressed), R+NS (B, restricted-non stressed), NR+S (C, non-restricted–stressed) and NR+NS (D, non-restricted-non stressed) rats. Data are expressed as mean ±SEM. **p<0,01; ***p<0,001

- **Cafeteria model**

Phase 1: food intake and body weight

The first phase of the experiment (days 1-40) was aimed to induce obesity in rats by hedonic overfeeding through the exposure to a cafeteria (CAF) diet, as explained in the materials and methods paragraph.

As expected, the exposure to CAF diet regimen caused rats to gain weight: since day 10 until the end of phase 1, there was a significant difference between the weight of CAF group compared to CHOW one. Two-way ANOVA for repeated measures analysis of body weight of phase 1 showed a significant effect of the diet (F_{diet}= 24,536, df= 1/19, p<0,001), a significant effect of the time (F_{time}= 381,917, df= 1/19, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 46,037, df= 1/19, p<0,001). Bonferroni's post-hoc analysis results are shown in Fig. 3.6.

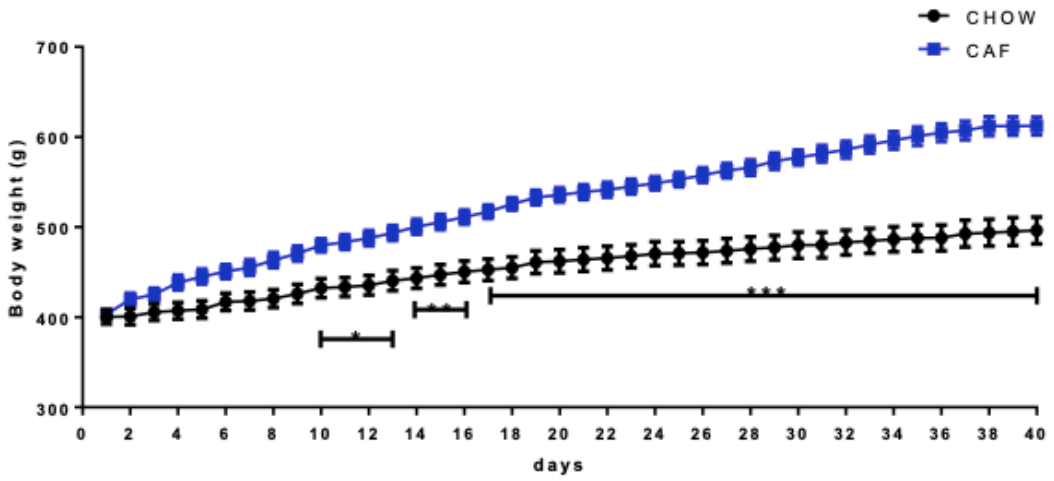

Fig. 3.6: Time course of body weight (g) during phase 1 of the experiment. Body weight was monitored every day. Results are expressed as mean ±SEM. *p<0,05; **p<0,01; ***p<0,001 in the same day (Bonferroni's post-hoc test for between-groups comparisons).

Significant body weight gain variation between the two groups was caused by significant variations of food intake: the analysis of daily caloric intake showed that animals exposed to CAF diet significantly consumed more

calories compared to CHOW group. In fact, two-way ANOVA for repeated measures analysis of normalized food intake of phase 1 showed a significant effect of diet (F_{diet}= 209,427, df= 1/19, p<0,001), a significant effect of time (F_{time}= 5,484, df= 1/19, p<0,001) and a significant effect of the interaction between the two factors was observed ($F_{interaction}$= 1,966, df=1/19, p<0,001). Bonferroni's post-hoc analysis results are shown in Fig. 3.7.

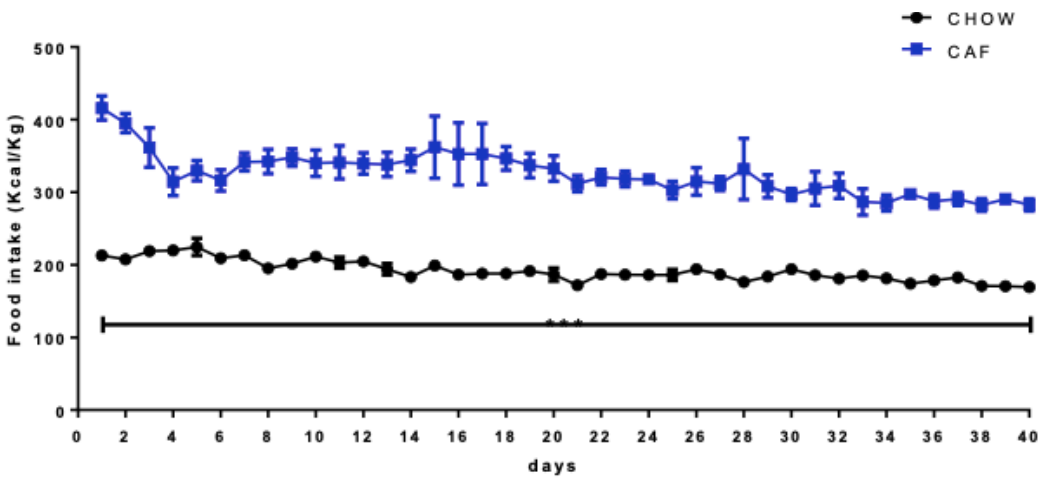

Fig.3.7: Time course of food intake (Kcal/Kg) during phase 1 of the experiment. Food intake was monitored every day. Results are expressed as mean ±SEM. ***p<0,001 on the same day (Bonferroni's post-hoc test for between-groups comparisons).

Phase 2: food intake and body weight

During phase 2 of the experiment (days 41-68) rats underwent an abstinence period from CAF diet. As expected, the withdrawal from CAF diet caused a significant reduction in both food intake and body weight: CAF rats, used to consume high palatable food, disliked consuming the standard pellet and reduced their caloric intake in the first twelve days of phase 2 (days 41-53). After this first period of adaptation, rats began to consume the same amount of food of the CHOW group, which was never exposed to CAF diet and, until the end of the experiment, no significant difference was detected in the body weight or in the food intake of animals from the different

groups. Interestingly, the chronic administration of PF-3845 (10 mg/kg s.c.) was unable to affect the body weight and food intake in both CHOW animals and in rats that underwent an abstinence period from CAF diet.

In fact, the two-way ANOVA for repeated measures of body weight of phase 2 between the groups CHOW VEH and CAF VEH showed a significant effect of the diet (F_{diet}= 9,927, df= 1/8, p<0,05), a significant effect of the time (F_{time}= 4,143, df= 1/8, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 41,967, df= 1/8, p<0,001). Differently, the two-way ANOVA between the groups CHOW PF and CAF PF did not show a significant effect of the diet (F_{diet}= 1045,712, df= 1/9, p>0,05), but a significant effect of the time (F_{time}= 9,320, df= 1/9, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 25,194, df= 1/9, p<0,001). Similarly, the two-way ANOVA analysis for the body weight between the groups CHOW VEH and CHOW PF did not show a significant effect of the treatment ($F_{treatment}$= 867,323, df= 1/9, p>0,05), but a significant effect of the time (F_{time}= 29,324, df= 1/9, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 5,394, df= 1/9, p<0,001). At the same time, the two-way ANOVA for repeated measures between the groups CAF VEH and CAF PF did not show a significant effect of both the treatment ($F_{treatment}$= 0,345, df= 1/8, p>0,05) and the interaction between the two factors ($F_{interaction}$= 0,394, df= 1/8, p>0,05) but a significant effect of the time (F_{time}= 38,228, df= 1/8, p<0,001).

Bonferroni's post-hoc analysis results are shown in Fig. 3.8.

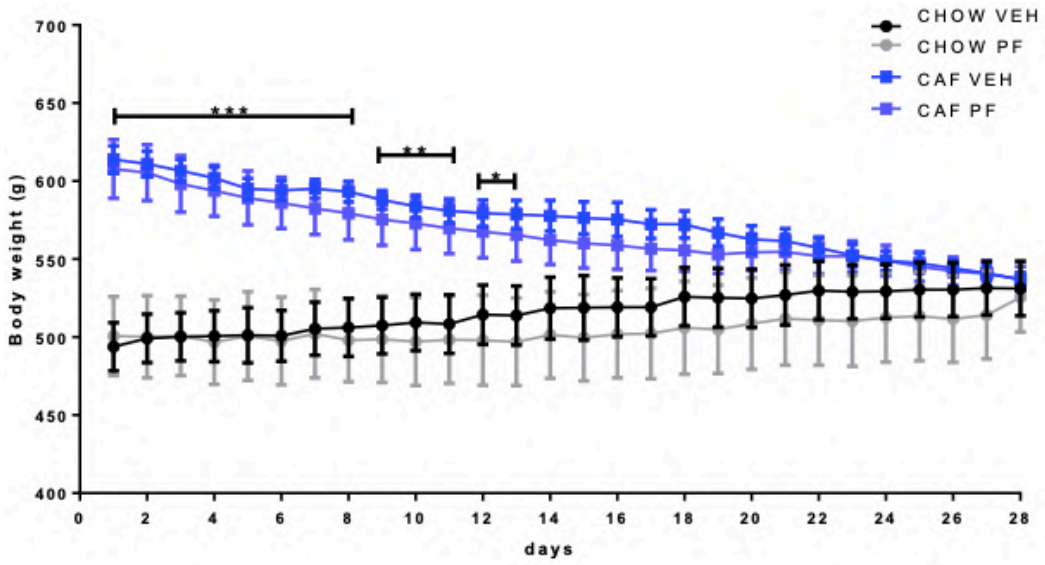

Fig. 3.8: Time course of body weight (g) during phase 2 of the experiment. Body weight was monitored every day. Results are expressed as mean ±SEM. *p<0,05; **p<0,01; ***p<0,001 in the same day between CHOW VEH and CAF VEH animals (Bonferroni's post-hoc test for between-groups comparisons).

On the other hand, in the analysis of food intake, the two-way ANOVA for repeated measures of phase 2 between the groups CHOW VEH and CAF VEH showed a significant effect of the diet (F_{diet}=16,872, df=8, p<0,01), a significant effect of the time (F_{time}=10,510, df=8, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$=11,550, df=8, p<0,001). The two-way ANOVA between the groups CHOW PF and CAF PF displayed a significant effect of the diet (F_{diet}=31,773, df=9, p<0,001), a significant effect of the time (F_{time}= 24,147, df= 1/9, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 27,998, df= 1/9, p<0,001). The same analysis for the food intake between the groups CHOW VEH and CHOW PF did not display a significant effect of the treatment ($F_{treatment}$= 0,342, df= 1/9, p>0,05), but a significant effect of the time (F_{time}= 1,546, df= 1/9, p<0,05) and a significant effect of the interaction between the two factors ($F_{interaction}$= 1,812, df= 1/9, p<0,05). At the same time, the two-way ANOVA for repeated measures between the groups CAF VEH and CAF PF

did not show a significant effect of the treatment ($F_{treatment}$= 4,372, df= 1/8, p>0,05), but a significant effect of the time (F_{time}= 73,804, df= 1/8, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 4,183, df= 1/8, p<0,001).

Bonferroni's post-hoc analysis results are shown in Fig. 3.9.

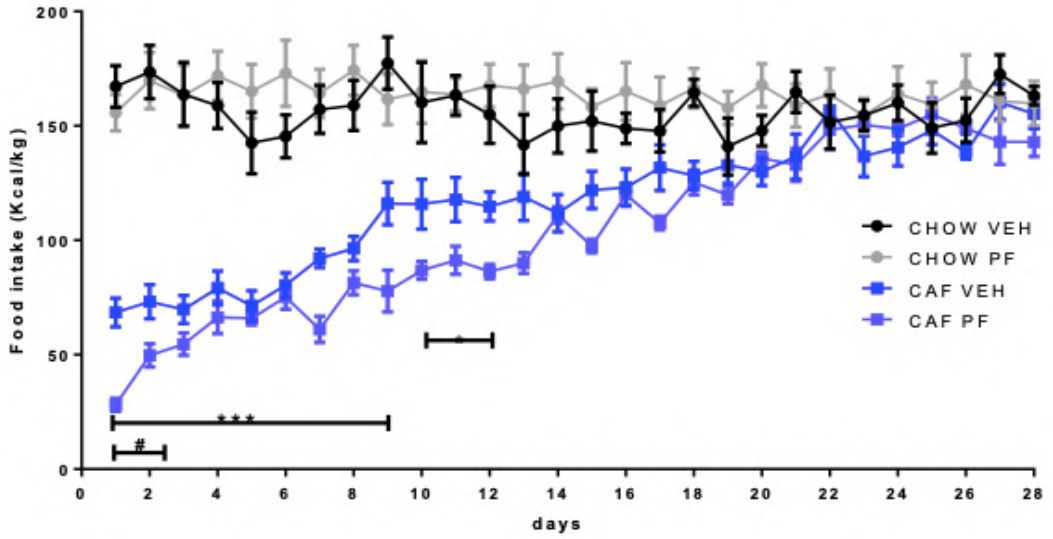

Fig. 3.9: Time course of food intake (kcal/kg) during phase 2 of the experiment. Food intake was monitored every day. Results are expressed as mean ±SEM. *p<0,05; ***p<0,001 in the same day between CHOW VEH and CAF VEH animals; #p<0,05 in the same day between CHOW PF and CAF PF animals (Bonferroni's post-hoc test for between-groups comparisons).

4.2. Effects of acute and chronic Oleoylethanolamide treatment on the expression of oxytocin and oxytocin receptor

- **Binge eating model**

Concerning the levels of OXY-R expression in the mPFC of binge eating rat model (Fig. 3.10-A), the results obtained by two-way ANOVA showed a significant effect of both treatment ($F_{treatment}$= 6,236, df= 1/11, p<0,05) and interaction between the two factors ($F_{interaction}$= 4,924, df= 1/11, p<0,05) and no significant effect of the food restriction ($F_{restriction}$= 1,684, df= 1/11, p=0,22). The results obtained from post-hoc Tuckey test showed a significant increase of the levels of OXY-R expression in R+S rats treated with OEA compared to VEH-treated R+S rats (p<0,01) (Fig. 3.10-B).

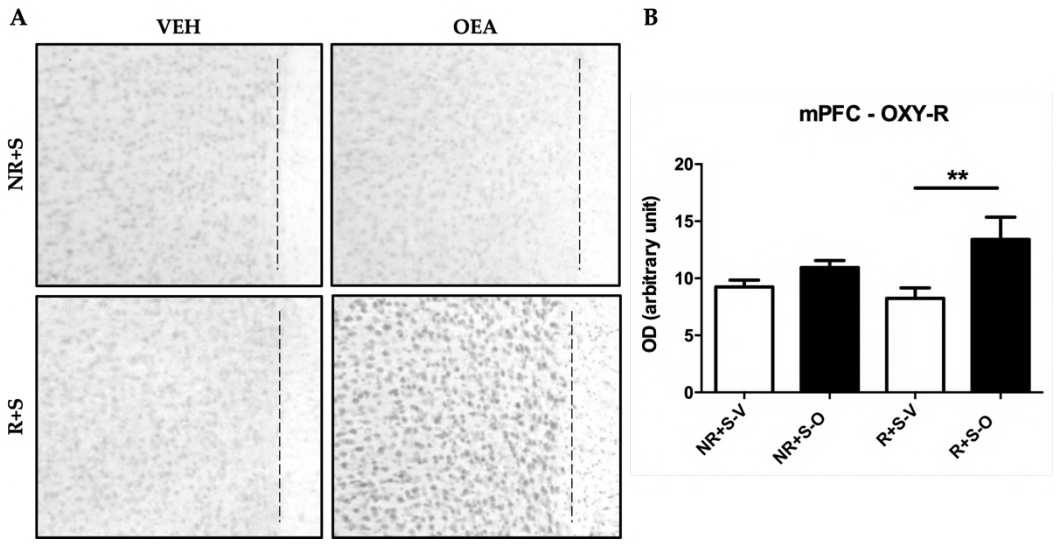

Fig. 3.10: Effects of acute OEA treatment on the expression of OXY-R in the mPFC of binge eating rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the mPFC. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the mPFC. Data are expressed as mean ±SEM. **p<0,01

Concerning the levels of OXY-R expression in the CPU and NAc of binge eating rat model (Fig. 3.11-A), the results obtained by two-way ANOVA showed a significant effect of interaction between the two factors in both

CPU ($F_{interaction}$= 8,479, df= 1/11, p<0,05) and NAc ($F_{interaction}$= 6,363, df= 1/11, p<0,05), a significant effect of treatment in NAc ($F_{treatment}$= 7,445, df= 1/11, p<0,05) but not in CPU ($F_{treatment}$= 1,440, df= 1/11, p=0,23) and no significant effect of food restriction in both the CPU ($F_{restriction}$= 0,481, df= 1/11, p=0,48) and the NAc ($F_{restriction}$= 2,047, df= 1/11, p=0,18).

The results obtained from post-hoc Tuckey test showed, in both CPU and NAc, a significant decrease of the levels of OXY-R expression in bingeing rats (R+S) treated with VEH compared to VEH-treated NR+S rats (CPU: p<0,05; NAc: p<0,01) and a significant increase of the levels of OXY-R expression in R+S rats treated with OEA compared to R+S rats treated with VEH (CPU: p<0,01; NAc: p<0,001) (Fig. 3.11-B).

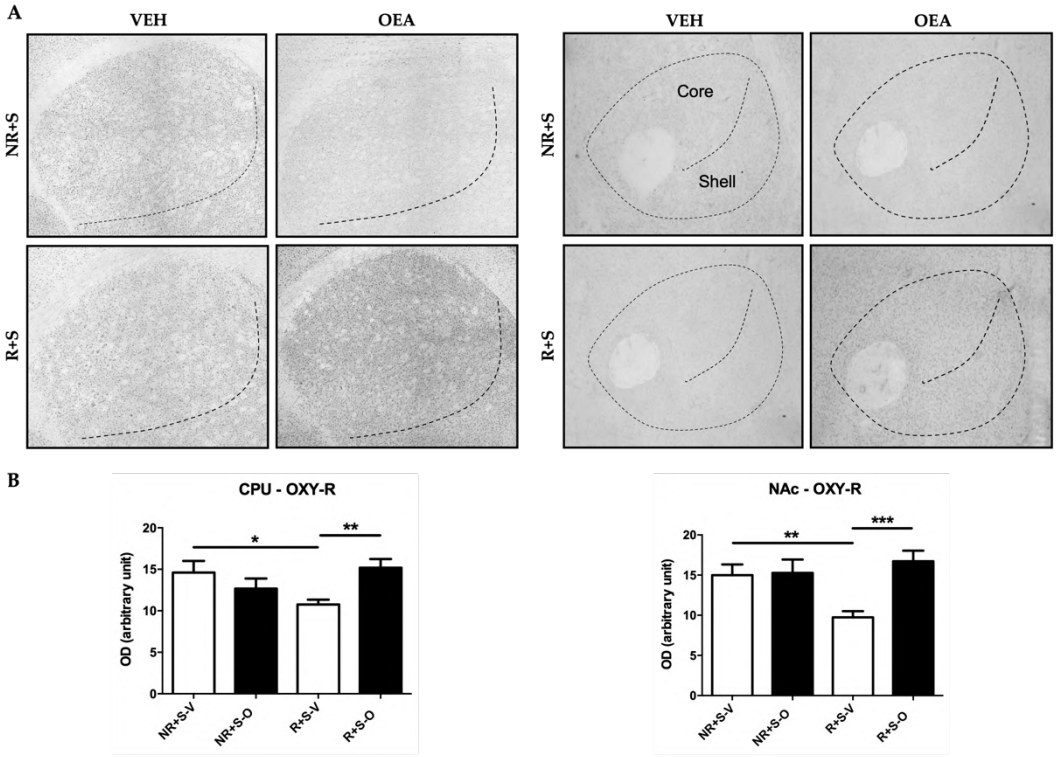

Fig. 3.11: Effects of acute OEA treatment on the expression of OXY-R in the CPU and NAc of binge eating rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the CPU and NAc. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the CPU and NAc. Data are expressed as mean ±SEM. *p<0,05 **p<0,01 ***p<0,001

- **DIO model**

Concerning the levels of OXY-R expression in the mPFC of DIO rat model (Fig. 3.12-A), the results obtained by two-way ANOVA showed a significant effect of the diet (F_{diet}= 12,816, df= 1/16, p<0,01), a significant effect of the treatment ($F_{treatment}$= 18,128, df= 1/16, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 10,145, df= 1/16, p<0,01).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to VEH-treated LF rats (p<0,001) and a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (HFO vs HFV p<0,01; SHIFTO vs SHIFTV p<0,001) (Fig. 3.12-B).

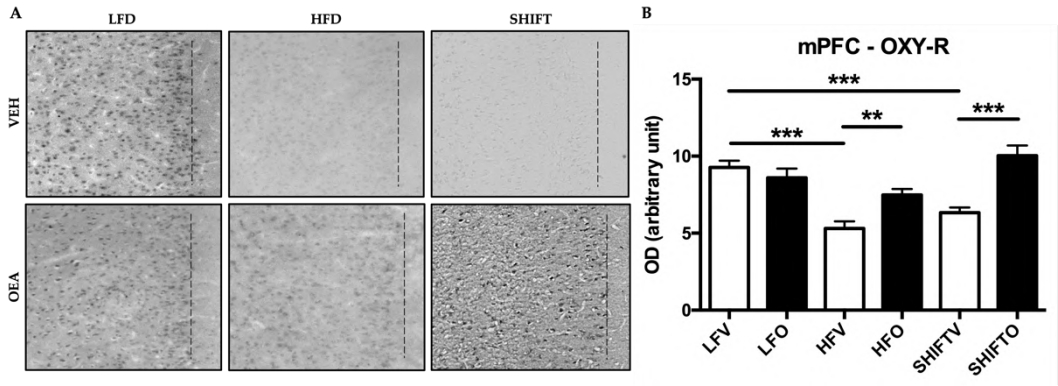

Fig. 3.12: Effects of chronic OEA treatment on the expression of OXY-R in the mPFC of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the mPFC. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the mPFC. Data are expressed as mean ±SEM. **p<0,01 *** p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the CPU of DIO rat model (Fig. 3.13-A), showed a significant effect of both the diet (F_{diet}= 72,761, df= 1/17, p<0,001) and the treatment

($F_{treatment}$= 17,111, df= 1/17, p<0,001) but no significant effect of the interaction between the two factors ($F_{interaction}$= 1,377, df= 1/17, p=0,25).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to VEH-treated LF rats (p<0,001), a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to OEA-treated LF rats (HFO vs LFO p<0,01; SHIFTO vs LFO p<0,001) and a significant decrease of the levels of OXY-R expression in SHIFT rats treated with both VEH and OEA compared to the HF rats belonging to the same treatment groups (p<0,001); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,01) (Fig. 3.13).

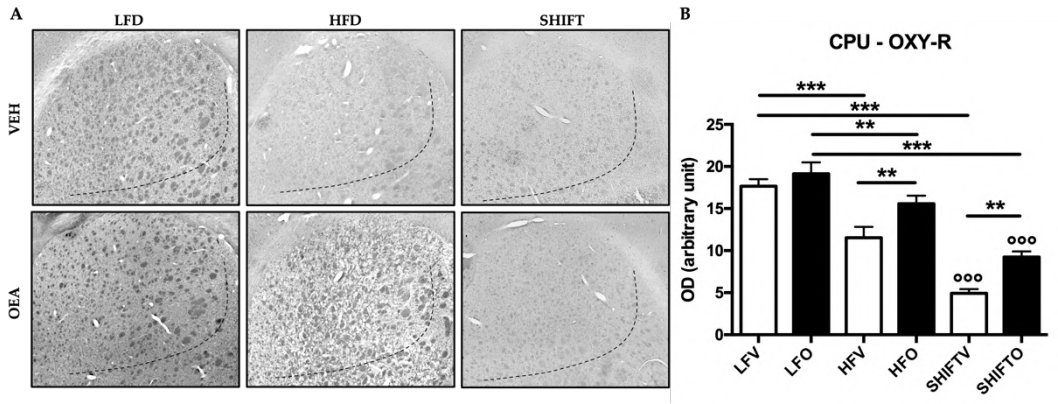

Fig. 3.13: Effects of chronic OEA treatment on the expression of OXY-R in the CPU of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the CPU. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the CPU. Data are expressed as mean ±SEM. °°° p<0,001 vs HF in the same treatment group; **p<0,01 *** p<0,001

Concerning the levels of OXY-R expression in the NAc of DIO rat model (Fig. 3.14-A), the results obtained by two-way ANOVA showed a

significant effect of both the diet (F_{diet}= 13,051, df= 1/17, p<0,01) and the treatment ($F_{treatment}$= 32,914, df= 1/17, p<0,001) but no significant effect of the interaction between the two factors ($F_{interaction}$= 1,033, df= 1/17, p=0,32).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in SHIFT rats treated with VEH compared to VEH-treated LF rats (p<0,001), a decrease, although not statistically significant, of the levels of OXY-R expression in HF rats treated with VEH compared to VEH-treated LF rats (p=0,056) and a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to OEA-treated LF rats (HFO vs LFO p<0,001; SHIFTO vs LFO p<0,01); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in LF, HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (LFO vs LFV p<0,001; HFO vs HFV p<0,05; SHIFTO vs SHIFTV p<0,001) (Fig. 3.14-B).

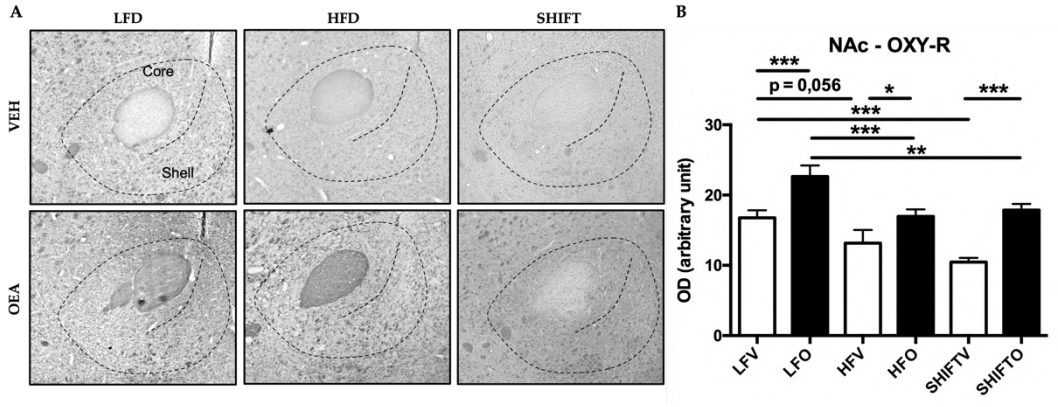

Fig. 3.14: Effects of chronic OEA treatment on the expression of OXY-R in the NAc of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the NAc. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the CPU. Data are expressed as mean ±SEM. *p<0,05 **p<0,01 ***p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the PVN of DIO rat model (Fig. 3.15-A), showed a significant effect of both the treatment ($F_{treatment}$= 40,210, df= 1/17, p<0,001) and the interaction between the two factors ($F_{interaction}$= 11,794, df= 1/17, p<0,01) but no significant effect of the diet (F_{diet}= 2,355, df= 1/17, p=0,14).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in HF rats treated with VEH compared to LF rats belonging to the same treatment group (p<0,05), a decrease, although not statistically significant, of the levels of OXY-R expression in SHIFT rats treated with VEH compared to VEH-treated LF rats (p=0,052), a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to OEA-treated LF rats (HFO vs LFO p<0,05; SHIFTO vs LFO p<0,001) and a significant increase of the levels of OXY-R expression in SHIFT rats treated with OEA compared to OEA-treated LF rats (p<0,05); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig. 3.15-B).

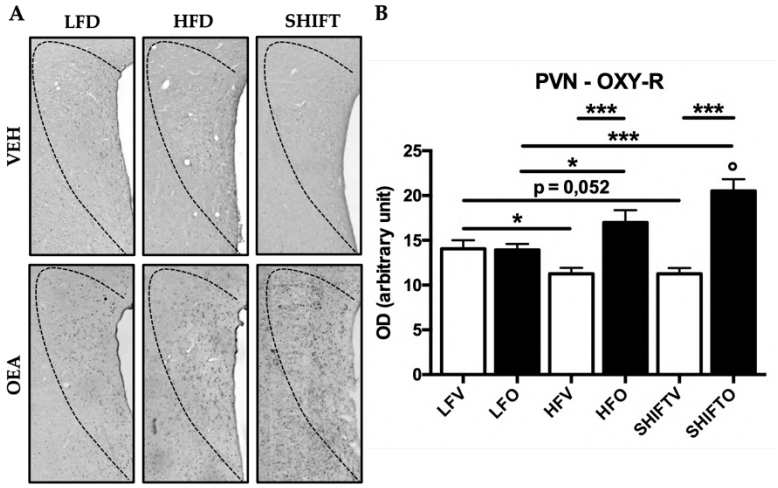

Fig. 3.15: Effects of chronic OEA treatment on the expression of OXY-R in the PVN of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the

PVN. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the PVN. Data are expressed as mean ±SEM. ° p<0,05 vs HF in the same treatment group; *p<0,05 *** p<0,001

Concerning the levels of OXY-R expression in the HYPO of DIO rat model, the results obtained by two-way ANOVA showed a significant effect of the diet (F_{diet}= 62,639, df= 1/17, p<0,001) a significant effect of the treatment ($F_{treatment}$= 33,406, df= 1/17, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 9,228, df= 1/17, p<0,01).

The results obtained from post-hoc Tuckey test showed a significant decrease the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to VEH-treated LF rats (HFV vs LFV p<0,001; SHIFTV vs LFV p<0,01), a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to OEA-treated LF rats (p<0,001) and a significant increase the levels of OXY-R expression in SHIFT rats treated with VEH compared to VEH-treated HF rats (p<0,001); moreover, the results obtained showed a significant increase the levels of OXY-R expression levels in both LF and HF rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig. 3.16).

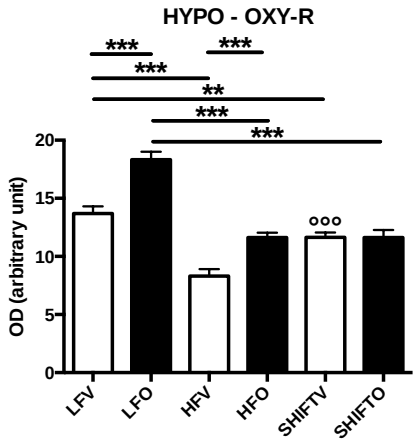

Fig. 3.16: Effects of chronic OEA treatment on the expression of OXY-R in the HYPO of DIO rat model. Representative images of OXY-R immunostaining in the HYPO are absent as it represents the

sum of the results obtained in its sub-nuclei. Data are expressed as mean ±SEM. °°°p<0,001 vs HF in the same treatment group. **p<0,01 ***p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the AP of DIO rat model (Fig. 3.17-A), showed a significant effect of the diet (F_{diet}= 5,644, df= 1/16, p<0,05) a significant effect of the treatment ($F_{treatment}$= 84,376, df= 1/16, p<0,001), and a significant effect of the interaction between the two factors ($F_{interaction}$= 24,284, df= 1/16, p<0,001).
The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (p<0,001), and a significant increase of the levels of OXY-R expression in SHIFT rats treated with OEA compared to LF rats belonging to the same treatment group (p<0,001); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig. 3.17-B).

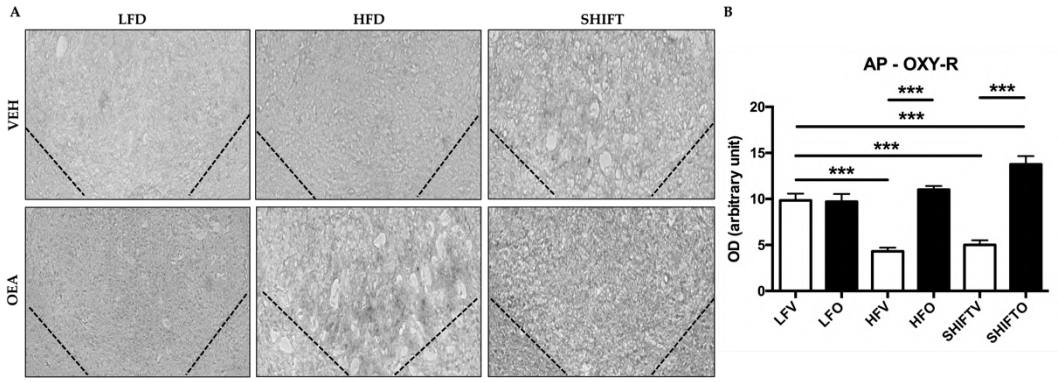

Fig. 3.17: Effects of chronic OEA treatment on the expression of OXY-R in the AP of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the AP. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the AP. Data are expressed as mean ±SEM ***p<0,001

Concerning the levels of OXY-R expression in the sub-nucleus SOLC of the NST of DIO rat model (Fig. 3.18-A), the results obtained by two-way ANOVA showed a significant effect of the diet (F_{diet}= 9,313, df= 1/16, p<0,01) a significant effect of the treatment ($F_{treatment}$= 61,246, df= 1/16, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 8,792, df= 1/16, p<0,01).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (HFV vs LFV p<0,001; SHIFTV vs LFV p<0,01) and a significant increase of the levels of OXY-R expression in SHIFT rats treated with OEA compared to LF rats belonging to the same treatment group (p<0,05); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig. 3.18-B).

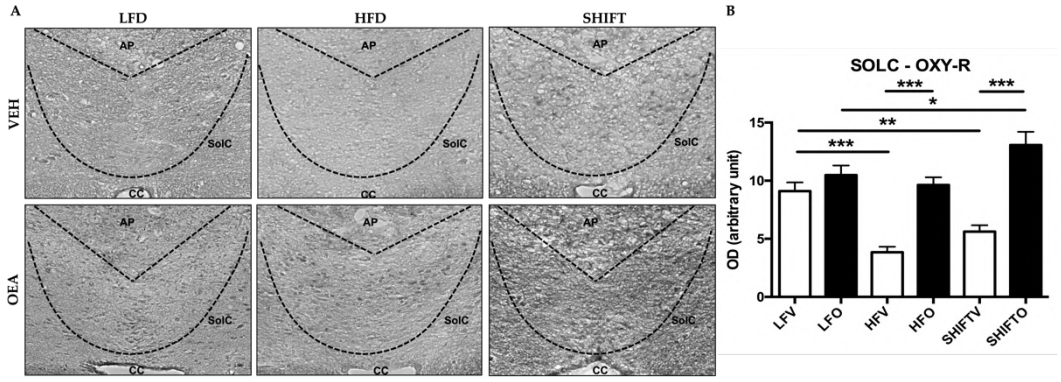

Fig. 3.18: Effects of chronic OEA treatment on the expression of OXY-R in the sub-nucleus SOLC of the NST of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the SOLC. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the SOLC. Data are expressed as mean ±SEM. *p<0,05 ** p<0,01 *** p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the sub-nuclei SOLDM and SOLVL of the NST of DIO rat model (Fig. 3.19-A), showed a significant effect of the treatment in both the SOLDM ($F_{treatment}$= 74,684, df= 1/16, p<0,001) and the SOLVL ($F_{treatment}$= 64,706, df= 1/16, p<0,001), a significant effect of the interaction between the two factors in both the SOLDM ($F_{interaction}$= 14,845, df= 1/16, p<0,01) and the SOLVL ($F_{interaction}$= 11,811, df= 1/16, p<0,01) and a significant effect of the diet in the SOLDM (F_{diet}= 5,280, df= 1/16, p<0,05) but no significant effect of the diet in the SOLVL (F_{diet}= 1,975, df= 1/16, p=0,17).

The results obtained from post-hoc Tuckey test showed, in both SOLDM and SOLVL, a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (p<0,001) and a significant increase of the levels of OXY-R expression in SHIFT rats treated with OEA compared to LF rats belonging to the same treatment group (p<0,05); moreover, the results obtained, from both SOLDM and SOLVL, showed a significant increase of the levels of OXY-R expression in both HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet groups but treated with VEH (p<0,001) (Fig. 3.19-B).

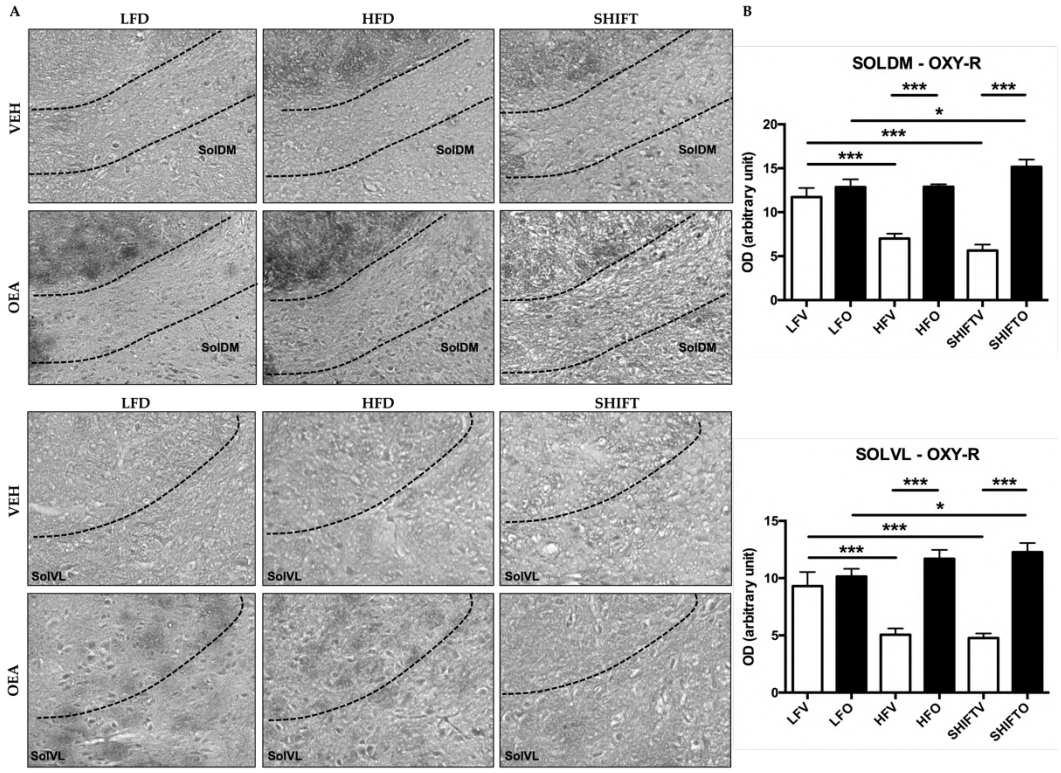

Fig. 3.19: Effects of chronic OEA treatment on the expression of OXY-R in the sub-nuclei SOLDM and SOLVL of the NST of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the SOLDM and SOLVL. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the SOLDM and SOLVL. Data are expressed as mean ±SEM *p<0,05 *** p<0,001

Concerning the levels of OXY-R expression in the sub-nucleus SOLM of the NST of DIO rat model (Fig. 3.20-A), the results obtained by two-way ANOVA showed a significant effect of the diet (F_{diet}= 10,584, df= 1/16, p<0,01), a significant effect of the treatment ($F_{treatment}$= 90,296, df= 1/16, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 6,401, df= 1/16, p<0,05).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (HFV vs LFV p<0,001; SHIFTV vs LFV p<0,01) and a significant increase of the levels of OXY-R expression in LF, HF and SHIFT rats treated

with OEA compared to the rats belonging to the same diet groups but treated with VEH (LFO vs LFV p<0,01; HFO vs HFV p<0,001; SHIFTO vs SHIFTV p<0,001) (Fig. 3.20).

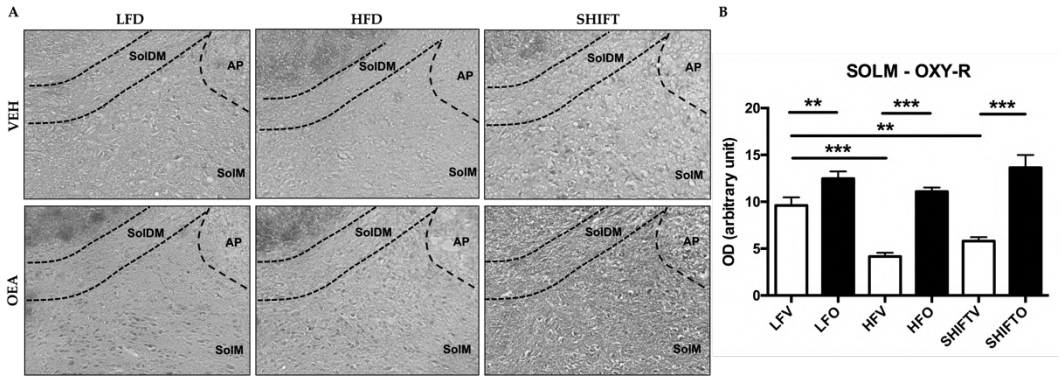

Fig. 3.20: Effects of chronic OEA treatment on the expression of OXY-R in the sub-nucleus SOLM of the NST of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the SOLM. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the SOLM. Data are expressed as mean ±SEM **p<0,01 *** p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the NST of DIO rat model, showed a significant effect of the diet (F_{diet}= 20,697, df= 1/16, p<0,001), a significant effect of the treatment ($F_{treatment}$= 264,803, df= 1/16, p<0,001) and a significant effect of the interaction between the two factors ($F_{interaction}$= 36,911, df= 1/16, p<0,001).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY-R expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (p<0,001) and a significant increase of the levels of OXY-R expression in SHIFT rats treated with OEA compared to LF rats belonging to the same treatment group (p<0,001); moreover, the results obtained showed a significant increase of the levels of OXY-R expression in LF, HF and SHIFT rats treated with OEA compared to the rats belonging to the same diet

groups but treated with VEH (LFO vs LFV p<0,01; HFO vs HFV p<0,001; SHIFTO vs SHIFTV p<0,001) (Fig. 3.21).

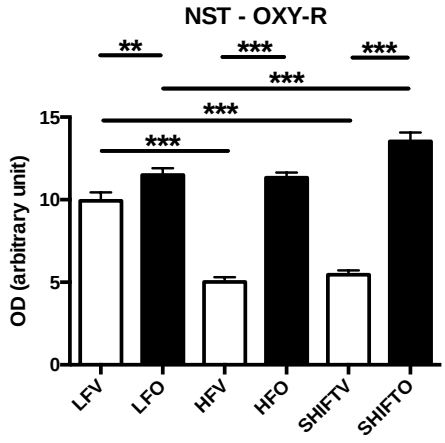

Fig. 3.21: Effects of chronic OEA treatment on the expression of OXY-R in the NST of DIO rat model. Representative images of OXY-R immunostaining in the NST are absent as it represents the sum of the results obtained in its sub-nuclei Data are expressed as mean ±SEM. **p<0,01 ***p<0,001

Finally, concerning the levels of OXY expression in the PVN of DIO rat model (Fig. 3.22-A), the results obtained by two-way ANOVA showed a significant effect of both the diet (F_{diet}= 16,173, df= 1/17, p<0,001), and the treatment ($F_{treatment}$= 90,296, df= 1/17, p<0,01) but no significant effect of the interaction between the two factors ($F_{interaction}$= 0,476, df= 1/17, p=0,49).

The results obtained from post-hoc Tuckey test showed a significant decrease of the levels of OXY expression in both HF and SHIFT rats treated with VEH compared to LF rats belonging to the same treatment group (HFV vs LFV p<0,01; SHIFTV vs LFV p<0,01) and a significant increase of the levels of OXY expression in SHIFT rats treated with OEA compared to HF rats belonging to the same treatment group (p<0,01); moreover, the results obtained showed a significant increase of the levels of OXY expression in both HF and SHIFT rats treated with OEA compared to the rats belonging

to the same diet groups but treated with VEH (HFO vs HFV p<0,01; SHIFTO vs SHIFTV p<0,05) (Fig. 3.22-B).

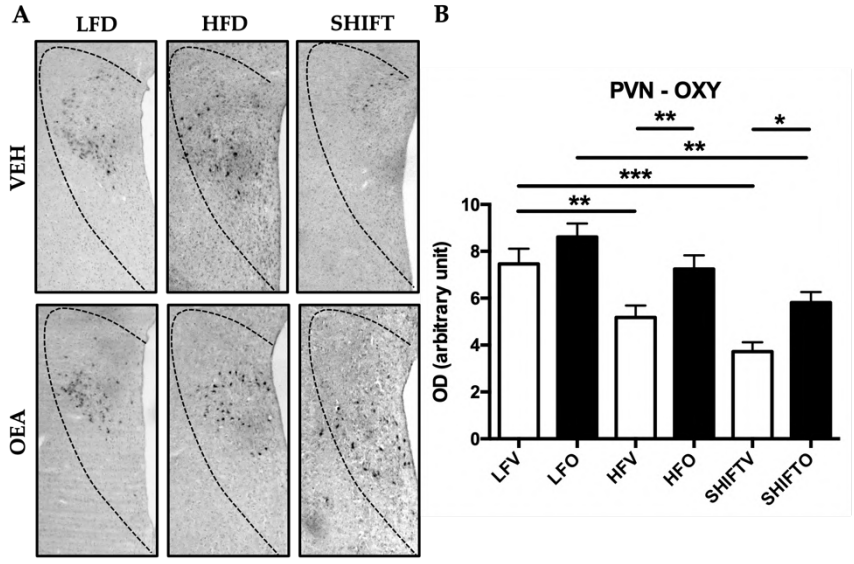

Fig. 3.22: Effects of chronic OEA treatment on the expression of OXY in the PVN of DIO rat model. (A) Representative photomicrographs (x20 magnification) of OXY immunostaining within the PVN. (B) Semiquantitative densitometric analysis of the expression of OXY within the PVN. Data are expressed as mean ±SEM. *p<0,05 **p<0,01 *** p<0,001

4.3. Effects of chronic PF-3845 treatment on the expression of oxytocin and oxytocin receptor

Concerning the levels of OXY-R expression in the mPFC of cafeteria rat model (Fig. 3.23-A), the results obtained by two-way ANOVA showed a significant effect of both treatment ($F_{treatment}$= 12,314, df= 1/11, p<0,01) and the interaction between the two factors ($F_{interaction}$= 6,634, df= 1/11, p<0,05) but no a significant effect of the diet (F_{diet}= 0,186, df= 1/11, p=0,67).

The results obtained from post-hoc Tuckey test showed a significant increase of the levels of OXY-R expression in rats abstinent from the cafeteria (CAF) diet treated with PF-3845 compared to VEH-treated rats abstinent from the CAF diet (p<0,001) (Fig. 3.23-B).

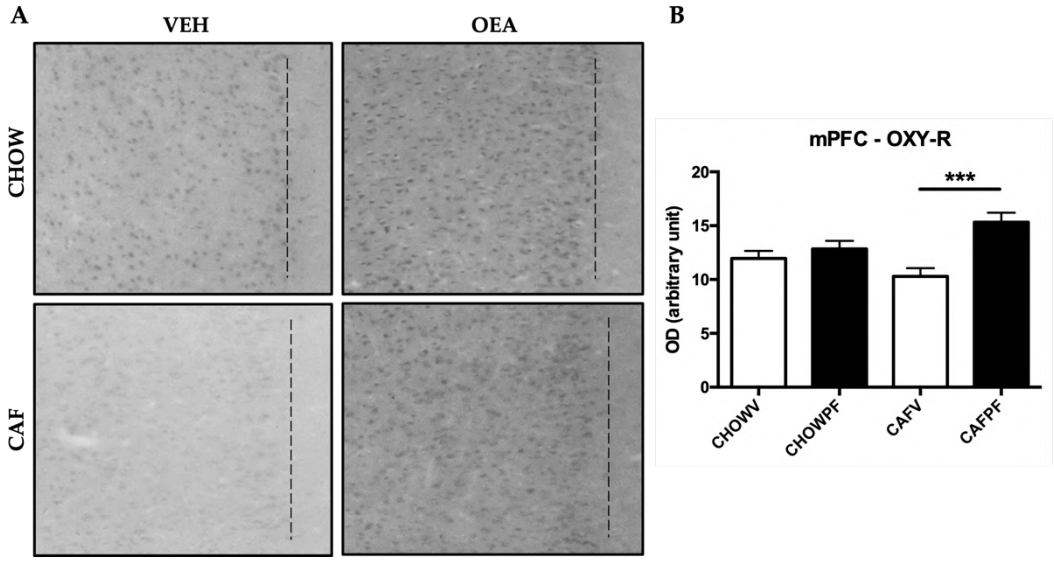

Fig. 3.23: Effects of chronic PF-3845 treatment on the expression of OXY-R in the mPFC of cafeteria rat model. A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the mPFC. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the mPFC. Data are expressed as mean ±SEM. ***p<0,001

The results obtained by two-way ANOVA, concerning the levels of OXY-R expression in the CPU and NAc of cafeteria rat model (Fig. 3.24-A), showed a significant effect of diet in both CPU (F_{diet}= 38,067, df= 1/11, p<0,001) and

NAc (F_{diet}= 24,357, df= 1/11, p<0,001) a significant effect of the treatment in both CPU ($F_{treatment}$= 36,921, df= 1/11, p<0,001) and NAc ($F_{treatment}$= 19,441, df= 1/11, p<0,01) and a significant effect of the interaction between the two factors in both CPU ($F_{interaction}$= 15,704, df= 1/11, p<0,01) and NAc ($F_{interaction}$= 11,799, df= 1/11, p<0,01).

The results obtained from post-hoc Tuckey test, in both CPU and NAc, showed a significant decrease of the levels of OXY-R expression in rats abstinent from the CAF diet treated with VEH compared to CHOW rats belonging to the same treatment group (p<0,001) and a significant increase of the levels of OXY-R expression in rats abstinent from the CAF diet treated with PF-3845 compared to VEH-treated rats abstinent from the CAF diet (p<0,001) (Fig. 3.24-B).

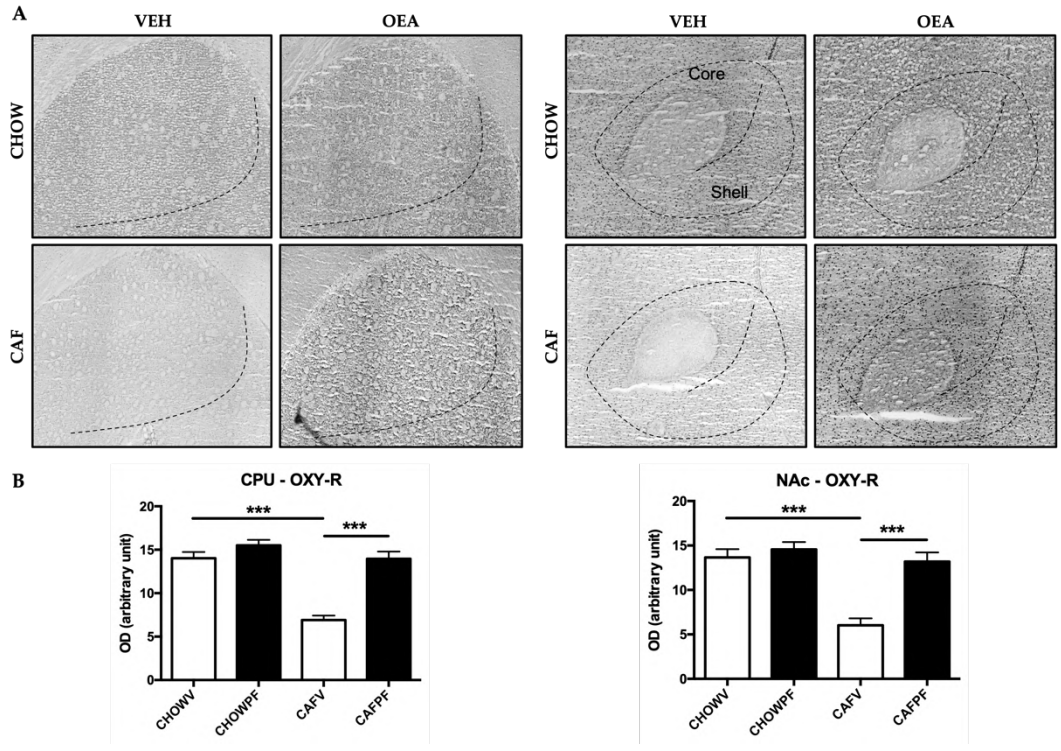

Fig. 3.24: Effects of chronic PF-3845 treatment on the expression of OXY-R in the CPU and NAc of cafeteria rat model. A) Representative photomicrographs (x20 magnification) of OXY-R immunostaining within the CPU and NAc. (B) Semiquantitative densitometric analysis of the expression of OXY-R within the CPU and NAc. Data are expressed as mean ±SEM. ***p<0,001

5. Discussion

The present study showed that the central oxytocinergic system is possibly one of the mechanisms that can coordinate several aspects of the energy balance at the crossroads between homeostatic and non-homeostatic mechanisms. Indeed, the results obtained suggest that OXY affects both the sensing of energy abundance/deficiency (homoeostatic aspects) and the motivation, as well as the salience and value of food (non-homeostatic aspects). In our models we hypothesize that, OXY integrates homeostatic signals derived from the HYPO with hedonic signals arising from the mesolimbic system and with inputs from superordinate decision-making centers, such as mPFC, to coordinate a harmonized response on feeding and energy metabolism.

However, as a peptide, OXY presents all the weaknesses associated with the administration of such compounds, comprising the chemical instability, the short half-life and the fast elimination [21]. Moreover, peptides are characterized by a scarce oral bioavailability, therefore the choice of a proper route of administration is crucial to evoke the desired effect.

In the present work we aimed to modulate the central oxytocinergic system, in different preclinical models of aberrant eating (binge eating, DIO and cafeteria models), directly by peripherally administering OEA (10 mg/kg i.p.) to bingeing and to DIO rats and indirectly by administering the fatty acid amide hydrolase (FAAH, the enzyme involved in the catabolism of the acylethanolamides) inhibitor, PF-3845 (10 mg/kg s.c.) to CAF rats.

The rationale of such hypothesis stems from the consideration that an interplay between OEA and OXY signaling, in the modulation of food intake, has been suggested by several studies [8–10,19]; particularly, it has been demonstrated that the anorexigenic effect of OEA is accompanied by

the stimulation of the central oxytocinergic system, in terms of increased secretion and expression of OXY peptide [8–10,19]. Moreover, it has been showed that the central administration of the selective OXY-R antagonist, L-368,899, completely abolishes OEA-induced satiety, supporting the pivotal role played by this neuropeptide in mediating OEA's effects [8].

In addition, in the pro-satiety effect evoked by OEA, a large body of evidence revealed the involvement of selected brain areas, which actively participate in the control of energy homeostasis and feeding behavior. Particularly, it has been observed that after being systemically administered, OEA (10 mg/kg i.p.) significantly increases the transcription of the early gene c-fos (marker of neuronal activation) in the NST and AP of brainstem and in the PVN, SON and tuberomammillary (TMN) hypothalamic nuclei [4,8–10].

Interestingly, in such nuclei, also the oxytocinergic system results particularly present. Indeed, the PVN is one of the hypothalamic nuclei strongly implicated in the synthesis of OXY; the HYPO and the brainstem (which includes AP and NST) represent the brain areas through which OXY regulate "homeostatic" aspects of food intake [3]. Moreover, OXY, in the non-homeostatic regulation of food intake, interacts with both the mesolimbic system (which includes CPU and NAc) and the superordinate decision-making centers, such as the mPFC [2]. These multitude of OXY functions are mediated by the specific oxytocin receptor (OXY-R), a member of the G-protein coupled receptor family, widely expressed in all the brain areas mentioned above [1,2].

Therefore, in this study we focused our attention on the expression of both OXY-R and OXY peptide within: the mPFC that participates to the mechanisms regulating eating on the bases of a conditioned response arising from previous eating-acquired experiences (superordinate decision-

making); CPU and NAc involved in the non-homeostatic regulation of food intake as part of the reward system; PVN, HYPO, AP and NST, brain areas involved in the synthesis of OXY and/or strictly involved in the OEA pro-satiety effect.

Binge eating and cafeteria models are both models of food addiction, in which the hedonic aspects of food play a key role. Indeed, excessive intake of HPF is associated with addiction-like induced deficits in brain reward system that, in turn, might drive overconsumption of this food to compensate for reward hyposensitivity [22,23].

Concerning cafeteria model, the results obtained from behavioral experiment of the phase 1, showed a difference, in terms of food intake and body weight, between rats exposed to CHOW diet and rats exposed to CAF diet. However, during abstinence phase, the result obtained showed that only in an initial stage food intake and body weight were affected in rats that underwent an abstinence period from CAF diet. This result suggests an effect of the abstinence period rather than of the chronic treatment with PF-3845. Concerning binge eating model, the results obtained from behavioral experiment, in agreement with previous studies published by my research group [12–14], showed that the combination of food restriction and stress is a trigger for the onset of binge-like behavior in rats, which consumed a large amount of HPF within a short period of time. Surprisingly, acute treatment with OEA (10 mg/kg i.p.), selectively prevented binge-like eating behavior only in bingeing rats suggesting that this anti-binge effect of OEA might likely be the consequence of the selective inhibition of hedonic hunger. This hypothesis seems to be supported by the results obtained in our immunohistochemistry experiments.

In binge eating and cafeteria models, immunohistochemistry experiments for OXY-R have been conducted only in the brain areas involved in the non-

homeostatic regulation of food intake: mPFC, CPU and NAc. The results obtained, within both the dorsal (CPU) and the ventral (NAc) striatum, revealed that both bingeing rats (R+S) and rats abstinent from the cafeteria diet (CAF) showed a reduction in the levels of OXY-R expression, suggesting a hypo-functionality of the oxytocinergic system. Surprisingly, OEA and PF-3845 treatments completely restored such decreases, reporting OXY-R immunoreactivity to the level observed in control groups.

The hypo-functionality of the oxytocinergic system can explain the onset of aberrant food intake. Animal studies support the role of OXY as a potent regulator of caloric intake, body weight and energy metabolism [24]. Indeed, it has been shown that OXY can acts as an anorexigenic hormone as both central and peripheral OXY administrations reduce food consumption, as well as sucrose [25,26] and saccharin [27] intake. Moreover, the deletion of genes encoding for OXY or OXY-R in mice promotes weight gain and/or dysregulated glucose regulation [28,29].

In addition, it has also been demonstrated that OXY participates to the control of reward-related behavior, by interacting with the central dopaminergic system. Particularly, the mesolimbic system is well known to play a pivotal role in the regulation of emotion, instinct, and reward-related behavior, including the food-associated reward [2]. Particularly, dopaminergic projections from the ventral tegmental area (VTA) to the striatum (CPU and NAc) are crucially involved in the reward processes associated to feeding behavior [30,31]. It has been demonstrated that OXY neurons of the PVN regulate the firing of dopaminergic neurons by projecting to the VTA and the striatum [32–36].

Therefore, we hypothesized that, at this level, the hypo-functionality of the oxytocinergic system was associated with an inability of OXY to decrease mesolimbic dopaminergic signaling and, consequently, to reduce

rewarding aspects of food intake, leading to an overconsumption of the HPF itself. In keeping with such hypothesis, it has been demonstrated that the central OXY administration into the VTA [33] and the NAc [27] induces a reduction of sucrose intake after food-deprivation [37] and that OXY attenuates consumption of fructose-sweetened beverages in monkeys [38]. Moreover, in 2013 Mullis and collaborators provided important evidence linking OXY to feeding reward [33]. The authors demonstrated that intra-VTA administration of OXY reduced deprivation-induced eating and palatability-driven sucrose intake; these effects were significantly abolished by the pretreatment with the selective OXY-R antagonist L-368,899. In addition, it has been demonstrated that OXY knockout mice showed an increased preference for sucrose [39,40], saccharin [41], and sodium compared to wild-type controls [42,43].

The results reported, suggested a possible neural mechanism, through which OXY may decrease mesolimbic dopaminergic signaling, by dampening dopamine release in the VTA and striatum, to reduce the reward value of HPF. The restoration of the functionality of the oxytocinergic system, exerted by OEA and PF-3845 (indirect increased of OEA tone) treatments, might enhance this neural mechanism and, consequently, might induce the reduction of the overconsumption of HPF, a well-known feature of obesity and eating-related disorders.

In the mPFC, the results obtained, revealed that OEA and PF-3845 treatments increased the levels of OXY-R expression in both bingeing rats (R+S) and rats abstinent from the cafeteria diet (CAF).

This enhancement of the oxytocinergic system, exerted by OEA and PF-3845 treatments, can be explained by the necessary involvement of higher-order brain centers in the regulation of feeding behavior [2]. Particularly, mPFC participates to the mechanisms regulating eating which takes into

consideration the cognitive expectations about the food. These expectations include taste, calorie content of the meal (hypocaloric vs hypercaloric), the satiating properties, and the healthy aspects [2]. OXY has been proposed to reduce eating by activating cognitive functions and shifting the attention from the short-term benefits derived from eating (for example rewarding properties) to the long-term consequences (such as increase of body weight and health problems) [44]. The results reported suggest that OXY increased the cognitive control of food addition, enhancing neural responses in superordinate decision-making regions. Therefore, in this scenario, the enhancement of the oxytocinergic system, exerted by OEA and PF-3845 treatments, suggested a dual role of this neuropeptide: on one hand OXY decreases the activity of networks that process the reward value of food and, on the other hand, OXY enhances the function of structures that mediate cognitive control of overconsumption behavior.

Concerning the pre-clinical model of DIO, immunohistochemistry experiments for OXY-R were conducted in different brain areas of both homeostatic and non-homeostatic systems regulating eating behaviors: mPFC, CPU and NAc mentioned above, PVN, HYPO, AP, NST and its sub-nuclei (SolC, SolDM, SolM and SolVL). Moreover, also immunohistochemistry experiment for OXY was conducted in the PVN of DIO rat model.

The results obtained, within the mPFC, CPU and NAc, revealed that also animals with an obese phenotype showed a reduction in the levels of OXY-R expression, suggesting a hypo-functionality of the oxytocinergic system. Surprisingly, OEA treatment completely restored such decreases. Also in the SHIFT group, introduced to mimic the fat and calories restriction observed in dieting individuals, the association between shift from HFD

and OEA treatment, in the mPFC, CPU and NAc, is associated with an increased OXY-R expression.

As mentioned above, decreased food intake appears to contribute to the ability of OXY to reduce body weight in rodents [45–49]. Administration of OXY to diet-induced rats [50] and mice [49] resulted in a decrease in body weight gain with a preferential reduction in fat mass in rats [51] as well as increased adipose tissue lipolysis, reduced glucose intolerance and insulin resistance in rats [45] and mice [46]. Moreover, Fritz and colleagues demonstrated that mice exposed to a high-fat, high-sugar "western diet" showed enhanced dopaminergic transmission throughout the dorsal striatum [52]. Therefore, these studies support the hypothesis that the hypo-functionality of the oxytocinergic system, observed in our results, is involved in the onset of the obese phenotype and that the excessive food intake observed in animals with an obese phenotype could be due to the inability of OXY to decrease mesolimbic dopaminergic signaling and, consequently, to reduce rewarding aspects of this type of food. In keeping with such hypothesis, it has been shown that OXY reduces the consumption of not only low fat/high carbohydrate diets, including sugars, but also high-fat diets (HFDs) [51]. Conversely, defective OXY signaling is linked to increased intake of fat [48,49] and carbohydrates, including sugar [33,39,53–55].

Altogether, the results reported suggest that the enhancement of the oxytocinergic system, exerted by OEA treatment, might positively affect food intake and body weight in part through impacting reward food-motivation brain regions; indeed, dietary choices and food intake emerge from a network of synergistically acting brain regions, including mPFC, CPU and NAc.

Concerning the homeostatic regulation of eating behaviors, the results obtained, showed in the animals with an obese phenotype a reduced OXY-R expression in all the brain areas analyzed: PVN, HYPO, AP, NST and its subnuclei; moreover, in the animals with an obese phenotype was also observed a reduced OXY expression in the PVN. Surprisingly, OEA treatment restored all these alterations. Moreover, also the association between dieting and OEA treatment in SHIFT group, was associated with an increase of both OXY expression in the PVN and OXY-R expression in all the brain areas analyzed, except for the HYPO in which no statistically significant variation was observed.

In the homeostatic control of eating, the oxytocinergic system has been reported to have a complex downstream signaling pathway, which comprise the involvement of a variety of different neuropeptides and hormones [2]. Once synthetized from magnocellular neurons of both PVN and SON nuclei OXY is released both locally within the HYPO [1] and distally, through axon terminals, within a variety of brain nuclei such as the arcuate nucleus (ARC), the VTA, the NAc, the amygdala, the HIPPO, and the PFC [1,56]. Moreover, oxytocinergic magnocellular neurons possess axonal projections to the neurohypophysis, which releases OXY into the blood system [2]. Oxytocinergic parvocellular neurons project toward various brainstem areas such as the NST and the AP [57]. OXY neurons of the PVN receive excitatory projections from ARC neuropeptide precursor pro-opiomelanocorticotropin (POMC) neurons (anorexigenic pathway) while receives inhibitory inputs from ARC neuropeptide Y/ agouti-related protein (NPY/AgRP) neurons (orexigenic pathway) [56,58–60]. This evidence underlies the importance of the projections of ARC POMC and AgRP/NPY first order neurons to PVN oxytocinergic second order neurons in the regulation of homeostatic feeding.

Furthermore, OXY might affect food intake also indirectly, by modulating the brain response to appetite-regulating hormones, including cholecystokinin (CCK) glucagon-like peptide type 1 (GLP-1) and ghrelin [61–67]. Indeed, parvocellular oxytocinergic projections within the descending pathway from the PVN to the NST are in the proximity of CCK-responsive neurons, responding to vagal-mediated peripheral CCK signals [2]. OXY induces a stimulation of such neurons that leads to an amplification of the CCK-inducing reduction of the meal size [68]. Moreover, the activation of GLP-1 signaling exerts a stimulatory effect on OXY release within the PVN (anorexigenic pathway) [62]. Also, the stomach-derived hormone ghrelin has been suggested to activate oxytocinergic neurons in the PVN of rats [69].

Therefore, the hypo-functionality of the oxytocinergic system, observed in our results, can cause an imbalance between orexigenic and anorexigenic pathways and, consequently, an altered homeostatic regulation of eating behaviors which can result in the onset of obesity.

Moreover, it has been demonstrated that the anorexigenic effect of OEA is associated to the activation of the central oxytocinergic system. Particularly, it has been demonstrated that peripherally administered OEA activates the AP [9], a brainstem region with fenestrated capillaries that lacks a functional blood-brain barrier (BBB). These properties allow the permeation of blood-born substances, which do not readily reach other areas of the brain parenchyma across the BBB [70–74]. At this level, OEA may cause the activation of noradrenergic neurons projecting to the NST. Noradrenergic neurons within the NST could, in turn, activate oxytocinergic neurons of the PVN [10], stimulating oxytocin expression and release [8,19] and, in addition, could presumably activate the TMN of the HYPO, evoking histamine release from neurons projecting to the PVN that indirectly

stimulate oxytocinergic neurons of this brain area [75]. Finally, OXY released from neurons of the PVN can centrally act to inhibit eating, mediating the OEA anorexiant actions [18]. These results support the key role of a functional oxytocinergic system in the hypophagic effect of OEA and support an interplay between OEA and OXY signaling in the regulation of obesity and eating related behaviors.

Therefore, the understanding of the biological substrates regulating feeding behavior is relevant to address the health problems related to food overconsumption. Several studies have expanded the conventional view of the homeostatic regulation of body weight mainly orchestrated by the HYPO, to include also the non-homeostatic control of appetite. The identification of endogenous systems acting as a bridge between homoeostatic and non-homeostatic pathways might represent a significant step toward the development of drugs for the treatment of aberrant eating patterns. Therefore, in the present paragraph has been summarized the role played by oxytocin in the control of both homeostatic and non-homeostatic eating, within cognitive, metabolic and reward mechanisms and it has been proposed that the modulation of the central oxytocinergic signaling by increasing directly or indirectly OEA tone might represent one of the mechanisms that coordinate food intake at the crossroads between homeostatic and non-homeostatic mechanisms in the treatment of aberrant eating behaviors.

6. References

1. Jurek, B.; Neumann, I.D. The Oxytocin Receptor: From Intracellular Signaling to Behavior. *Physiological Reviews* **2018**, doi:10.1152/physrev.00031.2017.

2. Romano, A.; Friuli, M.; Cifani, C.; Gaetani, S. Oxytocin in the Neural Control of Eating: At the Crossroad between Homeostatic and Non-Homeostatic Signals. *Neuropharmacology* 2020.

3. Liu, C.M.; Spaulding, M.O.; Rea, J.J.; Noble, E.E.; Kanoski, S.E. Oxytocin and Food Intake Control: Neural, Behavioral, and Signaling Mechanisms. *International Journal of Molecular Sciences* 2021, *22*.

4. Rodríguez De Fonseca, F.; Navarro, M.; Gómez, R.; Escuredo, L.; Nava, F.; Fu, J.; Murillo-Rodríguez, E.; Giuffrida, A.; Loverme, J.; Gaetani, S.; et al. An Anorexic Lipid Mediator Regulated by Feeding. *Nature* **2001**, doi:10.1038/35102582.

5. Fu, J.; Oveisi, F.; Gaetani, S.; Lin, E.; Piomelli, D. Oleoylethanolamide, an Endogenous PPAR-α Agonist, Lowers Body Weight and Hyperlipidemia in Obese Rats. *Neuropharmacology* **2005**, doi:10.1016/j.neuropharm.2005.02.013.

6. Serrano, A.; del Arco, I.; Javier Pavón, F.; Macías, M.; Perez-Valero, V.; Rodríguez de Fonseca, F. The Cannabinoid CB1 Receptor Antagonist SR141716A (Rimonabant) Enhances the Metabolic Benefits of Long-Term Treatment with Oleoylethanolamide in Zucker Rats. *Neuropharmacology* **2008**, doi:10.1016/j.neuropharm.2007.03.007.

7. Tellez, L.A.; Medina, S.; Han, W.; Ferreira, J.G.; Licona-Limón, P.; Ren, X.; Lam, T.K.T.; Schwartz, G.J.; de Araujo, I.E. A Gut Lipid Messenger Links Excess Dietary Fat to Dopamine Deficiency. *Science* **2013**, *341*, doi:10.1126/science.1239275.

8. Gaetani, S.; Fu, J.; Cassano, T.; Dipasquale, P.; Romano, A.; Righetti, L.; Cianci, S.; Laconca, L.; Giannini, E.; Scaccianoce, S.; et al. The Fat-Induced Satiety Factor Oleoylethanolamide Suppresses Feeding through Central Release of Oxytocin. *Journal of Neuroscience* **2010**, doi:10.1523/JNEUROSCI.0036-10.2010.

9. Romano, A.; Karimian Azari, E.; Tempesta, B.; Mansouri, A.; Micioni Di Bonaventura, M. v.; Ramachandran, D.; Lutz, T.A.; Bedse, G.; Langhans, W.; Gaetani, S. High Dietary Fat Intake Influences the Activation of Specific Hindbrain and Hypothalamic Nuclei by the Satiety Factor Oleoylethanolamide. *Physiology and Behavior* **2014**, doi:10.1016/j.physbeh.2014.04.039.

10. Romano, A.; Potes, C.S.; Tempesta, B.; Cassano, T.; Cuomo, V.; Lutz, T.; Gaetani, S. Hindbrain Noradrenergic Input to the Hypothalamic PVN Mediates the Activation of Oxytocinergic Neurons Induced by the Satiety Factor Oleoylethanolamide. *American Journal of Physiology - Endocrinology and Metabolism* **2013**, doi:10.1152/ajpendo.00411.2013.

11. Romano, A.; Tempesta, B.; Provensi, G.; Passani, M.B.; Gaetani, S. Central Mechanisms Mediating the Hypophagic Effects of Oleoylethanolamide and N-Acylphosphatidylethanolamines: Different Lipid Signals? *Frontiers in Pharmacology* 2015.

12. Cifani, C.; Polidori, C.; Melotto, S.; Ciccocioppo, R.; Massi, M. A Preclinical Model of Binge Eating Elicited by Yo-Yo Dieting and Stressful Exposure to Food: Effect of Sibutramine, Fluoxetine, Topiramate, and Midazolam. *Psychopharmacology* **2009**, doi:10.1007/s00213-008-1442-y.

13. di Bonaventura, M.V.M.; Ciccocioppo, R.; Romano, A.; Bossert, J.M.; Rice, K.C.; Ubaldi, M.; st. Laurent, R.; Gaetani, S.; Massi, M.; Shaham, Y.; et al. Role of Bed Nucleus of the Stria Terminalis Corticotrophin-Releasing Factor Receptors in Frustration Stress-Induced Binge-like Palatable Food

Consumption in Female Rats with a History of Food Restriction. *Journal of Neuroscience* **2014**, doi:10.1523/JNEUROSCI.1854-14.2014.

14. Micioni Di Bonaventura, M.V.; Ubaldi, M.; Giusepponi, M.E.; Rice, K.C.; Massi, M.; Ciccocioppo, R.; Cifani, C. Hypothalamic CRF1 Receptor Mechanisms Are Not Sufficient to Account for Binge-like Palatable Food Consumption in Female Rats. *International Journal of Eating Disorders* **2017**, doi:10.1002/eat.22767.

15. Lutz, T.A.; Woods, S.C. Overview of Animal Models of Obesity. *Current Protocols in Pharmacology* **2012**, doi:10.1002/0471141755.ph0561s58.

16. Johnson, P.M.; Kenny, P.J. Dopamine D2 Receptors in Addiction-like Reward Dysfunction and Compulsive Eating in Obese Rats. *Nature Neuroscience* **2010**, *13*, doi:10.1038/nn.2519.

17. Warfvinge, K.; Krause, D.; Edvinsson, L. The Distribution of Oxytocin and the Oxytocin Receptor in Rat Brain: Relation to Regions Active in Migraine. *Journal of Headache and Pain* **2020**, *21*, doi:10.1186/s10194-020-1079-8.

18. Romano, A.; Gallelli, C.A.; Koczwara, J.B.; Braegger, F.E.; Vitalone, A.; Falchi, M.; Micioni Di Bonaventura, M.V.; Cifani, C.; Cassano, T.; Lutz, T.A.; et al. Role of the Area Postrema in the Hypophagic Effects of Oleoylethanolamide. *Pharmacological Research* **2017**, doi:10.1016/j.phrs.2017.05.017.

19. Romano, A.; Cassano, T.; Tempesta, B.; Cianci, S.; Dipasquale, P.; Coccurello, R.; Cuomo, V.; Gaetani, S. The Satiety Signal Oleoylethanolamide Stimulates Oxytocin Neurosecretion from Rat Hypothalamic Neurons. *Peptides* **2013**, *49*, doi:10.1016/j.peptides.2013.08.006.

20. Paxinos, G.; Watson, C. The Rat Brain in Stereotaxic Coordinates Seventh Edition. *Elsevier Academic Press* **2014**, *170*.

21. Lawson, E.A. The Effects of Oxytocin on Eating Behaviour and Metabolism in Humans. *Nature Reviews Endocrinology* 2017.

22. Kenny, P.J. Reward Mechanisms in Obesity: New Insights and Future Directions. *Neuron* 2011.

23. Valdivia, S.; Patrone, A.; Reynaldo, M.; Perello, M. Acute High Fat Diet Consumption Activates the Mesolimbic Circuit and Requires Orexin Signaling in a Mouse Model. *PLoS ONE* **2014**, doi:10.1371/journal.pone.0087478.

24. Blevins, J.E.; Baskin, D.G. Translational and Therapeutic Potential of Oxytocin as an Anti-Obesity Strategy: Insights from Rodents, Nonhuman Primates and Humans. *Physiology and Behavior* **2015**, doi:10.1016/j.physbeh.2015.05.023.

25. Sinclair, M.S.; Perea-Martinez, I.; Abouyared, M.; st. John, S.J.; Chaudhari, N. Oxytocin Decreases Sweet Taste Sensitivity in Mice. *Physiology and Behavior* **2015**, *141*, doi:10.1016/j.physbeh.2014.12.048.

26. Zhou, L.; Ghee, S.M.; See, R.E.; Reichel, C.M. Oxytocin Differentially Affects Sucrose Taking and Seeking in Male and Female Rats. *Behavioural Brain Research* **2015**, *283*, doi:10.1016/j.bbr.2015.01.050.

27. Herisson, F.M.; Waas, J.R.; Fredriksson, R.; Schiöth, H.B.; Levine, A.S.; Olszewski, P.K. Oxytocin Acting in the Nucleus Accumbens Core Decreases Food Intake. *Journal of Neuroendocrinology* **2016**, doi:10.1111/jne.12381.

28. Camerino, C. Low Sympathetic Tone and Obese Phenotype in Oxytocin-Deficient Mice. *Obesity* **2009**, doi:10.1038/oby.2009.12.

29. Kasahara, Y.; Takayanagi, Y.; Kawada, T.; Itoi, K.; Nishimori, K. Impaired Thermoregulatory Ability of Oxytocin-Deficient Mice during Cold-Exposure. *Bioscience, Biotechnology and Biochemistry* **2007**, doi:10.1271/bbb.70498.

30. Adamantidis, A.R.; Tsai, H.C.; Boutrel, B.; Zhang, F.; Stuber, G.D.; Budygin, E.A.; Touriño, C.; Bonci, A.; Deisseroth, K.; de Lecea, L. Optogenetic Interrogation of Dopaminergic Modulation of the Multiple Phases of Reward-Seeking Behavior. *Journal of Neuroscience* **2011**, doi:10.1523/JNEUROSCI.2246-11.2011.

31. Watabe-Uchida, M.; Zhu, L.; Ogawa, S.K.; Vamanrao, A.; Uchida, N. Whole-Brain Mapping of Direct Inputs to Midbrain Dopamine Neurons. *Neuron* **2012**, doi:10.1016/j.neuron.2012.03.017.

32. Hung, L.W.; Neuner, S.; Polepalli, J.S.; Beier, K.T.; Wright, M.; Walsh, J.J.; Lewis, E.M.; Luo, L.; Deisseroth, K.; Dölen, G.; et al. Gating of Social Reward by Oxytocin in the Ventral Tegmental Area. *Science* **2017**, doi:10.1126/science.aan4994.

33. Mullis, K.; Kay, K.; Williams, D.L. Oxytocin Action in the Ventral Tegmental Area Affects Sucrose Intake. *Brain Research* **2013**, doi:10.1016/j.brainres.2013.03.026.

34. Peris, J.; MacFadyen, K.; Smith, J.A.; de Kloet, A.D.; Wang, L.; Krause, E.G. Oxytocin Receptors Are Expressed on Dopamine and Glutamate Neurons in the Mouse Ventral Tegmental Area That Project to Nucleus Accumbens and Other Mesolimbic Targets. *Journal of Comparative Neurology* **2017**, doi:10.1002/cne.24116.

35. Song, Z.; Borland, J.M.; Larkin, T.E.; O'Malley, M.; Albers, H.E. Activation of Oxytocin Receptors, but Not Arginine-Vasopressin (AVP) Receptors, in the Ventral Tegmental Area of Male Syrian Hamsters Is Essential for the Reward-like Properties of Social Interactions. *Psychoneuroendocrinology* **2016**, doi:10.1016/j.psyneuen.2016.09.001.

36. Tang, Y.; Chen, Z.; Tao, H.; Li, C.; Zhang, X.; Tang, A.; Liu, Y. Oxytocin Activation of Neurons in Ventral Tegmental Area and

Interfascicular Nucleus of Mouse Midbrain. *Neuropharmacology* **2014**, doi:10.1016/j.neuropharm.2013.10.004.

37. Klockars, A.; Levine, A.S.; Olszewski, P.K. Central Oxytocin and Food Intake: Focus on Macronutrient-Driven Reward. *Frontiers in Endocrinology* 2015.

38. Blevins, J.E.; Graham, J.L.; Morton, G.J.; Bales, K.L.; Schwartz, M.W.; Baskin, D.G.; Havel, P.J. Chronic Oxytocin Administration Inhibits Food Intake, Increases Energy Expenditure, and Produces Weight Loss in Fructose-Fed Obese Rhesus Monkeys. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2015**, doi:10.1152/ajpregu.00441.2014.

39. Amico, J.A.; Vollmer, R.R.; Cai, H.M.; Miedlar, J.A.; Rinaman, L. Enhanced Initial and Sustained Intake of Sucrose Solution in Mice with an Oxytocin Gene Deletion. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2005**, *289*, doi:10.1152/ajpregu.00558.2005.

40. Sclafani, A.; Rinaman, L.; Vollmer, R.R.; Amico, J.A. Oxytocin Knockout Mice Demonstrate Enhanced Intake of Sweet and Nonsweet Carbohydrate Solutions. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2007**, doi:10.1152/ajpregu.00826.2006.

41. Billings, L.B.; Spero, J.A.; Vollmer, R.R.; Amico, J.A. Oxytocin Null Mice Ingest Enhanced Amounts of Sweet Solutions during Light and Dark Cycles and during Repeated Shaker Stress. *Behavioural Brain Research* **2006**, doi:10.1016/j.bbr.2006.03.028.

42. Amico, J.A.; Morris, M.; Vollmer, R.R. Mice Deficient in Oxytocin Manifest Increased Saline Consumption Following Overnight Fluid Deprivation. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2001**, *281*, doi:10.1152/ajpregu.2001.281.5.r1368.

43. Puryear, R.; Rigatto, K. v.; Amico, J.A.; Morris, M. Enhanced Salt Intake in Oxytocin Deficient Mice. *Experimental Neurology* **2001**, *171*, doi:10.1006/exnr.2001.7776.

44. Striepens, N.; Schröter, F.; Stoffel-Wagner, B.; Maier, W.; Hurlemann, R.; Scheele, D. Oxytocin Enhances Cognitive Control of Food Craving in Women. *Human Brain Mapping* **2016**, doi:10.1002/hbm.23308.

45. Deblon, N.; Veyrat-Durebex, C.; Bourgoin, L.; Caillon, A.; Bussier, A.L.; Petrosino, S.; Piscitelli, F.; Legros, J.J.; Geenen, V.; Foti, M.; et al. Mechanisms of the Anti-Obesity Effects of Oxytocin in Diet-Induced Obese Rats. *PLoS ONE* **2011**, doi:10.1371/journal.pone.0025565.

46. Maejima, Y.; Iwasaki, Y.; Yamahara, Y.; Kodaira, M.; Sedbazar, U.; Yada, T. Peripheral Oxytocin Treatment Ameliorates Obesity by Reducing Food Intake and Visceral Fat Mass. *Aging* **2011**, doi:10.18632/aging.100408.

47. Morton, G.J.; Thatcher, B.S.; Reidelberger, R.D.; Ogimoto, K.; Wolden-Hanson, T.; Baskin, D.G.; Schwartz, M.W.; Blevins, J.E. Peripheral Oxytocin Suppresses Food Intake and Causes Weight Loss in Diet-Induced Obese Rats. *American Journal of Physiology - Endocrinology and Metabolism* **2012**, *302*, doi:10.1152/ajpendo.00296.2011.

48. Zhang, G.; Bai, H.; Zhang, H.; Dean, C.; Wu, Q.; Li, J.; Guariglia, S.; Meng, Q.; Cai, D. Neuropeptide Exocytosis Involving Synaptotagmin-4 and Oxytocin in Hypothalamic Programming of Body Weight and Energy Balance. *Neuron* **2011**, *69*, doi:10.1016/j.neuron.2010.12.036.

49. Zhang, G.; Cai, D. Circadian Intervention of Obesity Development via Resting-Stage Feeding Manipulation or Oxytocin Treatment. *American Journal of Physiology - Endocrinology and Metabolism* **2011**, doi:10.1152/ajpendo.00196.2011.

50. Roberts, Z.S.; Wolden-Hanson, T.; Matsen, M.E.; Ryu, V.; Vaughan, C.H.; Graham, J.L.; Havel, P.J.; Chukri, D.W.; Schwartz, M.W.; Morton, G.J.;

et al. Chronic Hindbrain Administration of Oxytocin Is Sufficient to Elicit Weight Loss in Diet-Induced Obese Rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2017**, *313*, doi:10.1152/ajpregu.00169.2017.

51. Blevins, J.E.; Thompson, B.W.; Anekonda, V.T.; Ho, J.M.; Graham, J.L.; Roberts, Z.S.; Hwang, B.H.; Ogimoto, K.; Wolden-Hanson, T.; Nelson, J.; et al. Chronic CNS Oxytocin Signaling Preferentially Induces Fat Loss in High-Fat Diet-Fed Rats by Enhancing Satiety Responses and Increasing Lipid Utilization. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2016**, doi:10.1152/ajpregu.00220.2015.

52. Fritz, B.M.; Muñoz, B.; Yin, F.; Bauchle, C.; Atwood, B.K. A High-Fat, High-Sugar 'Western' Diet Alters Dorsal Striatal Glutamate, Opioid, and Dopamine Transmission in Mice. *Neuroscience* **2018**, *372*, doi:10.1016/j.neuroscience.2017.12.036.

53. Herisson, F.M.; Brooks, L.L.; Waas, J.R.; Levine, A.S.; Olszewski, P.K. Functional Relationship between Oxytocin and Appetite for Carbohydrates versus Saccharin. *NeuroReport* **2014**, doi:10.1097/WNR.0000000000000201.

54. Lokrantz, C.M.; Uvnäs-Moberg, K.; Kaplan, J.M. Effects of Central Oxytocin Administration on Intraoral Intake of Glucose in Deprived and Nondeprived Rats. *Physiology and Behavior* **1997**, *62*, doi:10.1016/S0031-9384(97)00021-8.

55. Olszewski, P.K.; Klockars, A.; Olszewska, A.M.; Fredriksson, R.; Schiöth, H.B.; Levine, A.S. Molecular, Immunohistochemical, and Pharmacological Evidence of Oxytocin's Role as Inhibitor of Carbohydrate but Not Fat Intake. *Endocrinology* **2010**, doi:10.1210/en.2010-0151.

56. Maejima, Y.; Yokota, S.; Nishimori, K.; Shimomura, K. The Anorexigenic Neural Pathways of Oxytocin and Their Clinical Implication. *Neuroendocrinology* **2018**, doi:10.1159/000489263.

57. Spetter, M.S.; Hallschmid, M. Current Findings on the Role of Oxytocin in the Regulation of Food Intake. *Physiology and Behavior* 2017.

58. Schwartz, M.W.; Woods, S.C.; Porte, D.; Seeley, R.J.; Baskin, D.G. Central Nervous System Control of Food Intake. *Nature* 2000.

59. Maejima, Y.; Sedbazar, U.; Suyama, S.; Kohno, D.; Onaka, T.; Takano, E.; Yoshida, N.; Koike, M.; Uchiyama, Y.; Fujiwara, K.; et al. Nesfatin-1-Regulated Oxytocinergic Signaling in the Paraventricular Nucleus Causes Anorexia through a Leptin-Independent Melanocortin Pathway. *Cell Metabolism* **2009**, doi:10.1016/j.cmet.2009.09.002.

60. Yosten, G.L.C.; Samson, W.K. The Anorexigenic and Hypertensive Effects of Nesfatin-1 Are Reversed by Pretreatment with an Oxytocin Receptor Antagonist. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2010**, doi:10.1152/ajpregu.00804.2009.

61. Affleck, V.S.; Coote, J.H.; Pyner, S. The Projection and Synaptic Organisation of NTS Afferent Connections with Presympathetic Neurons, GABA and NNOS Neurons in the Paraventricular Nucleus of the Hypothalamus. *Neuroscience* **2012**, doi:10.1016/j.neuroscience.2012.05.070.

62. Katsurada, K.; Maejima, Y.; Nakata, M.; Kodaira, M.; Suyama, S.; Iwasaki, Y.; Kario, K.; Yada, T. Endogenous GLP-1 Acts on Paraventricular Nucleus to Suppress Feeding: Projection from Nucleus Tractus Solitarius and Activation of Corticotropin-Releasing Hormone, Nesfatin-1 and Oxytocin Neurons. *Biochemical and Biophysical Research Communications* **2014**, doi:10.1016/j.bbrc.2014.07.116.

63. Larsen, P.J.; Tang-Christensen, M.; Jessop, D.S. Central Administration of Glucagon-like Peptide-1 Activates Hypothalamic Neuroendocrine Neurons in the Rat. *Endocrinology* **1997**, doi:10.1210/endo.138.10.5270.

64. Saito, R.; So, M.; Motojima, Y.; Matsuura, T.; Yoshimura, M.; Hashimoto, H.; Yamamoto, Y.; Kusuhara, K.; Ueta, Y. Activation of Nesfatin-1-Containing Neurones in the Hypothalamus and Brainstem by Peripheral Administration of Anorectic Hormones and Suppression of Feeding via Central Nesfatin-1 in Rats. *Journal of Neuroendocrinology* **2016**, doi:10.1111/jne.12400.

65. Rinaman, L.; Rothe, E.E. GLP-1 Receptor Signaling Contributes to Anorexigenic Effect of Centrally Administered Oxytocin in Rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2002**, doi:10.1152/ajpregu.00008.2002.

66. Pekary, A.E.; Sattin, A. Rapid Modulation of TRH and TRH-like Peptide Release in Rat Brain and Peripheral Tissues by Ghrelin and 3-TRP-Ghrelin. *Peptides* **2012**, doi:10.1016/j.peptides.2012.04.021.

67. Wallace Fitzsimons, S.E.; Chruścicka, B.; Druelle, C.; Stamou, P.; Nally, K.; Dinan, T.G.; Cryan, J.F.; Schellekens, H. A Ghrelin Receptor and Oxytocin Receptor Heterocomplex Impairs Oxytocin Mediated Signalling. *Neuropharmacology* **2019**, doi:10.1016/j.neuropharm.2018.12.022.

68. Blevins, J.E.; Eakin, T.J.; Murphy, J.A.; Schwartz, M.W.; Baskin, D.G. Oxytocin Innervation of Caudal Brainstem Nuclei Activated by Cholecystokinin. *Brain Research* **2003**, doi:10.1016/j.brainres.2003.08.036.

69. Olszewski, P.K.; Bomberg, E.M.; Martell, A.; Grace, M.K.; Levine, A.S. Intraventricular Ghrelin Activates Oxytocin Neurons: Implications in Feeding Behavior. *NeuroReport* **2007**, doi:10.1097/WNR.0b013e328058684e.

70. Edwards, G.L.; Gedulin, B.R.; Jodka, C.; Dilts, R.P.; Miller, C.C.; Young, A. Area Postrema (AP)-Lesions Block the Regulation of Gastric Emptying by Amylin. *Gastroenterology* **1998**, *114*, doi:10.1016/s0016-5085(98)83064-3.

71. Fry, M.; Ferguson, A. v. Ghrelin Modulates Electrical Activity of Area Postrema Neurons. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* **2009**, *296*, doi:10.1152/ajpregu.90555.2008.

72. Punjabi, M.; Arnold, M.; Geary, N.; Langhans, W.; Pacheco-López, G. Peripheral Glucagon-like Peptide-1 (GLP-1) and Satiation. *Physiology and Behavior* **2011**, *105*, doi:10.1016/j.physbeh.2011.02.038.

73. Züger, D.; Forster, K.; Lutz, T.A.; Riediger, T. Amylin and GLP-1 Target Different Populations of Area Postrema Neurons That Are Both Modulated by Nutrient Stimuli. *Physiology and Behavior* **2013**, *112–113*, doi:10.1016/j.physbeh.2013.02.006.

74. Ritter, S.; McGlone, J.J.; Kelley, K.W. Absence of Lithium-Induced Taste Aversion after Area Postrema Lesion. *Brain Research* **1980**, *201*, doi:10.1016/0006-8993(80)91061-6.

75. Provensi, G.; Coccurello, R.; Umehara, H.; Munari, L.; Giacovazzo, G.; Galeotti, N.; Nosi, D.; Gaetani, S.; Romano, A.; Moles, A.; et al. Satiety Factor Oleoylethanolamide Recruits the Brain Histaminergic System to Inhibit Food Intake. *Proceedings of the National Academy of Sciences of the United States of America* **2014**, *111*, doi:10.1073/pnas.1322016111.

Chapter 4:

General discussion and conclusions

Obesity is a complex pathology that occurs when energy intake exceeds energy expenditure and is characterized by the accumulation of dysfunctional adipose tissue which triggers metabolic stress response and induces a low-grade inflammatory chronic state [1] at both central and peripheral levels.

The increased prevalence of overweight, obesity and eating related disorders is a serious medical and public health problem [2,3] and there is a particular interest in these pathologies, from both the scientific community and public health, because of the lack of effective pharmacological treatments.

Therefore, in the past years, research has focused on investigating the mechanisms involved in the control of feeding and energy balance to identify new pharmacological targets that could be potentially used in clinical practice for the treatment of pathological conditions characterized by multiorgan dysfunction, such as obesity.

Among possible novel pharmacological targets, oleoylethanolamide (OEA) seems to be of particular interest based on its anti-obesity, anti-inflammatory, and antioxidant effects [4–6]. OEA belongs to the lipid family of the N-acylethanolamines (NAEs) and is a potent endogenous ligand for peroxisome proliferator-activated receptor (PPAR)-α, a nuclear receptor involved in the transcriptional regulation of lipid metabolism, neuroplasticity and inflammation [4].

In the present work was investigated in depth the effect of exogenous administration of OEA on both molecular mechanisms involved in obesity-

related alterations and endogenous peptidergic system regulating eating behaviors. Particularly, our data demonstrated that OEA exert an anti-obesity effect that is accompanied, at central level by both a reduction of neuroinflammatory response and an increase of neuroplasticity and at peripheral level by an amelioration of fatty liver and oxidative stress parameters. Moreover, it has been summarized the role played by oxytocin in the control of both homeostatic and non-homeostatic eating, within cognitive, metabolic and reward mechanisms and it has been proposed that the modulation of the central oxytocinergic signaling by increasing directly or indirectly OEA tone might represent one of the mechanisms that coordinate food intake at the crossroads between homeostatic and non-homeostatic mechanisms in the treatment of aberrant eating behaviors.

In conclusions, the results reported in this work have the potential to unravel new targets for the development of pharmacological treatments for obesity and eating disorders, two major health problems worldwide, with important repercussions in the economic-industrial field. Although further studies are necessary, OEA treatment could represent an innovative approach for the treatment and/or prevention of multi-organ conditions, such as obesity, in humans. The translationality of the experimental data of the present study and, consequently, of the OEA treatment is supported by the FDA approved OEA-based supplement (RiduZone), available only in the USA, which have shown benefits in regulating body weight.

References

1. Klöting, N.; Blüher, M. Adipocyte Dysfunction, Inflammation and Metabolic Syndrome. *Reviews in Endocrine and Metabolic Disorders* 2014.

2. Sampey, B.P.; Vanhoose, A.M.; Winfield, H.M.; Freemerman, A.J.; Muehlbauer, M.J.; Fueger, P.T.; Newgard, C.B.; Makowski, L. Cafeteria Diet Is a Robust Model of Human Metabolic Syndrome with Liver and Adipose Inflammation: Comparison to High-Fat Diet. *Obesity* **2011**, *19*, doi:10.1038/oby.2011.18.

3. Hebestreit, A.; Börnhorst, C.; Barba, G.; Siani, A.; Huybrechts, I.; Tognon, G.; Eiben, G.; Moreno, L.A.; Fernández Alvira, J.M.; Loit, H.M.; et al. Associations between Energy Intake, Daily Food Intake and Energy Density of Foods and BMI z-Score in 2-9-Year-Old European Children. *European Journal of Nutrition* **2014**, *53*, doi:10.1007/s00394-013-0575-x.

4. Romano, A.; Tempesta, B.; Provensi, G.; Passani, M.B.; Gaetani, S. Central Mechanisms Mediating the Hypophagic Effects of Oleoylethanolamide and N-Acylphosphatidylethanolamines: Different Lipid Signals? *Frontiers in Pharmacology* 2015.

5. Gaetani, S.; Romano, A.; Provensi, G.; Ricca, V.; Lutz, T.; Passani, M.B. Eating Disorders: From Bench to Bedside and Back. *Journal of Neurochemistry* 2016.

6. Romano, A.; Micioni Di Bonaventura, M.V.; Gallelli, C.A.; Koczwara, J.B.; Smeets, D.; Giusepponi, M.E.; de Ceglia, M.; Friuli, M.; Micioni Di Bonaventura, E.; Scuderi, C.; et al. Oleoylethanolamide Decreases Frustration Stress-Induced Binge-like Eating in Female Rats: A Novel Potential Treatment for Binge Eating Disorder. *Neuropsychopharmacology* **2020**, doi:10.1038/s41386-020-0686-z.