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Endocannabinoids and Endocannabinoid-like Molecules
Restore Synaptic Homeostasis and Cognitive Functions in
Mouse Model of Obesity

Supervisor

Prof. Maria Pina Mollica

PhD Candidate

Dr. Brenda Marfella

Co-supervisor

Dr. Luigia Cristino

PhD Course Coordinator

Prof. Sergio Esposito

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1. Introduction

1.1 The Endocannabinoidome and the Endocannabinoid System

The endocannabinoidome is an expanded concept of the classical endocannabinoid system (ECS). While the ECS traditionally includes endocannabinoids (lipid-based signaling molecules like anandamide and 2-AG), Cannabinoid receptors (CB1 and CB2), Enzymes that synthesize and degrade endocannabinoids, the endocannabinoidome goes further. It encompasses: Additional lipid mediators beyond classical endocannabinoids (e.g., N-acylethanolamines, monoacylglycerols). A broader set of receptors, including: Cannabinoid receptors (CB1, CB2), Non-cannabinoid receptors (TRPV1, PPARs, GPR55, GPR119, etc.).

Metabolic enzymes involved in the synthesis and degradation of these mediators. Interactions with other signaling systems, such as the gut microbiome and immune pathways.

Essentially, the endocannabinoidome represents a complex lipid signaling network that influences numerous physiological processes, including appetite, metabolism, inflammation, and gut-brain communication. It is considered a key player in maintaining homeostasis and is a major target for therapeutic research (Simankowicz and Stępniewska et al., 2025)¹

From a physiological point of view, the endocannabinoidome plays a crucial role in the control of numerous processes: it contributes to the modulation of movement and motor coordination, participates in the maintenance of energy homeostasis and the regulation of nutrition, intervenes in higher cognitive functions (learning, memory, attention), in the perception and modulation of pain, in sexual behavior, in the protection of neurons from toxic damage or ischemic, in the regulation of immune system responses and in the balance of endocrine functions (Șerban et al., 2025)². In many of these functions, the endocannabinoid system acts as a "fine tuning" mechanism, i.e. a dynamic adjustment system that is recruited in response to changes in the internal and external environment, thus contributing to the maintenance of the body's physiological balance.

1.2 Cannabinoid Receptors

Cannabinoid receptors (CBs) are classified within the large family of metabotropic G-protein-coupled receptors (GPCRs) and share a typical structural architecture with them.

They consist of a single polypeptide chain that crosses the plasma membrane forming seven transmembrane α -helix domains; the amino end (N-terminal) is oriented towards the extracellular environment, while the carboxyl end (C-terminal) is oriented towards the cytoplasm. The loop segments connecting the transmembrane helices comprise three extracellular moieties, which together with the Nterminal host sites crucial for glycosylation and ligand interaction, and three intracellular moieties, which contain regulatory domains responsible for binding to G protein subunits. On the basis of pharmacological and tissue distribution characteristics, two main receptor subtypes have been distinguished: the CB1 receptor and the CB2 receptor.

The CB1 receptor was first identified as a cannabinoid-specific binding site in the late 1980s by Devane et al. (Devane et al., 1988)³ and, a few years later, its cDNA was cloned, allowing a detailed description of it in both rats (Matsuda et al., 1990)⁴ and humans (Gérard et al., 1991)⁵. CB1 represents one of the most abundant GPCRs in the mammalian central nervous system, as demonstrated by autoradiographic, immunohistochemical and in situ hybridization studies, which have shown a high expression in numerous brain areas: olfactory bulb, cerebellum, hippocampus, basal ganglia, cerebral cortex, amygdala and hypothalamus (Zou e Kumar et al., 2018; Zhao et al., 2024)^{6,7}. In more recent times, the presence of CB1 has also been documented on non-neuronal cells, such as astrocytes, and on postsynaptic compartments of specific neuronal populations, although its prevalent location remains the presynaptic termination, in line with the neuromodulatory role that this receptor plays on synaptic transmission. (Lutz et al., 2020; Saenz et al., 2025)^{8,9}.

In addition to the central nervous system, CB1 is also expressed in the peripheral nervous system and in numerous organs and tissues, such as the immune system, the gastrointestinal tract, several endocrine glands, the heart, the reproductive system, the bone marrow and the tonsils (Lu and Mackie et al., 2016)¹⁰. At the molecular level, CB1 can form homodimeric or heterodimeric complexes with other GPCR receptors, which contributes to modulating the specificity and intensity of the signaling response (Köfalvi et al., 2020; Navarro et al., 2025)^{11,12}. Signal transduction occurs mainly through coupling with Gi/o proteins: the activation of these proteins leads to an inhibition of adenylate cyclase, resulting in a reduction in intracellular levels of cyclic AMP (cAMP) and transient blockade of protein kinase A (PKA) activation (Kumar et al., 2019; Al-Zoubi et al., 2019; Yang et al., 2024; Gonzáles-Mariscal et al., 2018)^{13–16}. Moreover,

CB1 is able to activate MAP kinase pathways and modulate the activity of several ion channels, including potassium channels and N- and P/Q-type voltage-gated calcium channels, thereby (Andersen et al., 2024; Sitzia et al., 2022; Navarro et al., 2025)^{12,17,18}.

It was also observed that CB1 can interact with different classes of G proteins, such as Gq/11 and Gs, thus expanding the spectrum of possible intracellular outcomes. Such versatility could contribute to fine control of cellular excitability, resulting in a biphasic, excitatory or inhibitory response, depending on the cellular context and receptor activation patterns. In line with this complexity of signaling mechanisms and its wide distribution, the CB1 receptor participates in the regulation of numerous physiological processes: motor coordination and movement, attention, higher cognitive functions (learning, memory, emotion processing), modulation of nociception, energy balance and control of eating behavior (Pagotto et al., 2006)¹⁵. A particularly relevant role is attributed to CB1 in the maintenance of homeostasis, both at the central and peripheral level, where it contributes to the adaptive response to stressful stimuli and changes in the cellular microenvironment (Tansey et al., 2025)²².

The CB2 receptor, identified in 1993 by Munro and colleagues, shares important structural similarities and some aspects of the signaling cascade with CB1, but is clearly distinguished by its pattern of tissue expression (Munro et al., 1993)²³. CB2 shows, in fact, a high and widespread localization at the peripheral level, while in the central nervous system its expression is lower but still present extensively in specific neuronal populations, in microglia and astrocytes (Grabon et al., 2023; De Martin Esteban et al., 2022)^{24,25}. In the periphery, CB2 is particularly abundant in immune system cells, such as monocytes, macrophages, B and T lymphocytes, where it plays a key role in modulating inflammatory responses, regulating the release of proinflammatory cytokines and mediators involved in angiogenesis and lymphangiogenesis processes (Maia et al., 2023)

CB2 expression has also been documented in various immune-related districts, such as the spleen, tonsils, thymus, hematopoietic bone tissue, and keratinocytes, suggesting widespread involvement in the regulation of both innate and adaptive immunity (Ferranti et al., 2022; Turcotte et al., 2016)^{26,27}. Further studies have also highlighted the presence of CB2 in the pancreas and gastrointestinal tract, where its activation seems to contribute to the modulation of local metabolic and inflammatory processes (Lima et al., 2022)²⁸. Similarly to CB1, CB2 also exerts its action mainly through the activation of Gi and Go

proteins, resulting in a decrease in cAMP levels and influencing the activity of voltage-gated calcium channels (Saroj et al., 2019). Although the precise effects of CB2 activation in the central nervous system have not yet been fully elucidated, experimental evidence suggests a possible involvement of this receptor in the modulation of behavioral disorders and vulnerability to substance abuse addiction (Navarrete et al., 2021)²⁹.

The body of data accumulated since their discovery to date identifies CB1 and CB2 as the main mediators of the physiological effects of the endocannabinoid system. However, in recent years the hypothesis has emerged, supported by a growing number of studies, that other non-canonical receptors may also contribute to the regulation of endocannabinoidergic processes. In this context, the GPR55 receptor, in the past defined as an "orphan receptor" as it lacks a known endogenous ligand, has been proposed as a possible new cannabinoid receptor (Balenga et al., 2011; Lauckner et al., 2008; Ryberg et al., 2007)³⁰⁻³². GPR55 also belongs to the GPCR family and is expressed in different regions of the central nervous system, as well as in various peripheral districts, where it seems to participate in the regulation of lipid metabolism and vascular functions.

Lysophosphatidylinositol has been identified as the main endogenous ligand of GPR55 (Oka et al., 2007)³³; however, this receptor can also be activated by Δ^9 THC and some synthetic agonists, including CB1 inverse agonists, suggesting a functional interaction with classical cannabinoidergic signaling. Activation of GPR55 induces a number of intracellular responses, including the release of Ca^{2+} ions from intracellular stores, modulation of the activity of several protein kinases, and increased expression of specific transcription factors, with possible repercussions on processes such as cell proliferation, migration, and inflammatory response (Marichal-Cancino et al., 2017)³⁴.

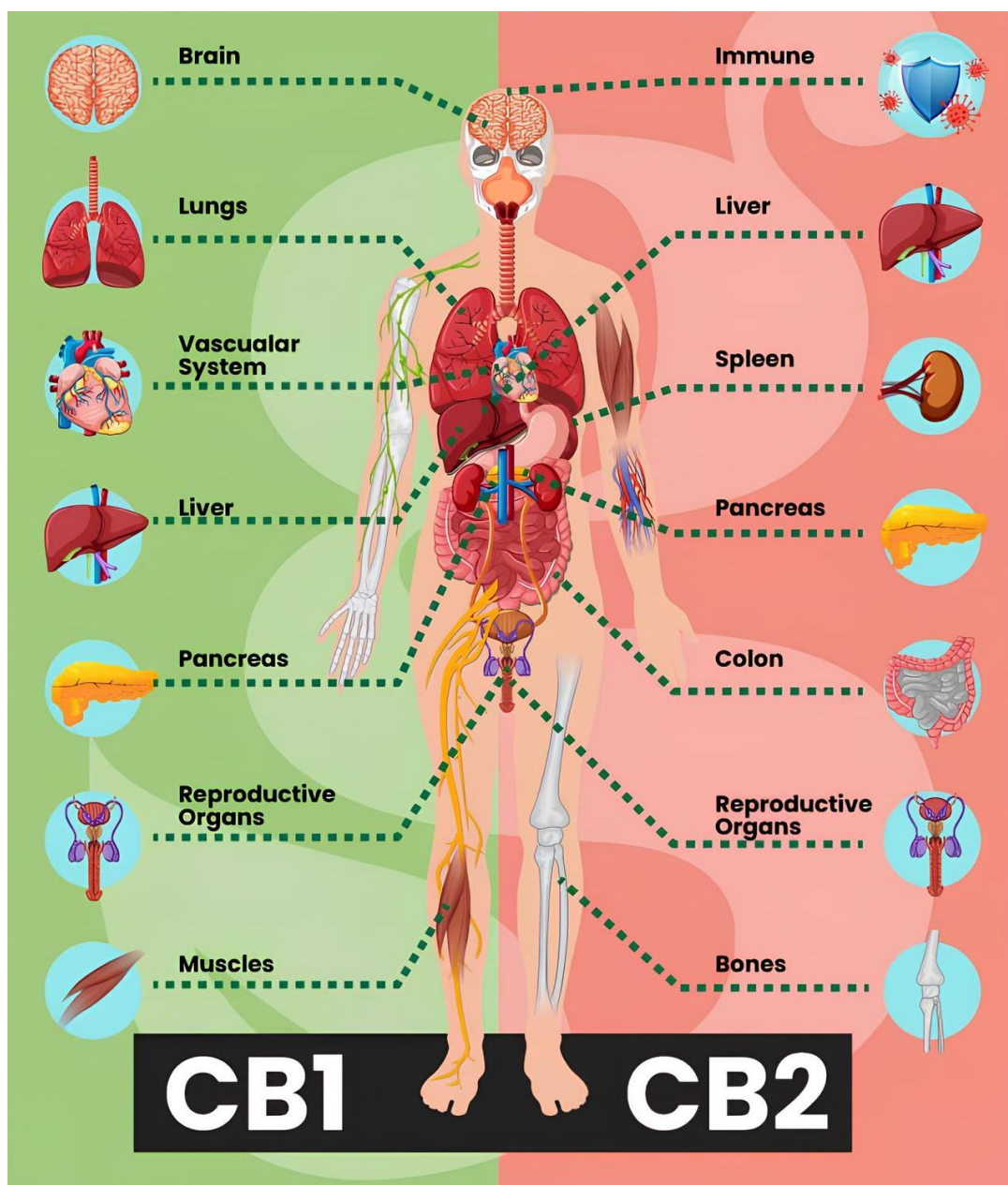


Figure 1. Distribution of cannabinoid receptors CB1 and CB2 in the human body.
Figure adapted from *GenoneEleven*, “Il sistema endocannabinoide e i recettori CB1 e CB2”³⁵

molecules capable of binding to these receptors in a similar way to Δ^9 THC. In this context, in 1992 Devane and collaborators isolated, from pig brain extracts, a lipid molecule identified as arachidonic acid amide (N-arachidonoyl ethanolamine), later named anandamide (AEA), from the Sanskrit term "ananda", meaning "bliss" or "state of grace" (Devane et al., 1992)³⁷. AEA showed a high affinity for CB receptors and was able to reproduce several behavioral effects induced by Δ^9 THC, thus configuring itself as the first endogenous cannabinoid ligand. A few years later, Mechoulam and colleagues, on the one hand, and Sugiura and collaborators, on the other, isolated a second lipid ligand, 2-arachidonoylglycerol (2-AG), from the dog intestine and rat brain, which was quickly recognized as another important endocannabinoid (Mechoulam et al., 1995; Sugiura et al., 1995; Tansey et al., 2025)^{22,38,39}.

With the characterization of AEA and 2-AG, the class of endogenous cannabinoids, or endocannabinoids, was defined. Subsequent studies have expanded this group to include several other compounds identified at the beginning of the new millennium, including 2-arachidonoylglycerylether (2-AGE or noladinether), o-arachidonoyl ethanolamine (virodamine) and n-arachidonoyldopamine (NADA), all capable of interacting with CB receptors, although AEA and 2-AG remain the most studied endogenous ligands with the best defined physiological role (Hanus et al., 2001; Porter et al., 2002; Huang et al., 2002; Sumanakowicz et al., 2025)^{1,40-42}.

Alongside the "classic" endocannabinoids, there are other endogenous molecules that, although not considered endocannabinoids in the strict sense, share structural and functional similarities with AEA that place them in the context of the endocannabinoid system. These are N-acyl ethanolamines (NAEs), a group of compounds congeners of anandamide, with which they share some biosynthetic and degradative pathways. Among the most representative NAEs are palmitoylethanolamine (PEA) and oleylethanolamine (OEA), whose effects – including some endocannabinoid effects – are not directly mediated by CB receptors, but are mainly attributable to the activation of nuclear receptors PPAR (peroxisome proliferator-activated receptors) and an "entourage" effect on the endocannabinoid system itself, enhancing the activity of endocannabinoids without acting as direct ligands of CB receptors (Iannotti and Vitale et al., 2021; Di Stefano et al., 2025)^{43,44}.

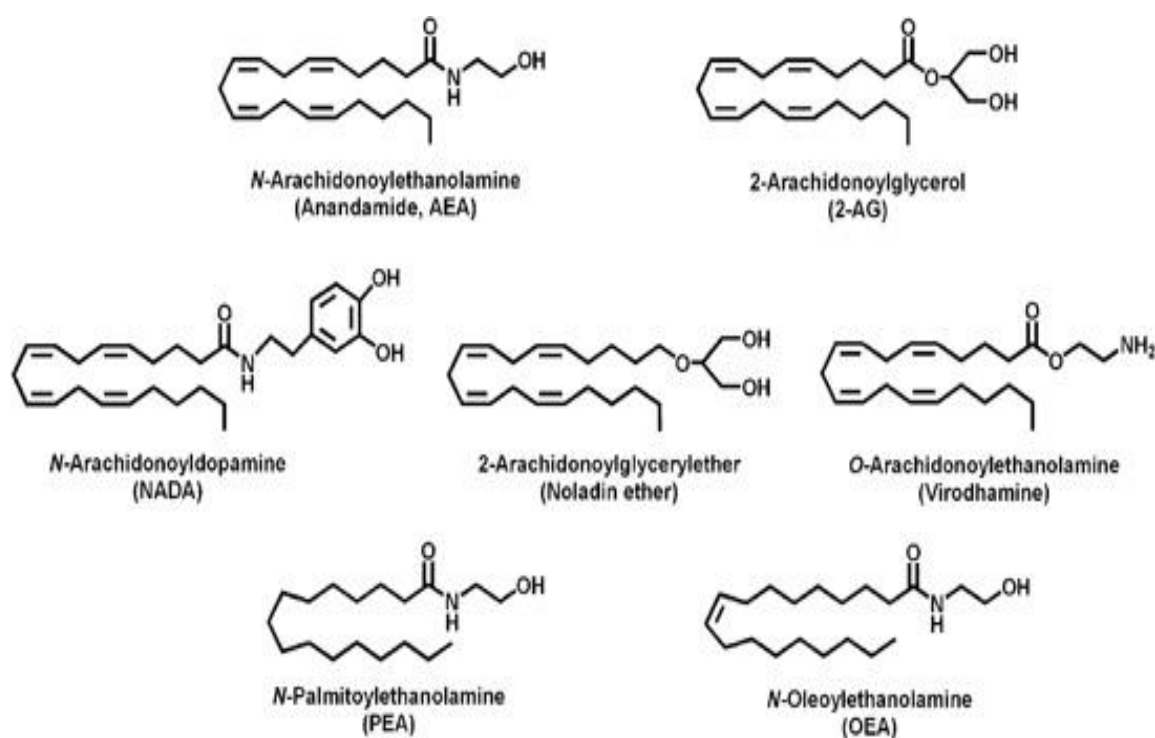


Figure 3. Structural formulas of the main endocannabinoids, highlighting the presence of the arachidonyl group and the different polar heads that determine their chemical-physical properties and receptor interaction profiles.

1.4 Metabolism

Endocannabinoids are considered real neurotransmitters, but they have peculiar characteristics that distinguish them from "classic" neurotransmitters. While respecting the fundamental criteria of the latter such as synthesis at the neuronal level, release in response to an appropriate stimulus, specific action on target receptors, removal by reuptake and subsequent degradation; Their lipid nature means that they are not stored in synaptic vesicles or released via conventional exocytosis. In contrast, endocannabinoids are produced "on demand" from lipid precursors located in the plasma membrane, and are rapidly released into the extracellular space immediately after their synthesis. (Alger

et al., 2011)⁴⁵.

A further peculiarity concerns the release site: endocannabinoids are generated and released mainly at the level of the postsynaptic terminal following specific physiological stimuli, such as the depolarization of the postsynaptic membrane and the consequent increase in intracellular Ca^{2+} . Once released, they diffuse retrograde to the presynaptic termination, where they bind to cannabinoid receptors expressed on the presynaptic membrane, thereby modulating the release of neurotransmitters and playing a key role in the fine regulation of synaptic transmission. (Cristino et al., 2020)³⁶.

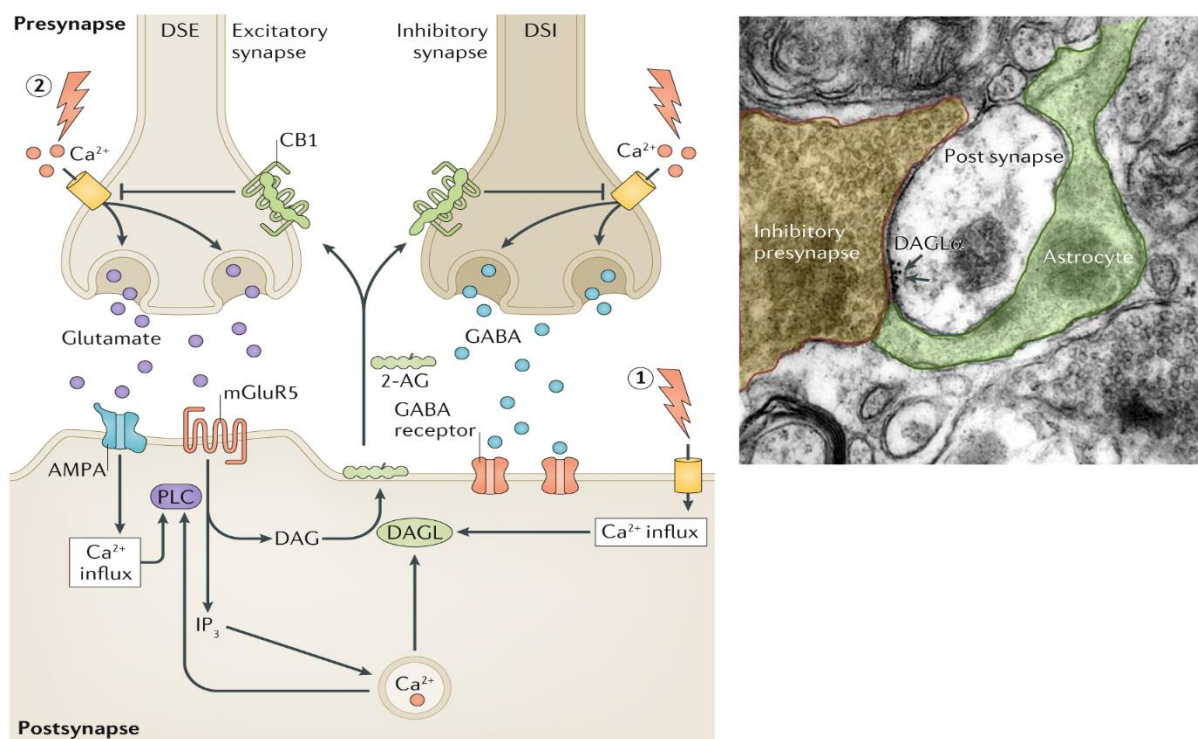


Figure 4. Retrograde neurotransmission of endocannabinoids.

Left: The role of endocannabinoid retrograde signaling in short-term plasticity, known as depolarization-induced suppression of excitation (DSE) or depolarization-induced suppression of inhibition (DSI). Two events can induce production of the endocannabinoid 2-arachidonoyl glycerol (2-AG). Postsynaptic step depolarization or an action potential induces Ca^{2+} influx via voltage-gated Ca^{2+} channels (stimulus 1) that is amplified by metabotropic receptor-induced intracellular Ca^{2+} release. Alternatively, brief tetanic stimulation of excitatory afferents (stimulus 2) leads to glutamate release, which stimulates the metabotropic glutamate receptor (mGluR5), thereby initiating 2-AG

biosynthesis. AMPA receptor activation can contribute to this effect. Other G_{q/11}-coupled receptors can also activate phospholipase C (PLC) and diacylglycerol lipase (DAGL), which are required for synthesis of 2-AG, and induce inositol-trisphosphate (IP₃) production that causes intracellular calcium mobilization. 2-AG activates the presynaptic cannabinoid receptor 1 (CB1), leading to depression of neurotransmitter release. **Right:** transmission electron micrograph (L.C., unpublished observations) of the tripartite synapse that shows DAGL α immunogold labelling (arrows) at the postsynaptic membrane of an active zone receiving a symmetrical synapse from a putative inhibitory neuron (orange) and enveloped by astroglial processes (green). DAG, *sn*-1 acyl-2-arachidonoyl-glycerol; GPR, G protein-coupled receptor. *Figure adapted from Cristino et al., 2020*³⁶

1.5 Mechanism of Action

Endocannabinoids act as local paracrine mediators, exerting their function by binding to CB receptors expressed on cells near the release site. Anandamide, Noleadineter and Narachidonoyldopamine show greater functional selectivity for the CB1 receptor, while virodamine seems to prefer the CB2 receptor; 2-AG, on the other hand, has a comparable potency on both receptor subtypes. CB receptors, being coupled to G proteins, activate the typical intracellular signaling processes associated with this type of receptor: among the main effects are the inhibition of adenylate cyclase, with reduction of the cAMP/protein kinase A cascade, the activation of mitogen activated protein kinases (MAPK), the inhibition of voltage-gated calcium channels of type N and P/Q, the stimulation of potassium channels TASK1 and the activation of phosphatidylinositol 3kinase, resulting in intracellular calcium mobilization (Ye et al., 2019)⁴⁶.

In the central nervous system, the high concentration of endocannabinoids and their receptors at the GABAergic and glutamatergic synapses indicates a central role in the activity-dependent modulation of synaptic transmission. The mechanism is peculiar: endocannabinoids are produced in the postsynaptic neuron and, once released, diffuse retrograde to the presynaptic termination, where they activate CB1, resulting in a temporary reduction in the release of GABA or glutamate. Prolonged excitation of the presynaptic neuron induces a depolarization of the postsynaptic neuron; Through the

opening of voltage-gated calcium channels and the activation of metabotropic receptors, the enzymes responsible for the synthesis of endocannabinoids from membrane lipid precursors are activated. The newly formed endocannabinoid, thanks to its lipophilicity, rapidly crosses the postsynaptic membrane, diffuses into the synaptic space, and reaches CB1 on the presynaptic side (Alger et al., 2011)⁴⁵.

The activation of CB1, endowed with 7 transmembrane domains and coupled to G proteins, leads to the dissociation of the β and γ subunits, which directly inhibit the entry of Ca^{2+} and reduce the release of calcium-dependent neurotransmitters. When this mechanism occurs in GABAergic synapses, it results in a reduction in GABA-mediated inhibition; this phenomenon, initially described by Llano and Pitler, is known as "depolarization induced suppression of inhibition" (DSI) (Llano et al., 1991; Pitler et al., 1992)^{47,48}. If, on the other hand, it occurs at the level of glutamatergic synapses, a decrease in excitatory transmission mediated by glutamate, called "depolarization induced suppression of excitation" (DSE), is observed (Bevan et al., 2002)⁴⁹. In the long term, endocannabinoidergic modulation of presynaptic release can influence long-term potentiation (LTP), a fundamental process for synapse remodelling and consolidation: the reduction of excitatory glutamatergic transmission tends to hinder the establishment of LTP, while the decrease of GABAergic inhibitory transmission can facilitate its induction (Diana et al., 2014)⁵⁰.

In addition to the action mediated by CB1 and CB2 receptors, more recent evidence shows that endocannabinoids can exert cannabinoidergic effects via other targets. Some neurobehavioral effects of anandamide are not completely abolished by selective CB1 antagonists and persist in CB1 (CB1/) knockout mouse models, suggesting the involvement of alternative receptors (Cristino et al., 2020)³⁶. In particular, AEA is able to activate the vanilloid receptor TRPV1, inducing Ca^{2+} ingress and depolarization, effects that contribute, among other things, to the vasodilating action of anandamide (Muller et al., 2020)⁵¹. In addition, endocannabinoids can interact directly with several ion channels, particularly calcium and potassium channels, adding additional layers of regulation to their action on neuronal tone and synaptic transmission.

1.6 Effects of Endocannabinoids on the Central Nervous System

The extremely wide distribution of CB1 receptors in the central nervous system and in numerous peripheral districts testifies to the involvement of the endocannabinoid system in a wide range of physiological processes, which are not limited to the sole modulation of synaptic transmission. In fact, it contributes to the control of synaptic plasticity mechanisms, the regulation of motor activity and eating behavior, learning and memory processes, as well as the modulation of nociception, thermoregulation and emetic reflexes, as well as exerting a significant role in the regulation of immune responses, cardiovascular and endocrine functions and in the critical phases of brain development. (Alger and Kim et al., 2011)⁴⁵

The complexity of these functions derives to a large extent from the "fine modulator" nature of the endocannabinoid system, which operates predominantly as a negative feedback system on demand, being recruited under conditions of increased neuronal activity or perturbation of homeostasis.

1.7 Endocannabinoids and Appetite Control

The control of appetite and energy balance is the result of a complex integration of central and peripheral signals, which converge on specific hypothalamic and mesolimbic circuits. Among the neuromodulation systems most involved in this process, the endocannabinoid (ECS) system occupies a prominent position. A growing number of experimental evidences indicate that the ECS contributes significantly to the control of energy balance and the modulation of eating behavior, acting through both central and peripheral mechanisms (Bisogno et al., 1999)⁵². ECS receptors are particularly abundant in the central nervous system, particularly in the hypothalamus and mesolimbic system, key regions for regulating food intake and the hedonic value of food.

Pharmacological manipulation of the ECS confirms its orexigenic role. The administration of exogenous cannabinoids such as Δ^9 tetrahydrocannabinol (THC) or synthetic agonists of CB1 receptors, such as CP55,940 and WIN55,2122, results in an increase in food intake in both humans and animal models (Hollister, 1971; Koch et al., 2001; Hart et al., 2002; Cota et al., 2003)^{53–56}. Conversely, the use of CB1 antagonists or reverse agonists, such as rimonabant or AM251, is associated with a reduction in intake, often more pronounced for highly palatable foods, and a loss of body weight (Carai et

al., 2006)⁵⁷. These data are corroborated by genetic studies: CB1 knockout mice (CB1KO) exhibit a phenotype characterized by thinness and reduced food intake, indicating that CB1 expression and functional activity are determinants of normal feeding behavior (Wiley et al., 2005)⁵⁸.

One of the most interesting aspects of the ECS is its ability to respond dynamically to nutritional status, so its mediators are particularly suitable for reflecting fasting or satiety conditions. AEA and 2-AG levels have been shown to increase significantly in the hypothalamus and nucleus accumbens under fasting conditions to return to baseline values after refeeding, without undergoing major changes in other areas not directly involved in food intake control (Kirkham et al., 2002)⁵⁹. These oscillations suggest that the ECS participates in the regulation of appetite both at the homeostatic level, responding to the body's energy state, and at the hedonic level, modulating the reward value associated with foods, especially the most palatable ones.

Receptor localization studies have shown a distribution of CB1 in the hypothalamus that allows a direct modulation of neurons expressing orexigenic and anorexigenic neuropeptides. It has been reported, for example, that CB1 receptor mRNA is co-expressed with transcripts encoding CRH, CART, MCH, and preproorexin (OX), suggesting a close interaction between endocannabinoid signaling and neuropeptidergic networks key to appetite control (Cota et al., 2003)⁵⁶. In an *ex vivo* model of murine hypothalamic explantation, AEA and the CB1 agonist CP55,940 significantly increase neuropeptide Y (NPY) release, while AM251, a CB1 antagonist, blocks its effect. In addition, AM251 alone is able to reduce the basal release of NPY, confirming that the ECS is tonically involved in the regulation of this orexigenic peptide (Gamber et al., 2005)⁶⁰. Similarly, rimonabant is able to prevent hyperphagia induced by central NPY administration, while the stimulating effect of NPY on food intake is absent in CB1KO mice (Poncellet et al., 2003)⁶¹. These data place the ECS on a par with other peptidergic signals in the regulation of appetite.

The ECS also interacts with peripheral hormones involved in appetite regulation, such as ghrelin and leptin. In fasting rats, rimonabant significantly reduces the increase in plasma ghrelin levels; in experimental models, ghrelin inhibits excitatory inputs in the paraventricular nucleus of the hypothalamus (PVN) through a mechanism that requires the integrity of endocannabinoid signaling, since the effect is abolished by AM251 or inhibition of endocannabinoid synthesis (Cani et al., 2004)⁶². In addition, both THC and

ghrelin increase AMPdependent protein kinase (AMPK) activity in the hypothalamus of wildtype mice but not in CB1KOs, indicating that the hyperphagic effect of these stimuli requires a functionally intact ECS (Tucci et al., 2004)⁶³. Conversely, acute leptin treatments reduce hypothalamic endocannabinoid levels, while in obese leptin-resistant models these levels are chronically elevated (Di Marzo et al., 2001)⁶⁴, suggesting that an ECS imbalance may contribute to pathological hyperphagia.

1.8 The Gut Microbiota

The gut microbiota, a complex ecosystem consisting of over 10^{14} bacteria belonging to hundreds of different species, colonizes the gastrointestinal tract and has a genetic heritage – the microbiome – about 150 times higher than that of humans (Bäckhed et al., 2007; Turnbaugh et al., 2006)^{65,66}. Commensal bacteria not only facilitate digestion and nutrient absorption, but produce essential metabolites, including SCFAs such as acetate (C2), propionate (C3), and butyrate (C4), generated by fermentation of non-digestible carbohydrates (Cummings et al., 1987; Jenkins et al., 1998)^{67,68}. These metabolites modulate intestinal pH, inhibit colonization by pathogens, provide energy to enterocytes, and influence the development and function of the immune system (Ley et al., 2006)⁶⁹.

The production of SCFAs is closely linked to the composition of the microbiota and the diet of the host. Studies in obese mouse models have shown increased levels of acetate and butyrate compared to lean subjects, suggesting a direct role in the greater efficiency of energy extraction from the diet (Turnbaugh et al., 2006)⁶⁶. In addition, dietary alterations, such as a high intake of fats and sugars, change the microbial composition by increasing Firmicutes at the expense of Bacteroidetes, while diets rich in polyunsaturated fatty acids and fiber favor beneficial bacteria such as *Bifidobacterium* and *Roseburia*, associated with positive metabolic effects (Arpaia et al., 2013; Vieira et al., 2017; Sun et al., 2018)^{70–72}.

In addition to energy metabolism, the microbiota exerts a direct influence on eating behavior. SCFAs, and in particular acetate, can enter the circulation and reach the hypothalamus, where they modulate the activity of orexinergic neurons, interacting with lipid signaling pathways such as the endocannabinoidome and regulating neuronal excitability and the profile of local lipid mediators (Kelly et al., 2015; Kim et al.,

2016)^{73,74}. Studies on germ-free models and fecal transplants confirm that the composition of the microbiota determines both energy accumulation and behavioral responses to food stimuli, suggesting a critical role of this gut-brain axis in the regulation of hunger, satiety and energy balance (Ridaura et al., 2013; Vrieze et al., 2012)^{75,76}.

These evidences outline a picture in which the microbiota, through the production of SCFAs and the modulation of hypothalamic circuits, becomes a central player in the integrated regulation of metabolism and eating behavior

1.9 Microbiota-Endocannabinoidome-Gut Axis

The gut microbiota is able to interact with the host through various intra- and inter-cellular signaling pathways. Among these, the intestinal endocannabinoidome is based on the use of endocannabinoids or endocannabinoid-like molecules, i.e. lipid signals of an endogenous nature derived from fatty acids (Di Marzo et al., 2018)⁷⁷. This connection emerged following the treatment of mouse models with pre- and probiotics that caused alterations in host eCB levels (Piscitelli et al., 2011)⁷⁸. In addition, some commensal bacteria produce metabolites similar to endocannabinoids, with which they also often share receptors and metabolic enzymes; could in fact be considered as part of this group (Cohen et al., 2017)⁷⁹. Studies show that GF mice differ dramatically from conventionally bred (CR) mice in the expression levels of receptors, enzymes, and intestinal concentrations of eCBome mediators. Most of these alterations were remedied, after only one week, following the transfer of the fecal microbiota from CR to GF mice; thus highlighting the crucial importance of commensal microorganisms in the direct control of intestinal eCBome signaling. We can therefore say that the microbiota-eCBome interaction seems to have a particular impact on CB1, PPAR α and GPR55 signaling. Conversely, genetic or pharmacological inactivation of biosynthesis or action of eCBome mediators has been shown to have influenced gut microbial composition and host metabolic response to high-fat diets (Mehrpouya-Bahrami et al., 2017; Everard et al., 2019; Geurts et al., 2015; Song et al 2017)⁸⁰⁻⁸³. Further evidence is offered by a study in mice fed with diet obesogenic diseases, characterized by the blockade of CB1 activity, which inhibited the development of obesity, improved glucose homeostasis and intestinal permeability by lowering circulating levels of LPS and inflammatory cytokines; leading,

in parallel, also to the increase of *Akkermansia muciniphila* and the decrease of *Lachnospiraceae* in the intestine (Mehrpouya-Bahrami et al., 2017)⁸⁰. Studies in the laboratory of Muccioli and collaborators have shown that CB1 agonism in wild-type mice increases intestinal permeability, while CB1 antagonism partially preserves the integrity of the tight junction in the intestinal epithelium thus reducing plasma levels of lipopolysaccharide (LPS), a structural macromolecule, a characteristic component of the outer membrane of Gram-negative bacteria, is considered one of the main molecular mediators of the interaction microbiota-host (Cani et al., 2016)⁶². LPS appears to have a close relationship with the endocannabinoid system, being able to modulate the levels of AEA and 2-AG, the main ligands of CB1 receptors, widely distributed in the intestinal wall (Wright et al., 2005)⁸⁴. A further role of CB1 receptors in the intestine concerns the permeability of the enteric barrier. This seems to depend on the portion of the intestine in which these receptors are expressed (Alhouayek et al., 2012)⁸⁵. It is precisely changes in the tone of the eCBs that can restore normal intestinal permeability. As in physiological conditions, LPS remains confined to the intestinal lumen and its passage into the systemic circulation is limited by the integrity of the latter; however, in conditions of dysbiosis or increased intestinal permeability, LPS can cross the epithelium and reach the bloodstream, resulting in a condition called metabolic endotoxemia (Cani et al., 2008; Membrez et al., 2008; Cani et al., 2019)^{86–88}. Related results were obtained by blocking CB1 receptors in obese mice, which improved the function of the intestinal barrier by increasing the expression of proteins responsible for the formation of tight junctions (Muccioli et al., 2010; Cani et al., 2007)^{89,90}. By virtue of this, given that obesity is commonly characterized by increased eCB tone, higher plasma LPS levels, an altered gut microbiota and metabolic dysfunction of adipose tissue, it is likely that all these changes may be triggered mainly by an alteration of the eCB system (Ridaura V.K. et al., 2013; Erny et al., 2015)^{75,91}. In another study, Mehrpouya-Bahrami and collaborators showed that CB1 antagonism in mice made obese by HFD counteracts dysbiosis and reduces LPS levels, thus helping to counteract the obese phenotype. Wanting to deepen the above, studies show that the concentration of AEA, both in the ileum and in the plasma, and of 2-AG, in the plasma, as well as the alterations in several other NAEs and 2-acylglycerols that have been reported with both the high-fat and sucrose diet (HFHS) are inversely correlated with the abundance of metabolically beneficial intestinal commensal microorganisms. such as *Akkermanisa*, *Barnesiella*, *Eubacterium*,

Adlercreutzia and *Propionibacterium* spp, in some cases regardless of changes in body weight. This study revealed the existence of interactions between the eCBome and different gut bacterial species during dysmetabolic events with potential impact on a host's ability to adapt to increased fat and sucrose intake. Therefore, a diet-microbiota-andCBome loop influences intestinal permeability and systemic inflammation in opposite ways, obesogenic diets create a vicious circle leading to an increase in endotoxemia and metabolic diseases (Jourdan et al., 2017)⁹², otherwise the consumption of diets associated with prebiotic fibers creates a virtuous circle, which reduces intestinal permeability, metabolic endotoxemia and systemic inflammation.

In this scenario the protagonist of our study is placed, acetate, which produced in the intestine and introduced into the circulation, reaches the hypothalamus, where it can be picked up by the neurons of the lateral hypothalamus. Here, in addition to being used as an energy substrate, acetate could intervene on cellular metabolism and lipid signaling pathways, including the endocannabinoidome, by modulating the excitability of orexinergic neurons and the profile of local lipid mediators. The following chapters of this paper explain the experimental results obtained, illustrating how this hypothesis is verified through a combination of behavioral studies, neuronal activation analysis, electrophysiological recordings and measurements of hypothalamic lipid mediators

1.10 Aim of the Thesis

The overarching aim of this PhD thesis is to investigate the role of the endocannabinoids and food-derived endocannabinoid-like molecules in the regulation of neuronal networks and behavior, with particular emphasis on how metabolic, environmental, and sensory signals are translated into changes in neuronal excitability, synaptic plasticity, and complex behavioral output. In this framework, the endocannabinoidome is conceived not as an isolated neuromodulatory system, but as a molecular and circuit-level platform that integrates information arising from the gut microbiota, diet and photic input to shape the functional organization of hypothalamic, mesolimbic, and hippocampal circuits.

To this purpose, the initial phase of the project was designed to extend this perspective to the domain of food reward and obesity, by investigating how olive oil-derived endocannabinoid-like mediators, in particular oleoylglycine (OlGly) and oleoylalanine

(OlaA), modulate the reinforcing properties of highly palatable food and the susceptibility to diet-induced obesity. Through the integration of conditioned place preference paradigms (CPP), in vitro electrophysiology of ventral tegmental area dopaminergic neurons, targeted lipidomics, and gut microbiota profiling, this Task aimed to define an olive oil–eCBome–PPAR–brain–microbiota axis as an endogenous brake on palatable food–induced reward and body weight gain (Task I – *Olive oil-derived endocannabinoid-like mediators inhibit palatable food-induced reward and obesity*).

Subsequently, the second part of the project was devoted to dissecting how a microbiota-derived short-chain fatty acid, namely acetate, can influence hypothalamic circuits involved in energy balance and feeding. By focusing on orexin/hypocretin neurons of the lateral hypothalamus, this part of the work aimed to characterize a novel mechanism by which the acetate–endocannabinoidome axis participates in the gut–brain regulation of energy homeostasis and food intake (Task II – *The Short-chain fatty acid acetate modulates Orexin/Hypocretin Neurons – a novel mechanism in gut–brain axis regulation of energy homeostasis and feeding*).

Finally, the last part of the thesis explored the interface between photic signaling and hypothalamic homeostatic regulation, focusing on a previously underappreciated lateral branch of the retinohypothalamic tract. This phase of the work characterized how intrinsically photosensitive retinal ganglion cells, selectively responsive to blue light, project to the perifornical hypothalamus and influence orexin neurons via glutamatergic inputs endowed with CB1 receptors. By integrating anatomical tracing, immunohistochemistry, time-resolved measurements of hypothalamic 2-AG levels, and analysis of endocannabinoid-mediated DSE at retinal terminals, this Task set out to demonstrate that blue light transiently activates orexinergic neurons while simultaneously engaging a local endocannabinoid feedback that dampens excitatory drive, thereby revealing a light-responsive, endocannabinoid-driven mechanism for the fine tuning of arousal and energy-related hypothalamic functions (Task III – *The Blue Light Responsive Lateral Pathway of the Retinohypothalamic Tract Promotes Endocannabinoid Driven Modulation of Orexin Neurons*).

Taken together, these three Tasks delineate a coherent research trajectory in which the endocannabinoidome emerges as a shared molecular language through which the central nervous system integrates metabolic, and photic cues to adapt neuronal network dynamics and behavior to internal and external demands. By bridging apparently

disparate domains, from microbiota–gut–brain communication to food reward and blue-light–driven arousal, this thesis proposes a unified view of the endocannabinoidome as a crucial hub at the crossroads between peripheral signals and brain homeostasis.

In continuity with this framework, an ongoing Task of the project is devoted to investigating how the endocannabinoidome – and in particular the CB2 receptor – contributes to preserving microglial and synaptic homeostasis in the context of high-fat-diet–induced obesity. In this study, currently under preparation as a manuscript on which I will be first author, CB2 receptor activation is examined as a strategy to reduce microglial lipid droplet accumulation and to restore hippocampal synaptic function and plasticity, thereby extending the concept of the endocannabinoidome as a protective interface between metabolic stress, glial fitness, and the integrity of neuronal networks (Task IV – *Studies of 2-Arachidonoyl-sn-glycerol-3-phosphate (2-AGP), a Lysophosphatidic Acid, Mediates High-Fat Diet–Induced Working Memory Impairment via Tau Hyperphosphorylation and Microglial Dysfunction*)

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3. Results

3.1 Task I – “Olive oil-derived endocannabinoid-like mediators inhibit palatable food-induced reward and obesity”

Obesity is a condition of chronic low-grade inflammation and energetic imbalance in which metabolic components and reward circuits converge to promote overconsumption of highly palatable foods. In this scenario, the endocannabinoidome plays a central role in modulating both hypothalamic energy homeostasis and hedonic responses mediated by mesolimbic circuits, thereby influencing vulnerability to the development of addiction-like eating behaviors. In parallel, epidemiological and experimental evidence attribute a protective effect against obesity and cardiometabolic diseases to olive oil, a cornerstone of the Mediterranean diet, yet the molecular links between this food, reward processing, dopaminergic activity, and the gut microbiota remain only partially understood.

From a biochemical standpoint, olive oil is rich in oleic acid, the precursor of a class of endocannabinoid-like lipid mediators, such as oleoylglycine (OlGly) and oleoylalanine (OlAla), already known for their ability to modulate dependence-related behaviors toward opioids, alcohol, and nicotine via activation of PPAR α and, in the case of OlAla, through inhibition of FAAH, with consequent alteration of the profile of other endocannabinoid mediators. Previous work indicates that OlGly and OlAla act as an endogenous brake on compulsive seeking of rewarding stimuli, suggesting that they may also operate as a physiological “brake” on reward driven by hypercaloric, highly palatable foods. The present study employs behavioral paradigms of conditioned place preference (CPP) for highly palatable food, electrophysiological recordings from ventral tegmental area (VTA) dopaminergic neurons, targeted lipidomics in key brain regions (VTA, nucleus accumbens, insula, hypothalamus), and metataxonomic analysis of the microbiota to test the hypothesis that these lipoamino acids constitute a functional axis linking diet, brain, and microbiota.

Specifically, the comparison between a lard-rich diet (HFDL) and an isocaloric high-olive oil diet (HFDO) allows the qualitative effect of fat composition on obesity

vulnerability to be isolated: while HFDL promotes weight gain, reduction of intestinal microbial diversity, and depletion of OIGly/OlAla in the brain, HFDO is associated with increased levels of these mediators in the VTA, insula, accumbens, and hypothalamus, with attenuation of weight gain and microbiota profiles more similar to those of lean animals. Use of the PPAR antagonist GW6471 demonstrates that protection from obesity and reduction of CPP for palatable food depend substantially on PPAR α activation, both for the direct pharmacological action of OIGly/OlAla and for the effects induced by the olive-oil-rich diet. At the neuronal level, OIGly and OlAla reduce the spontaneous firing rate of VTA dopaminergic neurons in a PPAR-dependent fashion, providing a plausible cellular substrate for the decrease in incentive value of food. This study aimed to determine whether OIGly, OlAla, and a high-olive oil diet can counteract reward induced by highly palatable food and diet-induced weight gain, by acting on PPAR signaling, modulating dopaminergic neuron activity, reshaping brain and intestinal lipid mediator profiles, and restructuring the microbiota toward configurations associated with obesity resistance.

Olive oil-derived endocannabinoid-like mediators inhibit palatable food-induced reward and obesity

Nicola Forte¹, Charlène Roussel^{2,3,11}, Brenda Marfella^{1,4,11}, Anna Lauritano¹, Rosaria Villano¹, Elvira De Leonibus^{5,6}, Emanuela Salviati⁷, Tina Khalilzadehsabet^{2,3}, Giada Giorgini^{2,3}, Cristoforo Silvestri^{2,3}, Fabiana Piscitelli¹, Maria Pina Mollica^{4,8,9}, Vincenzo Di Marzo^{1,2,3,10}✉ & Luigia Cristino¹✉

N-oleoylglycine (OIGly), a lipid derived from the basic component of olive oil, oleic acid, and *N*-oleoylalanine (OIAla) are endocannabinoid-like mediators. We report that OIGly and OIAla, by activating the peroxisome proliferator-activated receptor alpha (PPAR α), reduce the rewarding properties of a highly palatable food, dopamine neuron firing in the ventral tegmental area, and the obesogenic effect of a high-fat diet rich in lard (HFD-L). An isocaloric olive oil HFD (HFD-O) reduced body weight gain compared to the HFD-L, in a manner reversed by PPAR α antagonism, and enhanced brain and intestinal OIGly levels and gut microbial diversity. OIGly or OIAla treatment of HFD-L mice resulted in gut microbiota taxonomic changes partly similar to those induced by HFD-O. We suggest that OIGly and OIAla control body weight by counteracting highly palatable food overconsumption, and possibly rebalancing the gut microbiota, and provide a potential new mechanism of action for the obeso-preventive effects of olive oil-rich diets.

¹Institute of Biomolecular Chemistry, National Research Council of Italy, Via Campi Flegrei 34, 80078 Pozzuoli (NA), Italy. ²Heart and Lung Research Institute of Université Laval, Québec City, QC, Canada. ³Institute for Nutrition and Functional Foods, Centre NUTRISS, Université Laval, Québec City, QC, Canada. ⁴Department of Biology, University of Naples Federico II, 80126 Naples, Italy. ⁵Telethon Institute of Genetics and Medicine, Pozzuoli, Naples, Italy. ⁶Institute of Biochemistry and Cell Biology, Consiglio Nazionale delle Ricerche (CNR), Monterotondo Scalo, Rome, Italy. ⁷Department of Pharmacy, University of Salerno, Fisciano (SA), Italy. ⁸Centro Servizi Metrologici e Tecnologici Avanzati (CeSMA), Complesso Universitario di Monte Sant'Angelo, 80126 Naples, Italy. ⁹Task Force on Microbiome Studies, University of Naples Federico II, 80138 Naples, Italy. ¹⁰Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, Université Laval, Québec City, QC 61V0A9, Canada. ¹¹These authors contributed equally: Charlène Roussel, Brenda Marfella. ✉email: vdimarzo@icb.cnr.it; luigia.cristino@icb.cnr.it

The daily consumption of Western diets, consisting of highly palatable and caloric food, represents the main environmental cue triggering addictive-like behaviors and obesity^{1,2}. Highly palatable food (HPF) stimulates dopaminergic (DA) neurons in the VTA, thereby activating the reward circuit^{3,4}. Short-term exposure to HPF induces long-lasting synaptic plasticity in mesolimbic DA neurons⁵.

Lipoaminoacids, such as OIGly, are endogenous endocannabinoid-like mediators belonging to the large family of the endocannabinoids (eCBome)⁶. OIGly is endogenously synthesized from oleic acid⁷, the main monounsaturated long-chain fatty acid present in olive oil, a basic component of the Mediterranean diet. Since OIGly undergoes fast degradation by endogenous amidases, a more stable analogue, OIAla, was synthesized and tested, and found to be more efficacious than OIGly *in vivo* (see below)⁸. Interestingly, evidence exists for the presence also of endogenous OIAla in the brain⁹.

Indeed, OIGly and, particularly, OIAla reverse abuse-related effects of naloxone-precipitated withdrawal behaviors from chronic opiates in mice, while they do not modify tolerance to nociception, hyperthermia, and suppression of activity produced by morphine^{8–10}. Recently it was demonstrated that both OIGly and OIAla reduce alcohol intake and preference¹¹. More importantly for the purpose of this study, OIGly reduces nicotine preference and withdrawal in mice¹² by activating PPARα¹², and GW6471 (GW) (2 mg/kg), a selective PPARα antagonist, prevents OIGly-induced inhibition of nicotine conditioned place preference (CPP)¹². It was previously demonstrated that PPARα activation reduces VTA DA output^{13,14}.

Results from studies in both rodents and humans have suggested that the gut microbiome is modulated by the diet and intervenes in increased consumption of, and dependence from, HPF^{15,16}. Indeed, the gut microbiome is increasingly being proposed to play a role in obesity and its related disturbances, such as food addictive behaviors, and to interact with eCBome signaling^{17–19} in both the brain and gut^{20,21}. Nevertheless, the involvement in behavioral signs of food choice or preference in rodents of the gut microbiome, and the effects thereupon of eCBome mediators, such as OIGly and OIAla, have not yet been fully elucidated.

The current study aimed at assessing the role of OIGly and OIAla as endogenous, and possibly olive oil-derived, molecules counteracting food-related addictive behaviors and, consequently, high fat-induced increases of food intake and weight gain in mice.

Results

OIGly and OIAla do not alter food intake but counteract HPF-induced CPP via PPARα receptor activation. Before testing whether OIGly and OIAla are good candidates to counteract food craving induced by HPF, we first assessed the effect of their chronic treatment (7 weeks, 3 times per week; 50 mg/kg) on food (chow) intake and body weight in mice fed with a standard mouse diet. Neither parameter was affected by the treatments (Fig. 1A–D).

Since it was shown that OIGly counteracts nicotine-induced CPP¹², we tested if this endogenous mediator also affected the rewarding properties of HPF using the apparatus and protocol depicted in Fig. 2A. The results show that mice ate significantly more HPF than standard food regardless of treatment during the conditioning phase (Fig. 2A, B, D). However, during the place preference test phase (when no food was present), mice injected with vehicle spent significantly more time in the room where the HPF had been present, thus confirming the rewarding properties of HPF; in contrast the OIGly- (50 mg/Kg) or OIAla-treated (50 mg/Kg) group spent the same amount of time in both rooms (Fig. 2E).

Short consumption of HPF leads to long-lasting changes in food approach, even when alternated with a standard diet⁵. To test whether our protocol was able to induce these long-lasting effects on food preference (Fig. 2A), conditioned mice were kept for three further days under an *ad libitum* chow diet regimen (Fig. 2C) without pharmacological treatment. Subsequently, CPP was re-tested (CPP2). The results show the persistence of a CPP in mice, which confirmed their preference for the HPF-associated room despite not being under food deprivation. Also this long-lasting effect was prevented by OIGly or OIAla (Fig. 2F).

These data suggest that mice prefer HPF compared to chow and that OIGly or OIAla do not influence either the food choice or the amount of food consumed, indicating that the two drugs do not modify the hedonic value of food or the motivational drive of the animals. However, both compounds were able to counteract the reward-associative properties of HPF.

Our results point the attention to the capability of the OIGly and OIAla to counteract the “addictive” effect of HPF, recapitulating what had been demonstrated using nicotine¹². It was also previously demonstrated that OIGly and OIAla activate PPARα^{8,12} and that the selective PPARα antagonist GW6471 (GW) (2 mg/kg) prevents OIGly-induced inhibition of nicotine CPP¹². To test whether the effects described above were also mediated by PPARα, we coadministered either compound with GW (2 mg/kg). GW-treated mice, without and with OIGly or OIAla, still consumed significantly more HPF than chow (Fig. 2G). Furthermore, GW-treated mice still showed CPP for the HPF compartment. While GW was unable to significantly counteract the ability of OIAla to inhibit the rewarding properties of HPF in the CPP test, it did inhibit this effect of OIGly (Fig. 2H).

OIGly and OIAla decrease HFD-L induced weight gain. To test the hypothesis that OIGly and OIAla (50 mg/kg), by virtue of their inhibition of the CPP induced by HPF, could reduce weight gain associated with chronic consumption of an HPF, we treated two groups of mice fed with HFD-L. Both molecules reduced weight gain and food intake in these mice (Fig. 3A–D), while no difference in the weight increment was observed as compared to GW alone when mice were treated with GW, GW + OIGly or GW + OIAla (Fig. 3E). Interestingly, mice fed with an isocaloric HFD diet containing olive oil (HFD-O) instead of lard behaved as chow-vehicle mice after 7 weeks of treatment as far as weight gain (Fig. 3F). GW administration in mice fed with HFD-O, but not HFD-L, induced a significant increment in weight gain, supporting the possible existence of endogenous PPARα activators, possibly derived from olive oil, in the control of weight gain (Fig. 3F). The analysis of cumulative food intake revealed that the HFD-O mice ate overall slightly less than the mice fed with the chow diet (Fig. 3G), although their caloric intake was higher (Fig. 3H).

OIGly and OIAla reduce the action potential discharge of putative VTA DA neurons. HPF significantly activates the VTA and strengthens the dopamine reward circuitry^{3,4}. To analyze the effect of OIGly and OIAla on putative VTA DA neurons¹⁴, loose cell-attached recording was performed in brain slices and only neurons with electrophysiological and anatomical features specific of DA neurons were selected for the study (Supplementary Fig. 1A–C)^{14,22}. OIGly and OIAla (10 μM) were added to the recording chamber after 10 min of baseline measurement. Both molecules reduced the firing rate of putative DA neurons of mice under an *ad libitum* chow diet (Fig. 4A–C). However, pretreatment with GW (100 nM) inhibited the effect of both OIGly and OIAla on VTA DA neuron firing rate (Fig. 4D). Very similar

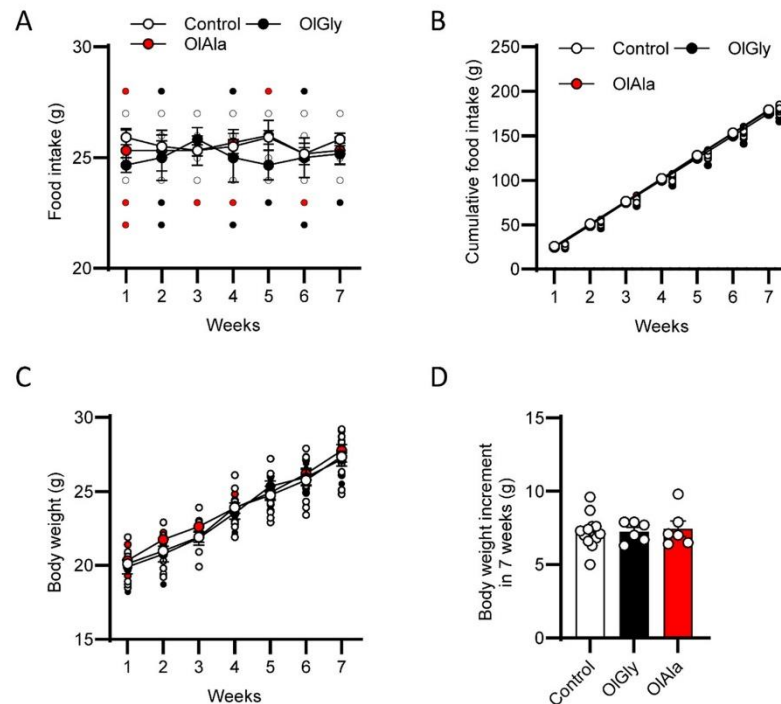


Fig. 1 OIGly and OIAla do not alter food intake and body weight by chronic treatment in mice under a standard chow diet. **A** Effect OIGly (50 mg/Kg) or OIAla (50 mg/Kg), on food intake of mice fed with chow. **B** Cumulative food intake (g). **C** Body weight changes following OIGly (50 mg/Kg) or OIAla (50 mg/Kg) administration. **D** Effect of OIGly or OIAla on the the weight of mice after 7 weeks of treatment. $n = 12$ vehicle, $n = 6$ OIGly, $n = 6$ OIAla.

results were obtained in slices from mice under HFD-L (Fig. 4E–H) or HFD-O (Fig. 4I–L).

These results point to PPAR α activation as a possible mechanism of action of OIGly and OIAla in the control of the addictive effects of HPF through the reduction of VTA DA neuron firing rate²³.

The concentrations of OIGly and OIAla in brain areas involved in reward are increased in HFD-O mice as compared to HFD-L and/or chow mice. We, therefore, next measured by IT-TOF MS-MS coupled to liquid chromatography^{9,12} the tissue concentrations of OIGly and OIAla in various brain regions involved in reward (VTA, accumbens, insula and hypothalamus) from mice fed with chow, HFD-O or HFD-L for 7 weeks. In chow mice, OIGly and OIAla were detected only in the insula and VTA. In these two regions, OIGly levels tended to be higher in the HFD-O group as compared to the other two groups, while, in the accumbens and, particularly, the hypothalamus, the HFD-O diet led to the appearance of detectable or even quite high concentrations of this mediator (Supplementary Fig. 2). Although, in HFD-O mice, OIAla levels in the VTA were not higher than the in chow mice, nor did they become detectable in the hypothalamus and accumbens, a trend towards an increase was found in the insula. Additionally, HFD-L mice exhibited significantly reduced or undetectable concentrations of this compound in the insula and VTA, as well as those of OIGly in all brain regions, as compared to chow and/or HFD-O mice (Supplementary Fig. 2). The levels of the well-established endogenous PPAR α agonist, N-oleylethanolamine (OEA)²⁴, were at least one order of magnitude higher than those of OIGly or OIAla in all brain regions and

were not significantly altered by the HFD-O diet in any of the four regions analysed, and in fact tended to be lower in the VTA, accumbens and insula of HFD-O mice (Supplementary Fig. 2).

In order to understand if endogenous intestinal signaling of the two mediators is increased following the intra-peritoneal (i.p.) administration of the exogenous compounds, we analysed OIGly and OIAla concentrations in the large intestine of chow and HFD-L mice w/o treatment with the exogenous compounds, and in HFD-O mice as a comparison. We found that: (1) the administration of the two compounds did not significantly alter their levels in chow mice, but it strongly increased them in HFD-L mice, possibly due to the fact that the intestine expresses high levels of amidases for endocannabinoid-like mediators, such as fatty acid amide hydrolase (FAAH), which can be inhibited by fats²⁵, and (2) the HFD-O diet led to levels that were much higher than those of HFD-L and, in the case of OIGly, comparable to those obtained with the exogenous administration of the two compounds in HFD-L mice (Supplementary Fig. 3).

OIGly, OIAla and olive oil affect intestinal microbiota composition in chow or HFD mice. Given the recently suggested important role of the gut microbiota in food addiction^{15,26,27}, we next investigated the effect of OIGly and OIAla administration on the relative taxonomic composition of the small and large intestinal microbiota in mice fed with either chow or HFD-L for 7 weeks and compared it to the effect of HFD-O. First, we observed that the two mediators did not affect α -diversity in chow mice, a measure of microbial diversity and metabolic “health”, assessed with the Shannon index (Fig. 5A), although a trend towards an increase was found with OIAla in the small intestine.

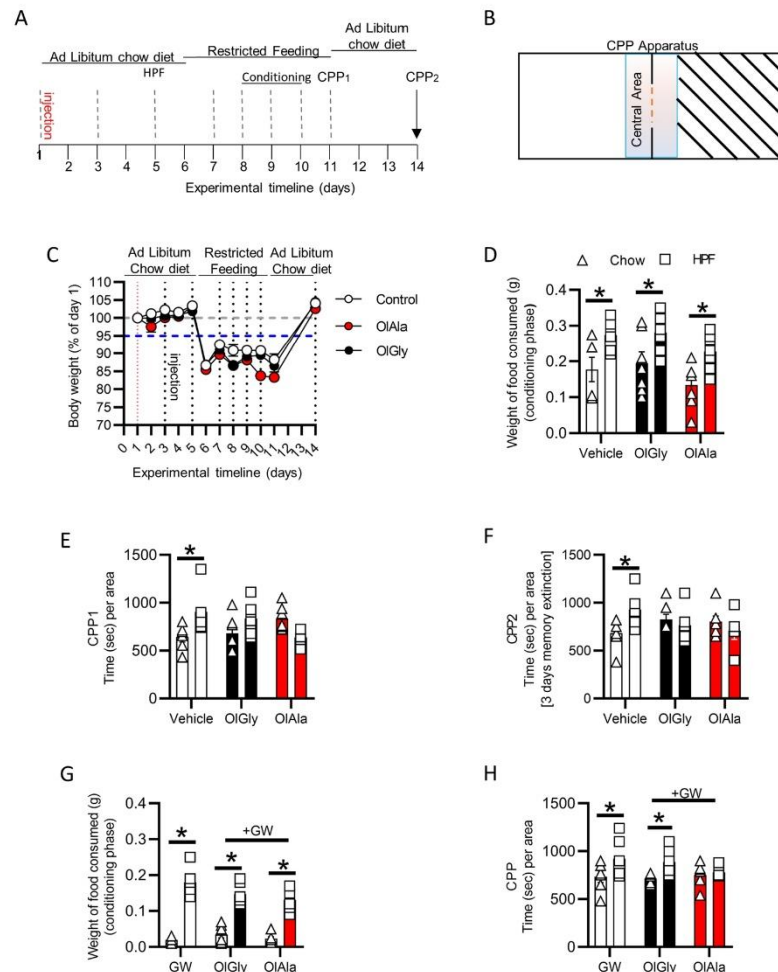


Fig. 2 OIGly and OIAla inhibit CPP induced by HPF in a manner prevented by GW in OIGly-treated mice. **A** CPP experimental timeline. **B** Schematic representation of the CPP apparatus. **C** Body weight changes during the CPP experiment in vehicle-, OIGly- (50 mg/Kg) or OIAla-injected (50 mg/Kg) mice. **D** Weight of food consumed (chow food or HPF) per mouse during the conditioning session of vehicle- (control), OIGly- (50 mg/Kg) or OIAla-injected (50 mg/Kg) mice. **E** CPP₁ test was performed after the conditioning session. **F** CPP₂ test performed after two days of ad libitum feeding. **G** Weight of food consumed (chow food or HPF) per mouse during the conditioning session in GW (2 mg/Kg), GW (2 mg/Kg) + OIGly (50 mg/Kg) and GW (2 mg/Kg) + OIAla (50 mg/Kg) group. **H** CPP test performed after a conditioning session. * $p < 0.05$. Two-way ANOVA and Sidak's multiple comparison test. $n = 6$ GW, $n = 7$ GW + OIGly and $n = 7$ GW + OIAla mice per group. * $p < 0.05$. Two-way ANOVA and Sidak's multiple comparison test. $n = 6$ vehicle-, $n = 7$ OIGly-, $n = 7$ OIAla-, $n = 6$ GW-, $n = 7$ GW + OIGly-, $n = 7$ GW + OIAla-injected mice.

Likewise, neither OIGly nor OIAla counteracted the reduced α -diversity typical of HFD-L obese mice, although again a trend was observed with HFD-O. On the other hand, feeding with HFD-O resulted in a significant increase in α -diversity in the large intestine compared to HFD-L mice, resulting in a value similar to that of chow mice, whereas in the small intestine, only a non-statistically significant increase was observed vs. the HFD-L diet (Fig. 5A, Supplementary Data 2). Based on the phyla and the 20 most abundant genera, each diet, i.e. chow, HFD-L and HFD-O, displayed distinct microbiota signature compositions, and only few changes seemed to be exerted by OIGly and OIAla in either HFD-L and, particularly, chow-fed

mice (Fig. 5B, C). PCoA analysis of bacterial genera based on Jaccard dissimilarity in the small (Fig. 5D) and large intestine (Fig. 5E) confirmed that the diet was the major determinant of differences between groups ($p \leq 0.05$). The heatmap in Fig. 6 based on the DESeq normalized counts of genera (Supplementary Data 3) that were significantly shifted between diets and/or treatments ($p \leq 0.05$), obtained using a Wald Test, further confirmed this conclusion.

A more in-depth analysis of the single genera, however, highlighted how OIGly and OIAla did induce changes in microbiota genera under HFD-L in both segments (Fig. 7). These changes were similar between the two compounds, although

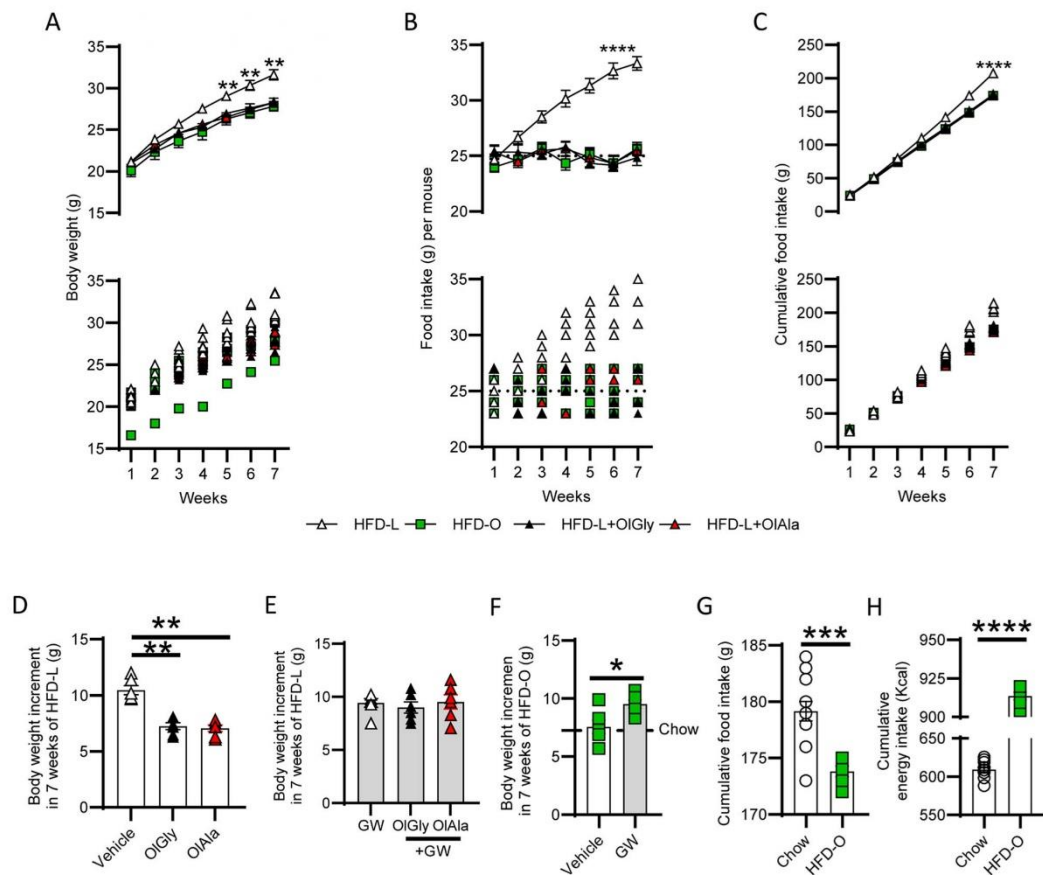
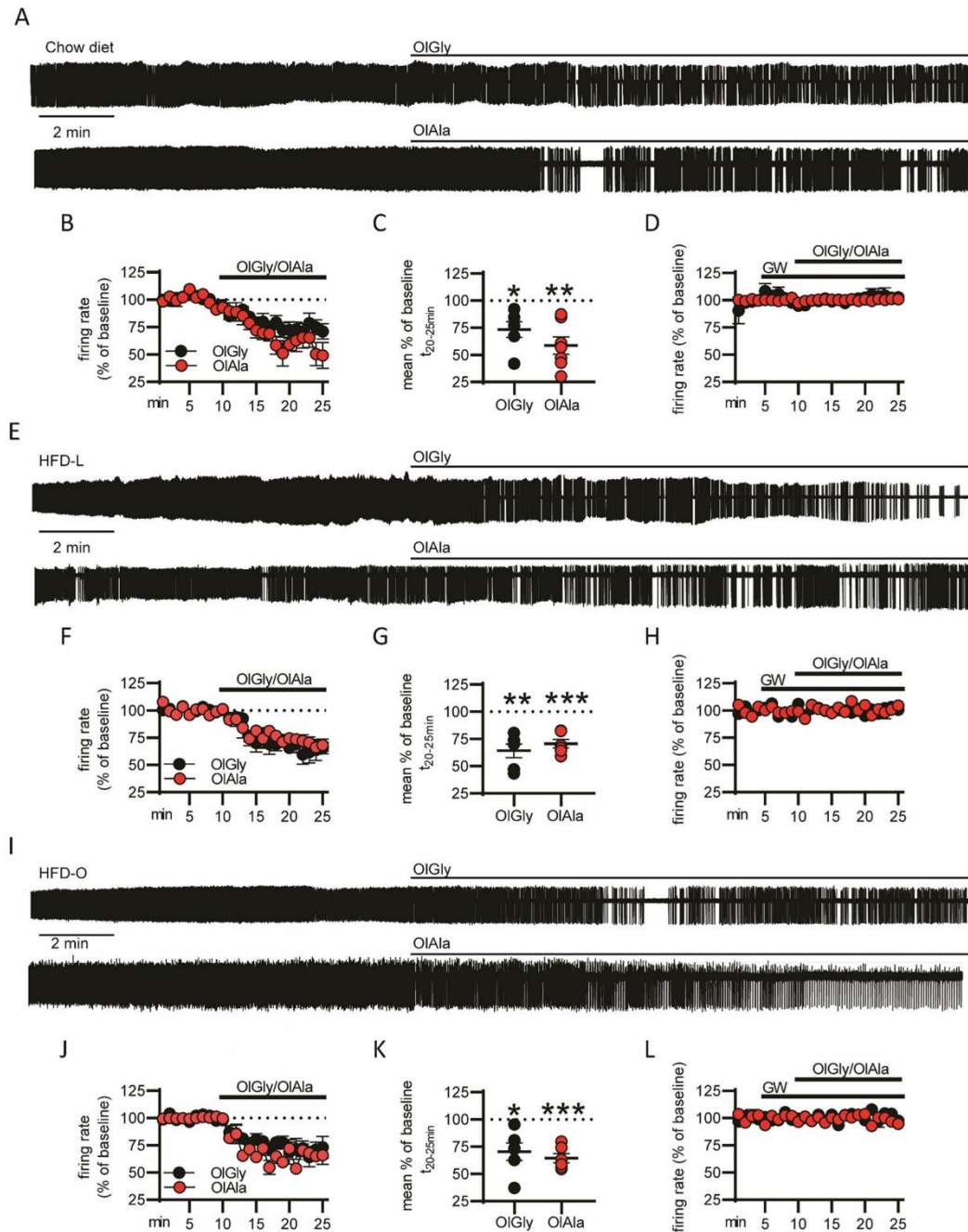


Fig. 3 OIGly and OIAla counteract the obesogenic effect of a HFD diet rich in lard. **A** Body weight changes following 7 weeks of HFD-L or HFD-O, and following OIGly (50 mg/Kg) or OIAla (50 mg/Kg) treatment of HFD-L mice. $^{**}p < 0.01$, one-way ANOVA and Bonferroni post hoc, the graphs show the mean + SEM (upper graph) and the individual data points (lower graph). **B** Effect of OIGly (50 mg/Kg) or OIAla (50 mg/Kg) treatment on mice fed with HFD-L and effect of HFD-O on food intake per week. $^{****}p < 0.0001$, one-way ANOVA and Bonferroni post hoc, the graphs show the mean + SEM (upper graph) and the individual data points (lower graph). **C** Effect of OIGly (50 mg/Kg) or OIAla (50 mg/Kg) treatment on mice fed with HFD-L and effect of HFD-O on cumulative food intake. $^{****}p < 0.0001$, one-way ANOVA and Bonferroni post hoc, the graphs show the mean + SEM (upper graph) and the individual data points (lower graph). **D** Weight increment after 7 weeks of HFD-L in the vehicle, OIGly and OIAla treated mice; $n = 6$ mice per group, $^{**}p < 0.01$, one-way ANOVA and Bonferroni post hoc. **E** Weight increment after 7 weeks in GW (2 mg/kg), GW + OIGly and GW + OIAla treated mice. **F** Weight increment after 7 weeks of HFD-O in the vehicle and GW (2 mg/kg) treated mice; $n = 6$ mice per group, $^{*}p < 0.05$, Unpaired t-test with Welch's correction. STD line indicates the mean of the weight increment in mice under standard diet (STD). **G** Cumulative food intake (g) in mice under chow diet and HFD-O; $n = 12$ chow, $n = 6$ HFD-O $^{***}p < 0.001$, Unpaired t-test with Welch's correction. **H** Cumulative energy intake (Kcal) in mice under chow diet and HFD-O; $n = 12$ chow, $n = 6$ HFD-O $^{****}p < 0.0001$, Unpaired t-test with Welch's correction.

OIAla produced additional changes in the large intestine ($p \leq 0.05$) and often produced quantitatively stronger changes (Supplementary Fig. 4, Supplementary Data 4). In particular: (1) *Akkermansia* and *Marvinbryantia* are two well established candidate indicators for persistent success in weight loss²⁸. *Akkermansia* was significantly increased by OIAla under chow and HFD-L in the large intestine. *Marvinbryantia*, known to be negatively associated with food addiction in obese women²⁹, was significantly increased by both OIGly and OIAla, mostly in the large intestine under both chow and HFD-L ($p \leq 0.05$) (Fig. 7); (2) *Desulfovibrio*, which also negatively correlates with body weight²⁹, and *Dubosiella*, for which contrasting results exist in this sense, but is considered a short chain fatty acid-producing

genus and as such would play beneficial actions in HFD-induced obesity and reward^{30,31}, tended to be increased, and significantly by OIAla ($p \leq 0.05$), in chow mice (Fig. 7); (3) *Faecalibaculum* has been associated with obesity³², but in the present study its relative abundance was strongly decreased by the HFD-L diet, and OIAla and OIGly tended to partially restore it to levels typical of chow mice in the large intestine and significantly for OIGly in the small intestine ($p \leq 0.05$, Fig. 7); (4) *Parasutterella*, for which contrasting results also exist, has also been inversely correlated to HFDs³³, as in the present study, and was again significantly increased by OIGly in the small intestine and OIAla in the large intestine ($p \leq 0.05$, Fig. 7); (5) *Rikenella* is associated with obesity^{34,35}, again as in our study, and tended to be decreased by OIGly/OIAla



in the small intestine, and significantly by OIGly in the large intestine ($p \leq 0.05$, Fig. 7); (6) the increase of *Peptococcus* in the small intestine by the two metabolites can also be considered beneficial³⁶ (Fig. 7); (7) *Tuzzerella*, which was significantly increased by HFD-L compared to chow ($p \leq 0.05$), and is known to be associated with metabolic disorders, tended to be reduced by

both molecules in the large intestine and significantly for OIAla in all gut regions ($p \leq 0.05$)³⁷ (Fig. 7); (8) *Streptococcus* and *Lachnospiraceae_UCG006*, two genera that were recently positively associated with food addiction in obese women²⁹, were significantly increased by HFD-L, mostly in large intestine compared to chow, and were significantly reduced by both OIGly

Fig. 4 OIGly and OIAla reduce the firing rate of putative DA neurons in the VTA of mice. **A** Representative trace from a putative DA neuron before and after OIGly and OIAla (10 μ M) treatment in mice under a chow diet regime. **B** Percentage of firing rate changes after addition of OIGly or OIAla in comparison to the baseline; statistical significance was calculated comparing the average of the firing rate in the last 5 min. **C** Plot of the mean firing rate in percentage of baseline calculated in the interval t_{20-25} . $n = 6$ neurons from $n = 3$ mice per group. $^*p < 0.05$ and $^{**}p < 0.01$ One sample T -test. **D** Percentage of variation of the firing rate in slices pretreated with GW6471 (100 nM) with respect to the baseline after the addition of OIGly or OIAla. $N = 3$ and 4 neurons, respectively, per group from 3 mice. **E** Representative trace from a putative DA neuron before and after OIGly and OIAla treatment in mice under a HFD-L. **F** Percentage of firing rate changes after addition of OIGly or OIAla in comparison to the baseline; statistical significance was calculated comparing the average of the firing rate in the last 5 min. **G** Plot of the mean firing rate in percentage of baseline calculated in the interval t_{20-25} . $n = 6$ neurons from $n = 3$ mice per group. $^{**}p < 0.01$, $^{***}p < 0.001$, One sample T -test. **H** Percentage of variation of the firing rate in slices pretreated with GW6471 (100 nM) with respect to the baseline after the addition of OIGly or OIAla. $N = 3$ neurons, respectively, per group from 3 mice each. **I** Representative trace from a putative DA neuron before and after OIGly and OIAla treatment in mice under a HFD-O. **J** Percentage of firing rate changes after addition of OIGly or OIAla in comparison to the baseline; statistical significance was calculated comparing the average of the firing rate in the last 5 min. **K** Plot of the mean firing rate in percentage of baseline calculated in the interval t_{20-25} . $n = 6$ neurons from $n = 3$ mice per group. $^*p < 0.05$, $^{***}p < 0.001$, One sample T -test. **L** Percentage of variation of the firing rate in slices pretreated with GW6471 vs the baseline after the addition of OIGly or OIAla. $N = 3$, respectively, per group from 3 mice.

and OIAla in the small intestine of chow mice (Fig. 7); (9) finally, *Muribaculum* is associated with antiobesity effects when increased³⁸, and was significantly increased by OIAla in the large intestine under HFD-L and by both compounds under chow ($p \leq 0.05$, Fig. 7).

The volcano plots of the above mentioned modulations, shown in Fig. 8 (Supplementary Data 5), which group both the small and large intestine, indicate that the HFD-O diet, similar to what described above for OIAla and/or OIGly using single genera comparisons, produced a significant albeit small decrease in the relative abundance of *Lachnospiraceae_UCG006* with respect to chow ($p \leq 0.05$, Fig. 8A), and the increase of *Parasutterella* and *Faecalibaculum* and the decrease of *Lachnospiraceae_UCG006* and *Rikenella* with respect to the HFD-L diet (Fig. 8B). Volcano plots also confirmed how OIGly and OIAla treatment led to significantly enhanced *Faecalibaculum* or *Parasutterella*, respectively, as compared to HFD-L ($p \leq 0.05$, Fig. 8E, F), while only OIAla imparted on chow mice significant differences (Fig. 8C, D, $p \leq 0.05$) that were not all discussed above but could still represent beneficial alterations, i.e. the increased abundance of *Prevotellaceae_UCG001* and the above-mentioned one of *Marvinbryantia* (Fig. 8F).

In summary, some of the alterations of intestinal microbiota genera induced by OIAla and OIGly, in either chow or, particularly, HFD-L mice, can be interpreted as beneficial against obesity and food addiction and were largely reproduced by HFD-O, which however produced many more changes. This is also exemplified by the volcano plots comparing the intestinal microbiota of HFD-O mice with those of HFD-L treated with either OIAla or OIGly, which showed several differences including a significant stimulation, under HFD-O, of *Bifidobacterium*, known for its health-related properties (Fig. 8G, H). In general, OIAla appeared to produce stronger effects on the gut microbiota (Fig. 7, Fig. 8C–F, Supplementary Fig. 4), possibly due to the fact that this molecule is more metabolically stable to enzymatic hydrolysis than OIGly⁸.

Discussion

In the present study, we aimed at assessing: (1) whether OIGly and OIAla, two oleic acid-derived mediators belonging to the large family of the eCBome and previously found to relieve either nicotine or alcohol preference or opiate dependence symptoms, or both (refs. ^{8,9,12,39,40}), are capable of interfering with food addiction in mice, and consequently with HFD-induced obesity, and through what neural and peripheral mechanisms, and (2) to what extent these putative properties could explain the protective effects of olive oil against HFD-induced obesity. In order to answer these questions, and also to assess to what extent the

intestinal microbiome is involved in the putative protective effects of OIGly, OIAla and olive oil, we applied pharmacological, electrophysiological and profiling (targeted lipidomics and metatranscriptomics) techniques to adult male mice fed with chow or with isocaloric 7-week diets with high fat content (60% of total daily calories) coming from either olive oil (HFD-O) or lard (HFD-L).

We first found that 7 weeks of treatment with 50 mg/kg of OIGly or OIAla did not alter weekly, final or cumulative food intake and body weight. Conversely, both molecules counteracted the CPP associated with a HPF (choco rice cereal), an effect that was not accompanied by inhibition of preference for HPF and, at least for OIGly, seemed to be exerted via PPAR α activation.

OIAla was previously shown to have a longer-lasting effect (60 min) in rats compared with OIGly due to its higher resistance to hydrolysis⁸. It is thus possible that the mere fact that OIAla is more metabolically stable than OIGly renders the effect of the former compound, at least during the CPP test, less amenable to be counteracted by the PPAR α antagonist. Additionally, OIAla was suggested to produce a stronger and more prolonged inhibition of FAAH than OIGly, and hence cause indirect activation of PPAR α as well as other receptors via elevation of the tissue levels of the endogenous substrates of this enzyme⁸. We may, therefore, hypothesize two effects: one in which OIAla acts directly on PPAR α , and a second one in which inhibition of FAAH, and subsequent indirect activation of more than one receptor by its endogenous substrates, plays a role. It is possible that GW6471, at the dose tested in this study, is unable to counteract the effect of OIAla because this compound, in the CPP test at least, is acting predominantly via the second, FAAH-mediated mechanism. Previous studies in rodents have shown that inhibition of FAAH can indeed impair the reward-related effects of nicotine⁴¹.

Regarding the apparent discrepancy between the absence of effect on HFP preference and the inhibition of CPP by OIGly and OIAla, this may be explained by two hypotheses. Food preference is based on food taste and metabolic/caloric needs. In contrast, the development and maintenance of CPP is based on associative learning between the unconditioned stimulus (US) and the context, which becomes the conditioned stimulus (CS) after pairing with the US. The simplest explanation for our results is that the two compounds block the formation of US-SC conditioning. However, given the current literature using different paradigms and showing that OIGly and OIAla cannot alter taste avoidance learning when administered alone^{8,39,40}, this explanation seems unlikely. Another possibility is that, although they do not alter preference for high-calorie foods, OIGly and OIAla do alter the ability of these foods to acquire incentive value, implying that food consumption might be driven by “homeostatic eating”

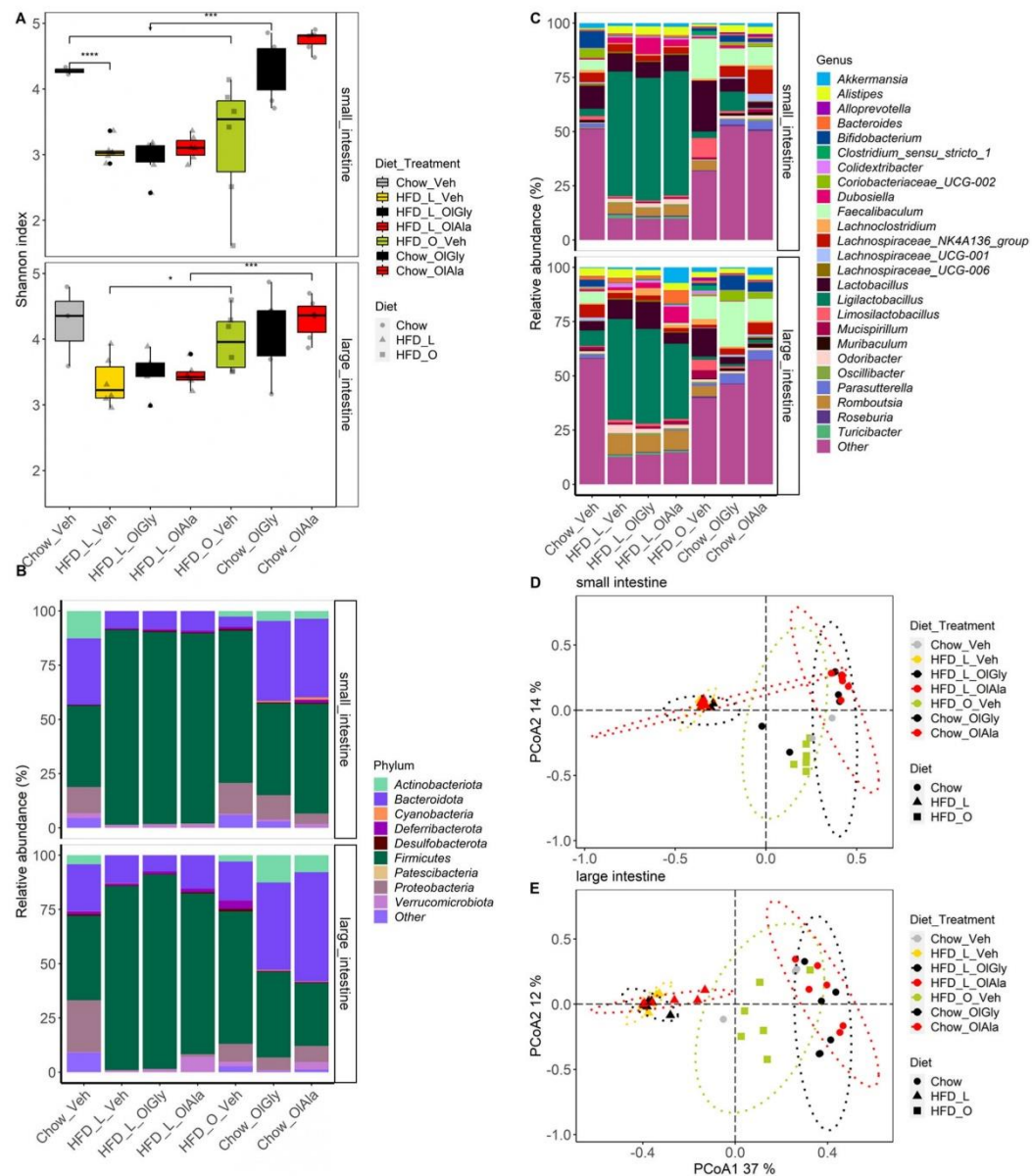


Fig. 5 Olive oil diet counteracts the decrease of microbiota diversity observed under HFD-L and displays a distinct microbiota composition at the interface between HFD-L and chow diets. **A** Shannon alpha diversity displayed for each diet and treatment combined condition. T-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. P-values were corrected for multiple comparisons using the Bonferroni method. Proportion of bacterial (b) phyla, (c) genera in each diet and treatment combined condition. Only the top 25 genera are shown in the figure. **D**, **E** PCoA of bacterial genera based on Jaccard dissimilarity in the small (d) and large (e) intestine. The ellipses were drawn for each Diet-Treatment condition.

rather than “hedonic eating”⁴² and thus HPF never acquires the properties of an US. Homeostatic eating could develop as a result of daily intake of a HFD, which gradually increases energy requirements while promoting weight gain. In humans, “hedonic eating” (and physiological mechanisms that may mediate it) can be defined as a subjective state, but not as actual “food intake”. In

rodents, it has been elegantly shown that the intake of standard chow in food-deprived animals, the motivation to seek HPF, and HPF preference can be independently modulated by different subregions of the nucleus accumbens shell⁴³. The results reported in this study suggest the intriguing hypothesis that it is possible to selectively alter these different processes involved in food

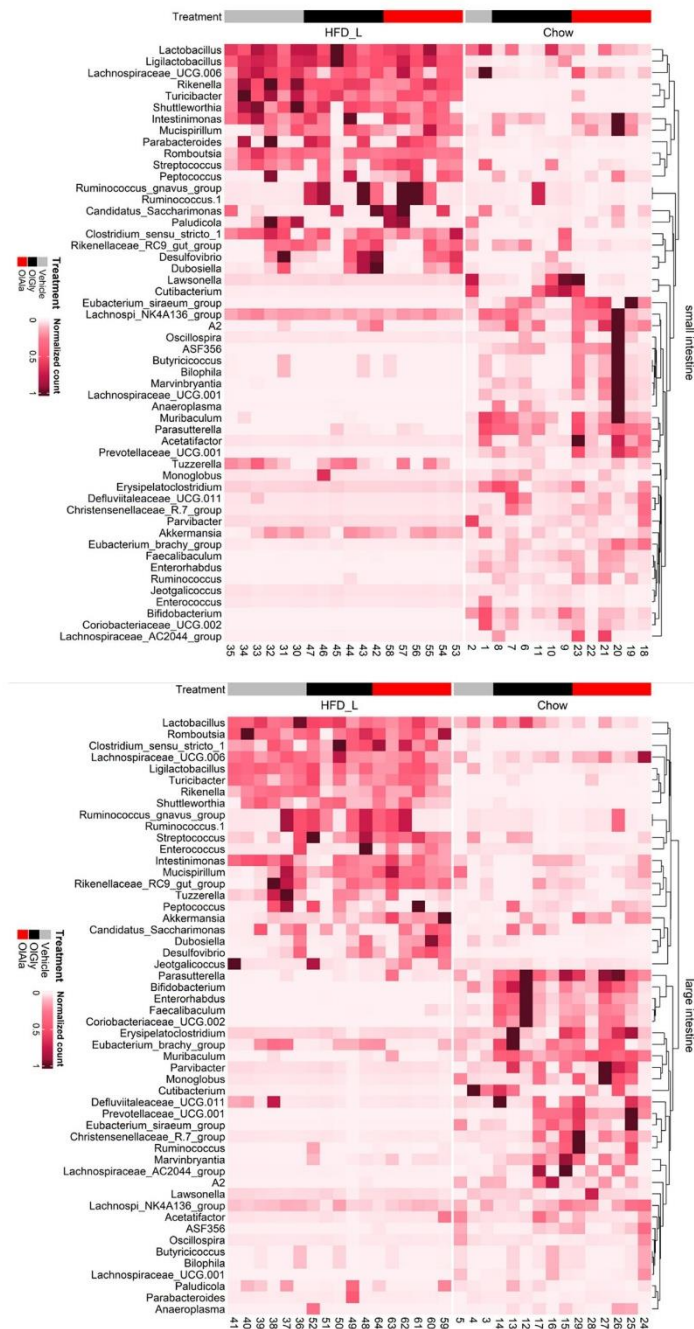


Fig. 6 Diet is the main driver of the overall microbial signature shift across gut regions regardless of the treatment. The heatmaps display the DESeq normalized counts of genera that are significantly shifted between diets chow and HFD-L, and/or treatments using a Wald Test. Columns are reordered according to an Unweighted Pair-Grouped Method using arithmetic Averages (UPGMA) clustering dendrogram at the genus level for each gut region.

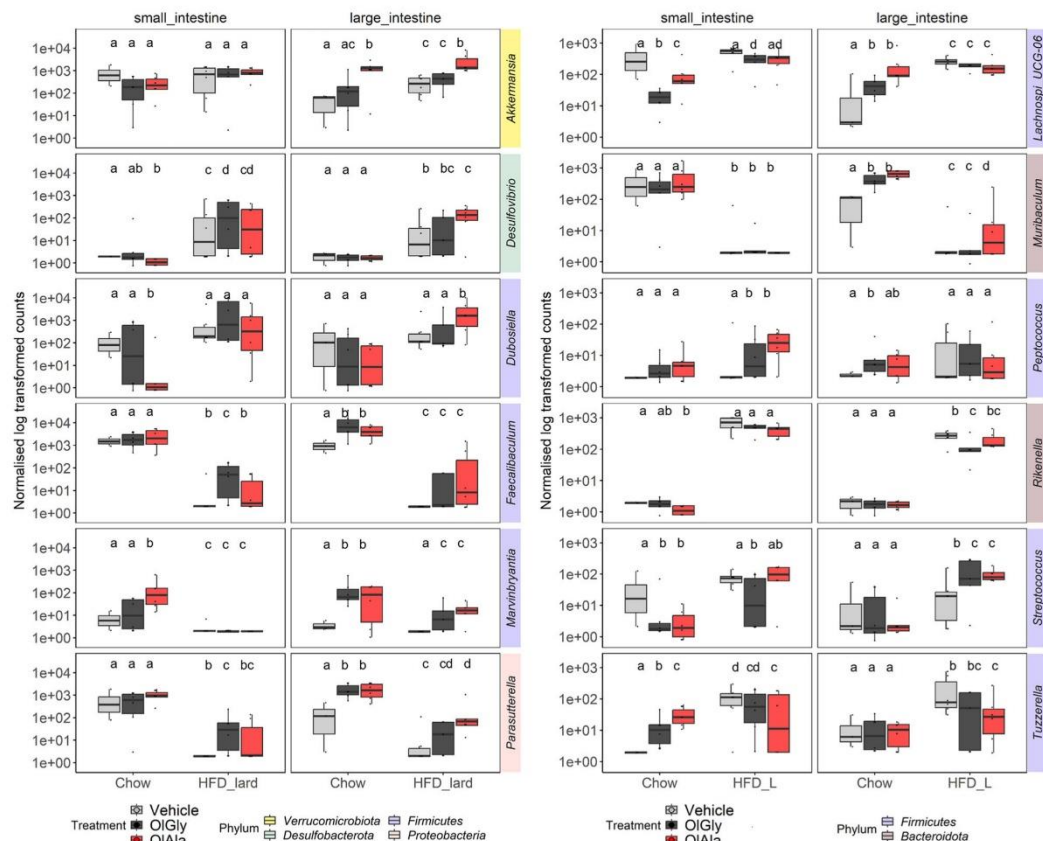


Fig. 7 Treatment with OIGly and OIAla induces similar changes in microbiota taxa under HFD-L, but OIAla produces additional changes in the large intestine. Selection of interesting taxa displaying significant changes in normalized genera level abundance between vehicle vs. OIGly/OIAla treatments and/or between diets chow vs. HFD lard as assessed by DESeq2 analysis in the different gut regions. Pairwise statistical differences per gut region were denoted by letters according to Wald tests. Identical letters indicated no significant differences ($p > 0.05$).

consumption by pharmacological treatments. Future studies using instrumental paradigms selectively targeting each of these three aspects will be necessary to dissect the precise motivational and consumption mechanisms regulated by the two compounds.

Electrophysiological recordings in brain slices of putative VTA-DA neurons, showed that OIGly and OIAla reduce the firing of these neurons, through the activation of PPAR α . It is important to underline the fact that not always there is a direct correlation between the firing rate and the release of dopamine in these neurons (see, as an example, the effect of cocaine on DA neurons⁴⁴). Therefore, the absence of direct data on dopamine release remains a limitation of this study.

OIGly and OIAla chronic treatment reduced cumulative body weight gain and food intake in mice fed on HFD-L, an effect that again was not seen when mice were co-treated with the PPAR α antagonist. Collectively, these results report, to the best of our knowledge, antiobesity and anti-food addictive properties for OIGly and OIAla and, more generally, their efficacy in the treatment of addictive behaviors consequent to a long consumption of HPF.

It is important to note that OIGly and OIAla may act also via peripheral PPAR α , which is highly expressed in duodenal and

jejunal enterocytes^{45,46}. Indeed, activating PPAR α receptors on vagal sensory afferences were shown to reduce fat intake through both epithelial cell-mediated and hypothalamic^{47,48} and striatal⁴⁹ feeding circuits.

Interestingly, the HFD-O diet induced an increase in weekly or cumulative body weight after 7 weeks very similar to chow, despite it being isocaloric with the HFD-L diet, and, accordingly, it slightly decreased cumulative food intake. The analysis of food intake underlines a decrease in these parameters, which could be explained also by a decrease in palatability. On the other hand, it has been reported that in mice the liking of olive oil decreases with concentration⁵⁰. In another study using BALB/c mice, it was reported that mice prefer 16- and 18-carbon unsaturated long-chain fatty acids such as oleic acid at low concentrations (1%)⁵¹. Our HFD-O diet contains 18% oleic acid, the maximum amount of olive oil that can be used for a solid diet. Additionally, although we did not measure energy expenditure changes induced by the diets, the HFD-O may have produced a positive effect on this parameter. Indeed, olive oil has been previously shown to increase energy expenditure in both rodents and humans^{52,53}.

At any rate, we observed that the effects of HFD-O on both food intake and body weight gain were undistinguishable from

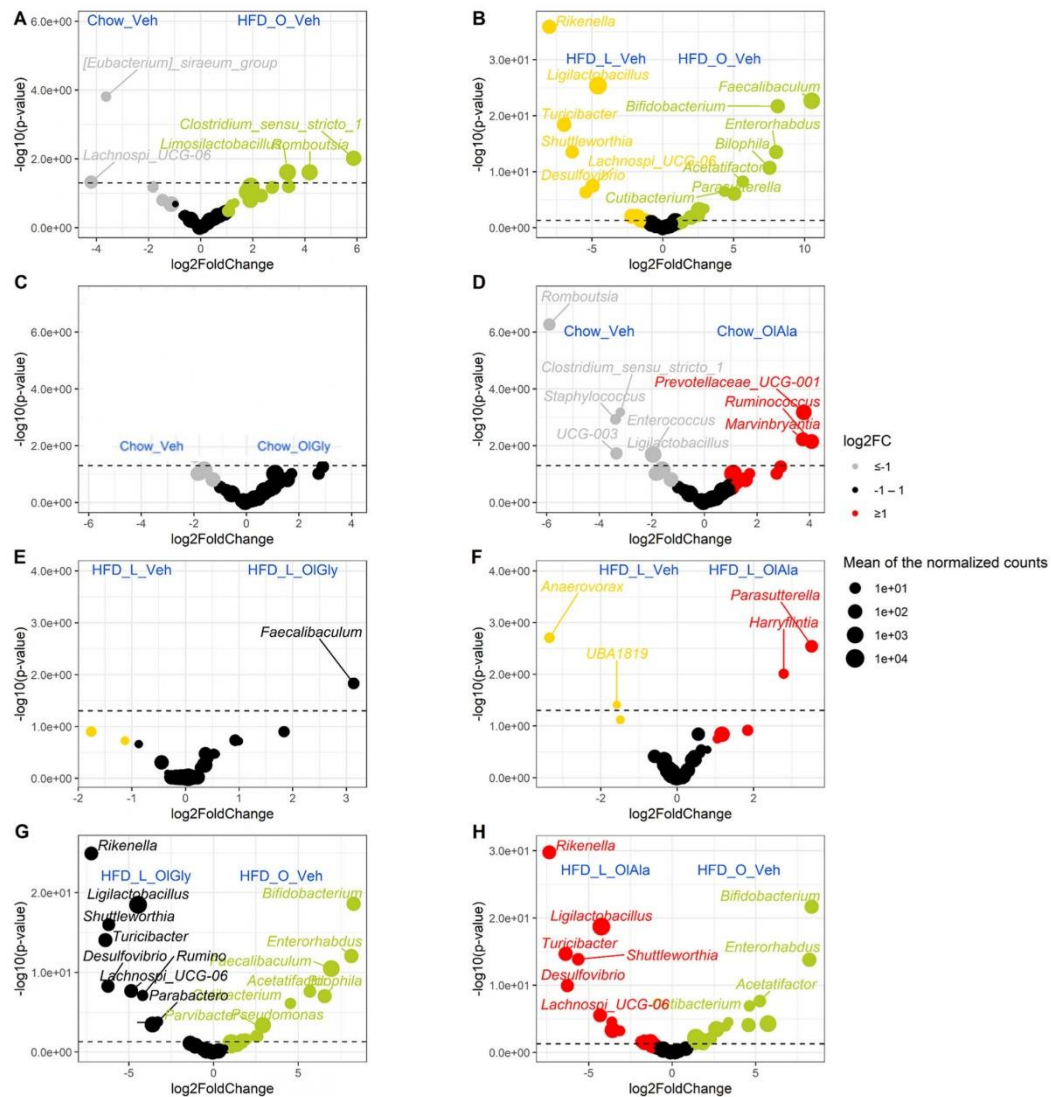


Fig. 8 Treatment with HDF-O OIGly and OIAla shows limited microbiota changes with chow diet while it displays increased contrasts with HFD-L. A positive log₂ fold-change (FC) indicates a stimulation of the genus under the olive oil diet (HDF-O) (in green) while a negative log₂ fold-change indicates a decrease of the genera compared to the chow (in gray) (A), as determined by Deseq2 analysis. Additional paired comparisons have been assessed as following: HFD-L-Veh vs. HFD-O (B), Chow-Veh vs. Chow-OIGly (C), Chow-Veh vs. Chow-OIAla (D), HFD-L-Veh vs. HFD-L-OIGly (E), HFD-L-Veh vs. HFD-L-OIAla (F), HFD-L-OIGly vs. HFD-O (G), and HFD-L-OIAla vs. HFD-O (H). Statistical differences were determined using Wald Tests. The log transformed adjusted *p*-value is displayed on the y-axis and the $\alpha = 0.05$ significance level is indicated by a dashed line.

those observed with an equicaloric HFD-L diet in the presence of either OIGly or OIAla, unless this diet was administered in the presence of the PPAR α antagonist. These findings raise the possibility that the HFD-O diet might act, in part, by providing dietary oleic acid necessary for the formation of endogenous PPAR α agonists, such as OIGly and OIAla, in brain areas involved in reward control. Indeed, by using a targeted lipidomics approach, we found that the concentrations of OIGly were, or tended to be, increased in all four brain areas investigated, including the VTA, shown above to respond to this compound

with a reduction in putative DA neuron firing in a PPAR α -mediated manner. This possibly indicates that the HFD-O, unlike the HFD-L, might counteract the rewarding-associative properties of the excess fat present therein partly by increasing OIGly levels in the brain and hence indirectly activating PPAR α in brain regions deputed to contribute to food addiction. Interestingly, the HFD-L diet decreased, instead, the levels of both OIGly and OIAla in the four analysed brain areas as compared to the HFD-O and/or chow diet, again suggesting that HFDs poor in oleic acid and rich in saturated fatty acids may owe their hedonic

and food intake-inducing properties in part to their inability to induce the formation of endogenous anti-food addiction OIAla and OIGly. This mechanism might be specific for OIAla and OIGly, since the levels of the best known endogenous, oleic acid-derived, PPAR α agonist, OEA, did not increase, following HFD-O, in the four brain areas analysed, in agreement with the proposed selective peripheral role (at the level of the small intestine) of OEA anorexigenic actions⁵⁴. However, to investigate if the mechanism of action of OIGly and OIAla, and their commonalities with that of the HFD-O diet, included also gut-mediated mechanisms, we examined their effects on the intestinal microbiota in both mice fed with chow and HFD-L. We found that the two compounds, unlike the HFD-O diet, did not significantly affect gut microbial diversity. However, OIGly and, particularly, OIAla did produce, in both the small and large intestine, several taxonomic shifts that could be reconciled with their anti-obesity and anti-food addiction actions, particularly in view of recent findings that have suggested a strong association between this latter pathological propensity and the composition of the gut microbiota^{15,26,27,55–57}. In particular, the increase and decrease, respectively, of three taxa that are known to be negatively (*Akkermansia*) and positively (*Streptococcus* and *Lachnospiraceae*) associated with food addiction²⁹, by the two compounds may have played a role in their anti-addictive actions. However, the HFD-O diet, beyond its positive effect on α -diversity, which has been recently associated with low food addiction and body weight in women⁵⁸, seemed to produce stronger, and potentially beneficial, eubiotic effects than OIAla and, particularly, OIGly. As an example, HFD-O significantly increased the relative abundance of *Bifidobacterium* compared to HFD-L-OIGly/OIAla. Accordingly, our clustering analysis clearly indicates that the diet, much more than the treatment with OIGly or OIAla, is the strongest determinant of gut microbiota compositional changes. This was not surprising for at least three reasons: (1) the HFD-O diet, and olive oil in particular, contain several components in addition to triglyceride-esterified oleic acid, such as polyphenols, which are known to affect gut microbiota composition⁵⁹; (2) olive oil could also act as a precursor of mediators other than OIGly and OIAla, and in tissues beyond the brain, such as intestinal OEA, which is known to produce beneficial effects on gut microbiota composition along with reduction of food intake and fat adsorption by the intestine^{60,61}—this could also explain the slight anorexic effects of the HFD-O, which was not observed with OIGly and OIAla; and (3) unlike the HFD-O diet, OIGly and OIAla had to be administered i.p. and not *per os*, and this could have attenuated their effects on the gut microbiota—indeed, in agreement with their stronger effects on the HFD-L mouse gut microbiota, we found that i.p. administration of OIGly and OIAla resulted in a strong increase of the intestinal concentrations of the two compounds (in the case of OIGly comparable to that obtained with HFD-O) only when administered together with the HFD-L. This finding is in agreement with previous reports showing that intestinal fat limits the inactivation by endogenous amidases of long chain fatty acid amides²⁵. Nevertheless, OIGly and, particularly, OIAla, on the one hand, and olive oil, on the other hand, did produce some common alterations in the gut microbiome, such as the small decrease in *Lachnospiraceae* *UCG006* with respect to chow, and the increase of *Parasutterella* and *Faecalibaculum* and the decrease of *Lachnospiraceae* *UCG006* and *Rikenella* with respect to the HFD-L diet. Due to the above-mentioned proposed involvement of these taxa in food addiction or obesity, these changes could have partly contributed to the similar effects of the three treatments on food intake and body weight gain under high-fat feeding.

In conclusion, we have shown here that two eCBome mediators, OIGly and OIAla, inhibit conditioned preference for HPE,

reduce the putative DA neuron firing in the VTA of brain slices, and reduce body weight and food intake in mice under an HFD rich in lard (i.e. mostly saturated fats). These effects appear to be mostly antagonised by PPAR α blockade, which instead increased body weight accumulation in mice under an HFD-O (i.e. richer in monosaturated fats, and oleic acid in particular), thus allowing us to suggest that the effect of the latter diet might be partly mediated by OIGly and OIAla, whose brain and intestinal levels were indeed modulated by HFD-O and HFD-L in opposing manners. Like with the HFD-O diet, the effects of OIAla and OIGly were also accompanied, in both chow-lean and HFD-L-obese mice, by potentially beneficial changes in the intestinal microbiota composition, although this study was not designed to assess the causal role of the gut microbiome in the actions of the two compounds. A parsimonious interpretation of our data is that OIGly and OIAla produce effects on food intake and body weight gain in mice under an HFD similar to those of olive oil, via partially overlapping mechanisms (i.e. PPAR α activation and intestinal microbiota modulation). However, it is important to note that our data do not allow us to exclude that other previously reported peripheral or central PPAR α -mediated mechanisms, proposed to mediate for example the action of the other oleic acid-derived endocannabinoid-like compound, OEA, such as the inhibition of intestinal fat adsorption, adipogenesis and insulin resistance, and the enhancement of satiety and energy expenditure, may mediate the anti-obesity effects of OIAla and OIGly^{62–65}. At any rate, the results of this study warrant further investigation on the capability of olive oil-rich diets, such as the Mediterranean diet, to produce beneficial effects via actions on food reward of oleic acid-derived eCBome mediators.

Methods

Animals and drugs. Experiments were performed following the European Union animal welfare guidelines [European Communities Council Directive of September 22, 2010 (2010/63/EU)] and the Italian Decree n.26/2014, authorization n. 152/2020-PR and n. 2013/0040360. The experiments were performed on 8–13-week-old mice. All the mice were housed in controlled temperature (20–23 °C) and humidity conditions (55 $\pm$ 5%) and fed *ad libitum* with a VFR1 standard diet (Sniff). For the HFD experimental group, C57Bl/6j were fed with an HFD-L where 60% kcal derived from Lard (233HFD-D12492, SAFE-Lab; 31.7%, 3.3% soya oil) for 8 weeks starting from 1 month of life or with an HFD-O where 17% of the total fat derived from olive oil (D12492, SAFE-Lab; lard 14.7%, 17% olive oil, 3.3% soya oil). The fatty acid composition of the two HFD is reported in Supplementary Data 6. Mice were housed one per cage to evaluate food consumption and body weight. A known amount of pellet per cage was positioned and the weight after a week was registered. The drug concentrations used for all the experiments were as follows OIGly and OIAla (50 mg/Kg *in vivo* i.p.; 10 μ M *in vitro*); GW6471 (2 mg/Kg *in vivo* i.p.; 100 nM *in vitro*).

Conditioned place preference. The experimental design is reported in Supplementary Fig. 1A. Animals were recorded using a video camera (PANASONIC WV-BP330) connected to a video-tracking system (ANY-MAZE 7.08, Stoelting, USA). CPP was carried in a plastic apparatus formed by two compartments of the same size (20 cm length, 10 cm width, 15 cm height)⁶⁶. One compartment had a white floor and the walls covered by black stripes and the other was white. During the conditioning session, the compartments remained closed by a removable door. Before starting the experiment mice were free to explore the two compartments for a 30-minutes period and behavior was monitored to calculate the time spent in each compartment to evaluate if

mice showed bias (Fig. 2). We did not observe any preference for one of the two compartments.

During the first 7 days of housing, C57Bl/6j mice at 2 months of age were injected on alternate days. OIGly and OIAla were injected 15–30 minutes before starting the experiment¹². GW6471 was administered 30 minutes before the administration of OIGly or OIAla¹². From the 8th to the 11th day, mice were injected every day, according to the protocol depicted in Fig. 2A. From the 5th day of the experiment, the HFP (choco rice cereals) was introduced in the diet of each experimental group for familiarization. On the 6th day, mice received limited access to food (HPF+standard pellet) to induce ~10–15% weight loss and to increase the mice's motivation to eat during the test. No differences were observed in the body weight between the experimental groups during the different experimental phases (Fig. 2C). On the 8th, 9th, and 10th day mice were conditioned into the CPP setup by performing two sessions per day (Fig. 2A, B). In particular, on the 8th day, mice were confined 15 min in the room containing chow food and 15 min in the other room containing HPF. The distribution of the chow or HPF in the white room or the room with black stripes (Fig. 2B) was equally subdivided and the mice were randomly assigned to one of the two conditions. On the day 9th and 10th, mice were subjected to a double session of conditioning for 30 minutes in the morning and 30 minutes in the afternoon. During these sessions, the connecting door between the room containing the HPF and that containing chow was opened allowing the mice to freely move in the apparatus and freely choose what to eat among the two types of food. On day 11th (CPP₁, Fig. 2A) CPP was tested under an extinction protocol: mice were free to move between the two rooms for a total duration of 30 minutes, no food was administered during this session and the time spent in the two rooms was calculated.

Electrophysiology. Preparation of VTA slices: C57BL/6J 8–9 weeks old of both sexes were used. After anesthesia with an overdose of isoflurane, the mice were decapitated, and the brains were quickly removed. Coronal slices (200 µm) containing the VTA were cut using a Leica VT1000 S Vibrating blade microtome at 3–5 °C in a solution containing (in mM): 87 NaCl, 25 NaHCO₃, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 10 glucose, 75 sucrose, and saturated with 95% O₂ and 5% CO₂. After cutting, we let the slices recover at least 30 min at 35 °C and for 10 min at room temperature in artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 25 NaHCO₃, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, and 1 MgCl₂ (bubbled with 95% O₂–5% CO₂).

Coronal slices were transferred to a recording chamber and continually perfused with gassed ACSF. The recording was performed with a Multiclamp 700B/Digidata1440A system in loose cell-attached configuration. The extracellular recording was performed from visualized putative DA neurons using a Leica DM6000 FS microscope equipped with a WAT-902H Ultimate camera. Recording pipettes pulled using a Sutter P-1000 puller had a resistance of 3–7 MΩ when filled with ACSF. Spontaneous action potentials were detected at 31 °C in voltage clamp (0 mV) lowering the pipette on the neuronal surface and applying a small suction until the series resistance reached a 35–50 MΩ resistance. Putative DA cells were recognized using the following criteria: (a) the anatomic localization and dimension of the cells, (b) the firing rate frequency between 1 and 5 Hz, (c) the action potential duration over 1 ms^{14,22} (Supplementary Fig. 1).

To stabilize the recording configuration, the recording was started 5 min after that the cell-attached configuration was established. One cell for each slice was recorded.

Data were acquired with pClamp 10.4 software (Molecular Devices) and analysed offline with Clampfit 11.1 (Molecular Device), Excel, and GraphPad Prism 8.0.2 (GraphPad Software, USA).

To analyse the variation of the firing rate we plotted the variation in the percentage of the firing rate with respect to the baseline of 10 min calculated as 100%.

LC/MS-MS analysis. Brain and intestinal tissues were frozen in liquid nitrogen immediately after dissection, which took place within 5 min from sacrifice. Tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal deuterated standards as described in⁹. Then, the lipid extract was dissolved in 100 µl of CH₃OH and analyzed by either LC-APCI-MS for OEA quantification, which was calculated based on their area ratio with the internal deuterated standard signal areas⁶⁷ and by LC-MS-IT-TOF (Shimadzu Corporation, Kyoto, Japan) for OIGly, OIAla and *N*-oleoylserine identification and quantification, using multiple reaction monitoring (MRM), as reported previously⁹. The chromatograms of the high-resolution [M-H][−] values were extracted and used for calibration and quantification. LC analysis was performed in the isocratic mode using a Phenomenex Kintex Polar C18 column (50 × 3 mm, 2.6 µm) and CH₃OH/water/formic acid (85:15:0.1 by vol.) as the mobile phase with a flow rate of 0.15 ml/min. Identification of OIGly, OIAla and *N*-oleoylserine was carried out using ESI ionization in the negative mode with a nebulizing gas flow of 1.5 ml/min and curved desolvation line temperature of 250 °C. The most dominant product ion for each lipid class (*m/z* 74 corresponding to glycine and serine loss or [M-H][−] *m/z* 88 corresponding to alanine loss) was selected for MRM.

After having ascertained the presence of OIGly and OIAla in the brain using a high resolution LC/MS-MS method, we proceeded to analyse the intestine of mice treated with OIGly and OIAla, or with the HFD-O, using a more sensitive but low resolution method. These analyses were acquired using a Shimadzu Nexera UHPLC (Kyoto, Japan) coupled online to a triple quadrupole LCMS-8050 (Shimadzu) equipped with an electrospray ionization (ESI) source operating in ESI positive mode. The separation was performed on a KinetexTM Biphenyl C18 100 × 2.1 mm, 2.6 µm (100 Å) (Phenomenex, Bologna, Italy). Flow rate was set to 0.5 mL/min, column oven was set at 45 °C. The mobile phases were respectively: (A) H₂O plus 1 mM CH₃COONH₄ + 0.05% acetic acid v/v and (B) ACN: H₂O 95:5 plus 1 mM CH₃COONH₄ + 0.05% acetic acid. The following gradient was used: 0 min, 20%B, 0–5 min 100%B, 5–7 min, 100% B, returning to 20% B in 0.1 MS source parameters: Desolvation Line (DL) 250 °C, Interface: 300 °C, Block heater 350 °C, Nebulizing (N₂), Drying (N₂), and Heating (Air) gas pressures: 3, 10, 10 L/min. Each analyte was optimized by infusing standard solution at 0.2 mg/mL, multiple reaction monitoring (MRM) mode. The following parameters were used, d4-OIAla: 358.1000 – 94.1500, Q1 prebias –11 V, CE –17 eV, Q3 prebias –15 V; d2-OIGly: 342.0000 – 78.2000, Q1 prebias –19 V, CE –16 eV, Q3 prebias –30 V, OIGly: 340.0000 – 76.2000, Q1 prebias –19 V, CE –15 eV, Q3 prebias –30 V, OIAla: 354.1000 – 90.1000, Q1 prebias –11 V, CE –17 eV, Q3 prebias –15 V. Dwell time was set to 50 ms for all transitions. Samples were injected in randomized order, a pooled quality control sample was inserted during the batch to monitor system stability over time.

Gut microbiota analysis

DNA extraction and sequencing. The DNeasy 96 PowerSoil Pro QIAcube HT (QIAGEN) was used for fecal DNA extraction.

Based on the manufacturer's protocol, up to 200 mg of frozen stool was aliquoted into PowerBead Pro Tubes (QIAGEN, Hilden, Germany), followed by sample homogenization in lysis buffer, and transfer to the QIAcube HT robot (QIAGEN) for further processing. The V3-V4 hypervariable region of 16S rRNA was amplified using primer pairs F (5'-TCGTCGCGCAGCGTCA-GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG- 3') and R (5'-GTCTCTGTTGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC- 3'). According to the Illumina 16S metagenomic sequencing library protocols (Illumina), the Nextera XT Index kit (Illumina Inc) was applied for the amplicon library preparation. The final PCR products were pooled, followed by paired-end sequencing using the MiSeq 600 cycles Reagent Kit V3 by an Illumina MiSeq System (Illumina, San Diego, CA).

Bioinformatics analysis of 16S rRNA gene amplicon data. Demultiplexed raw data files covering all the samples were imported into R studio environment (version 4.2.2, R Core Team)⁶⁸. Amplicon sequence variants (ASVs) were inferred using the DADA2 R package version 1.26.0, applying the recommended workflow⁶⁹. Briefly, sequence reads were first filtered and trimmed with the following parameters: truncQ = 2, truncLen = c(275,195), and maxEE = c(2,2). Filtered reads were denoised using the DADA2 algorithm, which infers the sequencing errors. After removing chimeras, ASVs sequences were subsequently merged and classified using the SILVA database SSU Ref NR 99 release 138 using default parameters⁷⁰. Unassigned taxa and singletons were removed. Sequences detected in <5% of all samples were filtered out with PoolTestR package (0.1.2). Rarefaction curves were constructed to ensure that the samples were sequenced at sufficient depth. To deal with differences in sampling depth, the data were rescaled to proportions for further analysis.

Microbiome statistical analysis. Statistical and microbiome analyses were performed in R (version 4.2.2) using ggplot2 (3.4.0) for data visualization; phyloseq (1.22.3) for exploring microbiome profiles⁷¹; vegan (2.6-4) for computing alpha and beta-diversity indexes⁷²; ggpvr (0.5.0) for means statistics comparisons; DESeq2 (1.38.1)⁷³ for differential analysis of normalized count data between conditions⁷⁴; and ComplexHeatmap (2.14.0) packages. All statistical tests used were two-sided. All statistical tests were followed by multiple-testing correction using the Benjamini-Hochberg method when testing more than two features. Data distribution was assumed to be normal, but if this was not the case, nonparametric testing or data transformation was applied.

Statistics and reproducibility. Statistical analysis was carried out using the GraphPad Prism Software version 8.0.2. Tests were significant when $p < 0.05$. Data are expressed as mean $\pm$ SEM. Sample sizes for experiments were determined based on sample sizes used in similar experiments reported previously in the literature. The statistical test used for each comparison is described in the figure legends. The normality distribution of the data was confirmed using D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov test. When comparing two unpaired groups, data were analyzed by two-tailed Student's *t*-test (parametric) or a two-tailed Mann-Whitney U test (nonparametric). When two paired groups, data were analyzed by Wilcoxon matched-pairs signed-rank test, two-tailed. When comparing three or more groups, data were analyzed by One-way ANOVA/Bonferroni, Kruskal-Wallis/Dunn's tests, or 2-way ANOVA with Tukey post hoc were used to analyze data appropriately. Fisher's LS post hoc tests were run when applicable to identify differences

among groups. Pearson's correlation coefficient was used to analyze the correlation graphs.

Reporting summary. Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Numerical source data are provided in Supplementary Data 1. Raw 16S rRNA gene amplicon sequencing data were deposited under the BioProject accession number PRJNA925185 (NCBI SRA).

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Author contributions

N.F. performed and analyzed the electrophysiological, the CPP and the food intake experiments; C.R., T.K., G.G. performed the experiments on the gut-microbiota, analyzed the data and prepared the figures; B.M., A.L. performed the CPP; E.D.L. conceptualized and supervised the CPP experiments; R.V. performed the synthesis of analytic standards and chemical analysis; F.P. and E.S. performed LC-MS analysis, analyzed the data and prepared the figures; C.S. conceptualized and supervised the experiments on the gut-microbiota; N.F. wrote a draft of the paper and prepared the figures; M.P.M. writing—review & editing the paper; V.D. and L.C. conceptualized the study and wrote the final version of the manuscript; All approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Vincenzo Di Marzo or Luigia Cristino.

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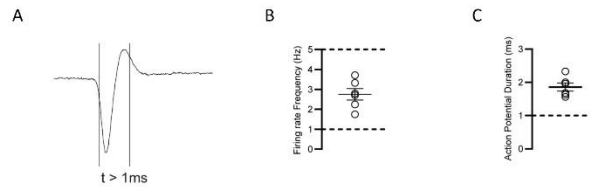
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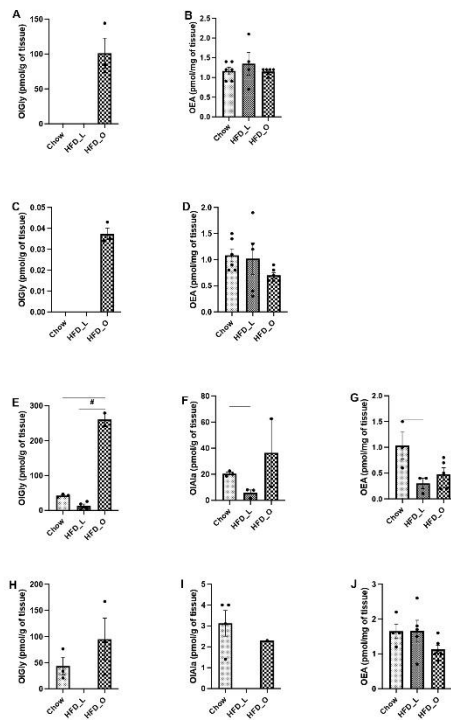
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Supplementary Data

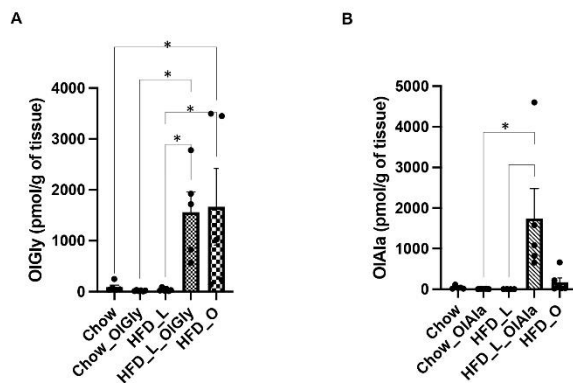


Supplementary Figure 1. Electrophysiological parameters were used to identify putative dopaminergic cells in VTA; (A-C) firing rate was between 1 and 5 Hz, and action potential duration was > 1 ms. OIGly and OIAla reduce the firing rate of putative dopaminergic neurons in the VTA via PPAR α .

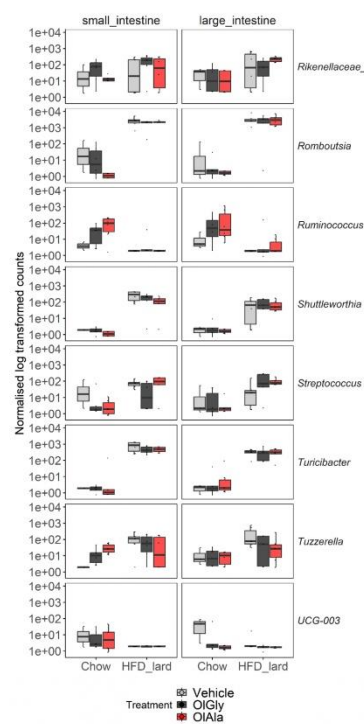
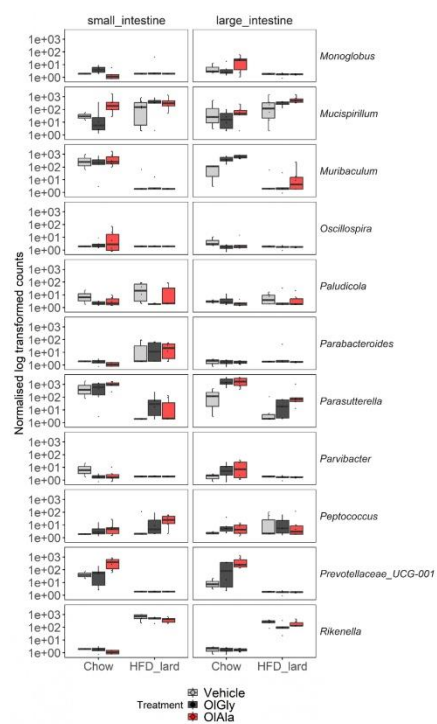


Supplementary figure 2. Oleic acid derivative concentration in brain regions. (A,B) OIGly and OEA levels in the hypothalamus. (C, D) OIGly and OEA levels in the nucleus accumbens. (E-G) OIGly, OIAla and OEA levels in the insula. (H-J) OIGly, OIAla and OEA levels in the ventral tegmental area.

* $p < 0.05$, ** $p < 0.01$ Unpaired t-test, Chow vs HFD_L or HFD_O; # $p < 0.05$ HFD_L vs HFD_O



Supplementary Figure 4. (Part 1) Taxa that are significantly modulated between diets and or treatments in each gut region using DESeq analysis.



Supplementary Figure 4. (Part 2) Taxa that are significantly modulated between diets and or treatments in each gut region using DESeq analysis.

3.2 Task II – “The Short-chain fatty acid acetate modulates Orexin/Hypocretin Neurons: a novel mechanism in gut- brain axis regulation of energy homeostasis and feeding”

The control of appetite and energy homeostasis arises from the integration of peripheral and central signals converging onto specific hypothalamic and mesolimbic circuits, within which the microbiota–endocannabinoidome–brain axis is emerging as a key regulatory node. In this framework, the intestinal microbiota constitutes a complex ecosystem capable of producing a wide range of metabolites, including short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, derived from the fermentation of nondigestible fibers. These metabolites modulate intestinal pH, barrier permeability, immune responses, and energy metabolism, and have been linked both to obesity and to central regulation of caloric intake. Studies in obese and germ-free mouse models have shown that changes in microbiota composition lead to profound alterations in SCFA profiles, in the distribution of bacterial phyla (increased Firmicutes, reduced Bacteroidetes), and in behavioral responses to food stimuli, suggesting that the production of acetate represents a privileged route through which the microbiota influences hypothalamic circuits controlling hunger and satiety.

Within this context, acetate, the simplest and most abundant SCFA, emerges as a key mediator of the gut–brain axis. It can reach the central nervous system, accumulate in specific hypothalamic regions, and modulate circuits that are sensitive to the organism’s energetic state. In particular, orexin/hypocretin neurons of the lateral hypothalamus and perifornical area represent a crucial hub where metabolic, hormonal, and motivational signals are integrated: these neurons are predominantly active during wakefulness, coordinate arousal, motivation, and feeding behavior, and are tightly connected to dopaminergic circuits and to locally expressed endocannabinoid systems. It has been shown that CB1 receptors and other components of the endocannabinoidome are abundantly expressed on afferent synapses targeting orexin neurons, enabling fine retrograde control of glutamatergic and GABAergic transmission via 2-AG and AEA, and thereby predisposing this circuit to respond plastically to changes in lipid and metabolic tone.

At the anatomical level, this work documents the presence of the SCFA receptor GPR43

in periventricular structures surrounding the third ventricle, suggesting an indirect route by which acetate, after entering the cerebrospinal compartment, can modulate GABAergic microcircuits projecting onto orexin neurons rather than acting directly on their somata. Functionally, acetate exerts an anorexigenic effect in both lean and obese mice, reduces the expression of the activation marker cFOS in orexin neurons, enhances synaptic GABAergic transmission, and reshapes hypothalamic endocannabinoid tone, in particular normalizing excessively high 2-AG levels in the ob/ob model, a murine model of monogenic obesity caused by inactivating mutations in the leptin gene, which result in absence of circulating leptin, hyperphagia, a marked increase in adipose mass, and insulin resistance with hyperglycemia and hyperinsulinemia⁹³. In parallel, acetate increases POMC expression in the arcuate nucleus, favoring the recruitment of the anorexigenic POMC–MSH circuit and counteracting the orexigenic drive of the orexin pathway, with an integrated impact on energy balance. This study aimed to ascertain whether, once produced by the microbiota and released into the circulation, acetate reaches the hypothalamus, specifically modifies the activity of orexin/hypocretin neurons, remodels local endocannabinoid tone, and induces a measurable change in food intake, with particular attention to differences between lean and obese animals.



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The short-chain fatty acid acetate modulates orexin/hypocretin neurons: A novel mechanism in gut-brain axis regulation of energy homeostasis and feeding

Nicola Forte^a, Brenda Marfella^{a,b}, Alessandro Nicois^{a,c}, Letizia Palomba^{a,c}, Debora Paris^a, Andrea Motta^a, Maria Pina Mollica^{b,d,e}, Vincenzo Di Marzo^{f,*}, Luigia Cristino^{a,*}

^a Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli, Naples, Italy

^b Department of Biology, University of Naples Federico II, Naples, Italy

^c Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy

^d Centro Servizi Metrologici e Tecnologici Avanzati (CeSMA), Complesso Universitario di Monte Sant'Angelo, 80126 Naples, Italy

^e Task Force on Microbiome Studies, University of Naples Federico II, 80138 Naples, Italy

^f Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, Faculty of Medicine and Faculty of Agricultural and Food Sciences, Université Laval, Québec City, QC, Canada

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ABSTRACT

The short-chain fatty acids (SCFAs) acetate, propionate and butyrate, the major products of intestinal microbial fermentation of dietary fibres, are involved in fine-tuning brain functions via the gut-brain axis. However, the effects of SCFAs in the hypothalamic neuronal network regulating several autonomic-brain functions are still unknown. Using NMR spectroscopy, we detected a reduction in brain acetate concentrations in the hypothalamus of obese leptin knockout *ob/ob* mice compared to lean wild-type littermates. Therefore, we investigated the effect of acetate on orexin/hypocretin neurons (hereafter referred as OX or OX-A neurons), a subset of hypothalamic neurons regulating energy homeostasis, which we have characterized in previous studies to be over-activated by the lack of leptin and enhancement of endocannabinoid tone in the hypothalamus of *ob/ob* mice.

We found that acetate reduces food-intake in concomitance with a reduction of orexin neuronal activity in *ob/ob* mice. This was demonstrated by evaluating food-intake behaviour and orexin-A/c-FOS immunoreactivity coupled with patch-clamp recordings in *Hcrt-eGFP* neurons, quantification of prepro-orexin mRNA, and immunolabeling of GPR-43, the main acetate receptor. Our data provide new insights into the mechanisms of the effects of chronic dietary supplementation with acetate, or complex carbohydrates, on energy intake and body weight, which may be partly mediated by inhibition of orexinergic neuron activity.

1. Introduction

The bidirectional communication between the central nervous system (CNS) and the gastrointestinal tract, modulated by hormones and vagal afferents, results in an intricate anatomical-morpho-functional system that promotes or inhibits food-intake [1]. This interaction forms a network in which signals from the CNS influence the motor and secretory functions of the gastrointestinal tract, which in turn, affect cerebral functions [2,3]. Dereglulation of this gut-brain axis contributes

to the development of eating disorders [4] and metabolic diseases such as obesity [5]. The latter pathological condition is characterized by hyperleptinemia, an increase in peripheral and cerebral tissue inflammation, particularly in the hypothalamic region [6,7], and gut dysbiosis, a disruption of the taxonomic composition and function of the gut microbiota [8,9]. The gut microbiota supports gut-brain communication and contributes to many physiological processes by regulating, among others, immune system function and hormone secretion [10,11]. Obesity is accompanied by gut dysbiosis, with profound functional

Abbreviations: 2-AG, 2-arachidonoylglycerol; DSE, depolarization-induced suppression of excitation; DSI, depolarization-induced suppression of inhibition; eCB, endocannabinoid; eCBome, endocannabinoidome; HFD, high-fat diet; HCRT, Hypocretin; OX-A, orexin A; LH, Lateral Hypothalamus.

* Corresponding authors at: Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli, Napoli, Italy (Luigia Cristino); Université Laval, Québec City, QC, Canada (Vincenzo Di Marzo).

E-mail addresses: vincenzo.dimarzo@criucpq.ulaval.ca (V. Di Marzo), luigia.cristino@icb.cnr.it (L. Cristino).

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changes in the metabolites produced by commensal microorganisms acting on the host [12]. Accordingly, faecal transplantation from obese mice and humans to germ-free mice has obesity-promoting effects [13], although the underlying mechanisms remain unclear [11,14].

The intestinal microbiota metabolize complex indigestible carbohydrates as a primary source of energy. The main products of this fermentative process are short-chain fatty acids (SCFAs) acetate, propionate, and butyrate. The molar ratio of these molecules in healthy rodents is 60:20:20 [15], making acetate the most abundantly produced SCFA in these animals. However, because of gut dysbiosis, profound changes in SCFA levels are found in both the feces and plasma of obese rodents [16,17].

Importantly, the absence and subsequent reintroduction of the gut microbiota in germ-free mice profoundly alters the levels of the lipid mediators and receptors belonging to another major player in energy metabolism and the gut-brain axis, the extended endocannabinoid (eCB) system, or endocannabinoidome (eCBome), in both the intestine and brain [18,19]. Accordingly, some of these mediators and receptors, including the eCBs anandamide and 2-arachidonoylglycerol (2-AG), and/or one of their main molecular targets, the CB1 receptor, are positively correlated with, or negatively regulated by SCFAs, in inflammatory conditions [20,21]; in contrast, the expression of eCB biosynthetic enzymes were found to be inhibited by SCFA-producing bacteria in epithelial intestinal cells [22].

Acetate has been reported to have opposing effects on appetite via the two G-protein-coupled receptors GPR41 (appetite stimulation) and GPR43 (appetite inhibition) [11]. However, data are lacking on the distribution of GPR41/GPR43 in the brain of lean and obese mice and the direct effect of acetate on the brain. Moreover, conflicting results have been reported on the association between acetate and obesity [11]. For example, some studies have indicated a positive correlation between faecal acetate concentrations and obesity, while others have demonstrated a negative relationship [23]. One study reported that acetate suppresses appetite by increasing pro-opiomelanocortin (POMC) mRNA in the hypothalamus [24] and exerts an anti-inflammatory effect that accompanies weight and visceral fat mass loss in male rats with high-fat-diet (HFD)-induced obesity [25]. However, another study showed that acetate induces overeating in rodents consuming an HFD by promoting hyperphagia and weight gain [26]. Contrasting results have also been reported in obese leptin knockout ob/ob mice, a genetic model of obesity that cannot produce leptin, where elevated or unchanged levels of acetate were found in the appendix compared to the same tissue of normal-weight mice [13,27].

The hypothalamus acts as a hub in the gut-brain axis, as it receives and integrates peripheral signals from the gastrointestinal tract as well as other brain regions, and modulates responses to appetite and food-intake [28]. Orexins (OXs, also called hypocretins, HCRTs) are neuropeptides found in two isoforms (OX-A and OX-B) and involved in several essential physiological functions, such as energy homeostasis, sleep-wake cycle, and somatic motor control [29]. OX-A and B are synthesized in neurons located exclusively in the lateral hypothalamus (LH) and projecting to several brain areas [30]. Orexin levels exhibit a robust diurnal fluctuation since they slowly increase during the dark period, or active phase (i.e., ZT13-24), and decrease during the light period, or rest phase (ZT0-ZT12), in the microdialyzed fluid collected from the LH in rodents, according to their physiological implication in arousal and wakefulness states [31].

In HFD and ob/ob obese mice, upregulation of OX signalling occurs, supported by factors such as impaired leptin signalling and subsequent excessive eCB levels and/or misplaced CB1 receptor localization. Indeed, acute or chronic deficiency of leptin signalling associated with fasting or severe obesity leads to activation of OX neurons with increased OX mRNA and protein levels [32,33]. On the other hand, it was demonstrated by our group and others that OX-A induces the biosynthesis of the eCB, 2-AG [34], which plays an important role as a potent modulator of synaptic transmission through activation of CB1

receptors by either enhancing or reducing excitatory or inhibitory drive on neurons, ultimately affecting their excitability [35]. The eCBs are synthesized following neuronal depolarization or metabotropic receptor activation of post-synaptic neurons and retrogradely activate CB1 receptors expressed at presynaptic terminals [35,36]. When CB1 receptors are activated in glutamatergic terminals, they trigger a depolarization-induced suppression of excitation (DSE), which causes a reduction in excitatory synaptic transmission. On the other hand, activation of CB1 receptors in GABAergic terminals leads to depolarization-induced suppression of inhibition (DSI), resulting in the decrease of inhibitory synaptic transmission [36,37]. Both these forms of synaptic plasticity have been shown to regulate OX-A release in different energy states [32,38]. Thus, eCB signalling at CB1 receptors, which is only one of the ways through which the larger eCBome participates in the control of food-intake, is regulated by, and in turn regulates, OX-A signalling in the hypothalamus and other brain areas.

Although a central role of OX neurons in the regulation of feeding behaviour [39] and the control of the gut-brain axis [40] has been demonstrated, data on the potential role of acetate in regulating OX neuron activity, and subsequently eCB levels, in the hypothalamus of lean and obese mice are lacking. To fill this knowledge gap and better understand the mechanisms behind the effect of acetate on appetite and energy homeostasis, we investigated here: (i) the effect of acetate on food-intake in Wt and ob/ob mice and on the functional activity of OX neurons; and (ii) the anatomical distribution and expression of the SCFA receptor GRP43 in the brain of Wt and obese mice before and after administration of acetate.

2. Materials and methods

2.1. Animals and drugs.

The experiments were carried out on 8- to 12-week-old male mice with spontaneous non-sense mutation of the ob gene for leptin (ob/ob, JAX mouse strain, B6.Cg-Lepob/J) and wild type ob gene expressing homozygous siblings obtained by breeding ob gene heterozygous mice and genotyping with PCR. In an experimental subset 8- to 12-week-old male Prepro-orexin-eGFP Tg(Hcrt-eGFP/Rpl10a) JD218Jdd were used.

All experiments were carried out according to the guidelines established by the European Communities Council (Directive 2010/63/EU of 22 September 2010) and approved by the Italian Ministry of Health (authorization n. 152/2020-PR and 589/2018). All the mice were housed under controlled temperature (20–23 °C) and humidity conditions (55 ± 5 %) and received standard chow ad libitum. All animals were used in scientific experiments for the first time and were not previously exposed to any pharmacological treatments. Each figure legend and statistical analysis indicates the number of mice used in each experiment. Since orexin levels exhibit a robust diurnal fluctuation (levels slowly increase during the dark period or active phase (i.e., ZT13–24) and decrease during the light period or rest phase, all the mice were housed under 12 h light:12 h dark cycle, light on at 8:00 PM, i.e. ZT0, and acetate (500 mg/kg) injected for 3 days every 12 h starting from ZT2 and the brain removed 1 h after the last injection for the biochemical NMR and immunohistochemical studies.

2.2. Food-intake measurements

Mice were isolated one per cage and fed ad libitum. Food-intake was measured for 2 days to obtain an average of the food consumed daily. To increase the motivation to eat during the test, lean and obese mice were subjected to 50 % caloric restriction the day before the test. During the first test session the mice were injected with a vehicle and 2 g of food were administered and the food-intake was assessed after 1 h, 2 h, and 4 h after acetate injection (500 mg/kg) at ZT2 [24].

2.3. NMR sample preparation and spectra acquisition

To extract the metabolites of interest, frozen brain tissues were mechanically disrupted. Then, the combined extraction of polar and lipophilic metabolites was carried out by using the methanol/water/chloroform protocol [41]. This procedure separates three phases: water/methanol at the top (polar fraction, containing polar metabolites), denatured proteins and cellular debris in the middle, and chloroform at the bottom (nonpolar fraction, containing lipophilic metabolites). Polar and nonpolar fractions were transferred into glass vials and the solvents were removed by using a rotary vacuum evaporator at room temperature and stored at -80°C until measurements. For NMR analysis, the polar fractions were resuspended in 630 μl of phosphate buffer saline (PBS, pH 7.4), adding 70 μl of $^2\text{H}_2\text{O}$ solution [containing 1 mM sodium 3-trimethylsilyl [2,2,3,3- $^2\text{H}_4$] propionate (TSP) as a chemical shift reference for ^1H spectra] to provide a field frequency lock, reaching 700 μl of total volume. The samples were then transferred to NMR tubes for analysis. One-dimensional (1D) spectra were acquired at 600.13 MHz on a Bruker Avance III-600 spectrometer equipped with a TCI Cryo-ProbeTM fitted with a gradient along the Z-axis, at a probe temperature of 27°C , using the excitation sculpting sequence for solvent suppression [42]. Spectra were referred to internal 0.1 mM sodium trimethylsilyl-propionate (TSP), assumed to be at $\delta = 0.00$ ppm. These 1D spectra were used for multivariate statistical analysis. In addition, two-dimensional (2D) clean total-correlation spectroscopy (TOCSY) and heteronuclear single quantum coherence (HSQC) experiments were acquired to help metabolite identification and signal assignment. 2D spectra were referenced to the lactate doublet assumed to resonate at $\delta = 1.33$ ppm for ^1H , and $\delta = 20.76$ ppm for ^{13}C . Metabolites were assigned to NMR profiles by comparison of chemical signal shifts (^1H and ^{13}C nuclei) with literature data [43] and/or an online database [44].

2.4. Hypothalamic quantitative RT-qPCR

This was carried out using the methods described by Bewick et al. [45]. Quantitative reverse transcriptase PCR (RT-qPCR) was used to study the expression of the different target genes. Total RNA was extracted from the whole hypothalamus using TRIzol (Invitrogen) according to the manufacturer's protocol. All samples were treated with DNaseI (Invitrogen) before the reverse transcription. First-strand cDNAs were prepared using 1 μg RNA and SuperScriptII Reverse Transcriptase (Invitrogen) in the presence of random hexamer and oligo(dT) primers (Promega, Charbonnières-les-Bains, France). The qPCRs were performed using the Light Cycler Fast Start DNA Master SyBR Green I kit (Roche, Meylan, France) in the presence of specific primer pairs selected to amplify small fragments (100–200 bp). PCR products were checked for specificity by measuring their melting temperature. Samples (in duplicate) were quantified by comparison with a standard curve obtained by dilutions of purified-specific cDNAs.

2.5. Measurements of POMC mRNA expression

RNA was extracted from the dissected hypothalamus using an Absolutely RNA micro prep kit from Stratagene (La Jolla, CA, USA). The gene transcription for POMC in the arcuate nucleus (ARC) of the hypothalamus was determined using real-time RT-PCR, and results were expressed as a ratio to the expression of the constitutive gene cyclophilin. The sequences of TaqMan probes and primers for cyclophilin (GenBank accession no. M15933) were: forward primer 5'-CCCACCGTGTCTTCGACAT; reverse primer 5'-TGCAAA-CAGCTCGAAGCAGA-3'; and probe 5'-CAAGGGCTCGCCATCAGCCG-3'. The probe and primers for POMC (assay identification no. Rn00595020_ml) were purchased from Applied Biosystems.

2.6. Lipid extraction and endocannabinoids measurement

Tissue samples were pooled and analyzed using liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS; LabSolution Shimadzu). The eCB 2-AG was extracted from tissues and then purified and quantified as previously described [46]. First, hypothalamic tissues were pooled and homogenized in 5 vol chloroform/methanol/Tris-Cl 50 mM pH 7.5 (2:1:1 by volume) containing 50 pmol of d5-2-AG as internal deuterate standard. Homogenates were centrifuged at $13,000 \times g$ for 16 min (4°C), and the aqueous phase plus debris were collected and four times extracted with 1 vol chloroform. The lipid-containing organic phases were dried and pre-purified by open-bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for 2-AG measurement were obtained by eluting the columns with 9:1 (by volume) chloroform/methanol and then analyzed by LC-APCI-MS. LC-APCI MS analyses were carried out in the selected ion monitoring mode, using m/z values of 384.35 and 379.35 (molecular ions + 1 for deuterated and undeuterated 2-AG). 2-AG levels were therefore calculated based on their area ratios with the internal deuterated standard signal areas. Values are expressed as pmol per mg of wet tissue extracted.

2.7. Immunohistochemistry

Mice injected with acetate (i.p., 500 mg/kg every 12 h for 3 days starting from ZT2) were used for immunohistochemical studies. c-FOS immunohistochemistry was performed by following the analysis of the food-intake behaviours in mice after 1 h of acetate or vehicle injection (500 mg/kg) at ZT2.

Animals were euthanized under isoflurane anaesthesia and perfused transcardially with 0.1 M phosphate buffer saline (PBS) followed by 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brain was removed, fixed and cryoprotected with 30 % sucrose in PB. Brain sections were cut by Leica CM3050S cryostat into 10 μm -thick through the coronal plane, collected in three alternate series and maintained frozen until being processed immunofluorescence.

For immunofluorescence labelling, the brain sections were incubated overnight in a mixture of the following primary antibodies diluted in PB-T (0.3 % PB-Triton): goat anti-OX-A (1:100; SC-8070; Santa Cruz Biotechnology, Inc. Dallas, Texas – U.S.A.); rabbit anti-c-FOS (1:100; SC-52; Santa Cruz Biotechnology, Inc., Dallas, Texas); rabbit anti-GPR43 (1:100; bs-13536R; Bioss Inc, Beijing, China). Then the sections were treated for three hours with a mixture of appropriate Alexa anti-IgGs secondary antibodies (Invitrogen, ThermoFisher Scientific, France) diluted 1:50 in PB-T as Alexa-488 donkey anti-goat (A11055), Alexa-594 donkey anti-rabbit (A21207) and Alexa-488 donkey anti-rabbit (A21206). Sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich S.r.l., Milan, Italy) to detect nuclei, and coverslipped with aqueous mounting medium Aquatex (Merck, Darmstadt, Germany).

Controls of immunolabeling specificity in multiple fluorescence experiments were performed by omission of primary or secondary antibodies or by pre-absorption of primary antibodies with the respective blocking peptides. Immunofluorescence was analyzed by the confocal microscopy Nikon Eclipse Ti2, and images were acquired with the digital camera DS-Qi2 (Nikon) and processed by Image analysis software NIS-Elements C (Nikon, Florence, Italy). $N = 6-10$ z-stacks were collected through each analyzed section every 0.5 μm throughout the area of interest to be processed by the MetaMorph imaging deconvolution software (Leica®, Germany). One focus image of serial Z plane for each channel was selected and merged to realize an optimal multi-channel image. Micrographs were saved in TIFF format and adjusted for light and contrast before being assembled on plates. The brain regions of interest were identified by referencing the Allen Mouse Brain Atlas [47].

2.8. Analysis of the immunohistochemical experiments

For the quantitative evaluation of immunolabeling serial Z plane images were collapsed into two-dimensional images ($n \leq 30$ planes with an increment 0.5 μm). The initial parameters assessed included the measurement of the percentages of c-FOS co-localization in OX-A immunoreactive areas of LH, an unbiased physical disector-based counting of double immunoreactive puncta performed following the protocols described in Bolte and Cordelières [48]. Additionally, we determine the percentage of GPR43-labeled cells in LH and around the 3 V, defined as the area expressing GPR34 immunolabeling. All analyses were conducted by an observer blinded to the experimental design.

2.9. Electrophysiological recordings

Tg(Hcrt-eGFP/Rpl10a)JD218Jdd 8–12 weeks old mice were deeply anesthetized with isoflurane and the brain was removed. LH containing coronal slices (250 μm) were cut using a Leica VT1000 S Vibrating blade microtome at 3–5 $^{\circ}\text{C}$ in a solution composed of (in mM): 87 NaCl, 25 NaHCO_3 , 2.5 KCl, 0.5 CaCl_2 , 7 MgCl_2 , 10 glucose, 75 sucrose, and saturated with 95 % O_2 and 5 % CO_2 . After cutting, we let the slices recover 1 h at 37/38 $^{\circ}\text{C}$ and 30 min at RT in artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 25 NaHCO_3 , 10 glucose, 2.5 KCl, 1.25 NaH_2PO_4 , 2 CaCl_2 , and 1 MgCl_2 (bubbled with 95 % O_2 -5 % CO_2). Coronal slices were transferred to a recording chamber and continually perfused with gassed ACSF. Hcrt-eGFP neurons were visualized using a 470 nm light source (CoolLED pE-100).

The recording was performed with a Multiclamp 700B/Digidata1440A system and a Leica DM6000 FS microscope equipped with a WAT-902H Ultimate camera. Pipette, pulled using a Sutter P-1000 puller, had a resistance of 3–7 $\text{M}\Omega$ when filled with ACSF/KCl or K-gluconate solution. Data were acquired at 10 kHz sample frequency and filtered at 2 kHz with a low-pass Bessel filter.

Spontaneous action potentials were detected in loose cell configuration in voltage clamp (0 mV) lowering the pipette on the neuronal surface and applying a small suction until the series resistance reached a 35–50 $\text{M}\Omega$ resistance. In an experimental subset the inhibitor of GPR43-GLPG0974 (Tocris) (100 nM) was used, while to inhibit CB1R was used AM251 (Tocris) at 4 μM . The inhibitory synaptic activity was recorded in whole cell patch clamp configurations ($V_c = -60$ mV) using a high chloride intracellular solution containing (in mM): 126 KCl, 4 NaCl, 1 MgSO_4 , 0.02 CaCl_2 , 0.1 BAPTA, 15 glucose, 5 HEPES, 3 ATP, and 0.1 GTP. pH 7.3 with KOH, 290 mOsm/L. Spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded in the presence of 50 μM D-(2R)-amino-5-phosphonovaleric acid (D-APV) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris Bioscience, Ellisville, MO), to inhibit NMDA and AMPA receptors.

The excitatory synaptic activity was recorded using a K-gluconate-based intracellular solution containing (in mM): 126 Kgluconate, 4 NaCl, 1 MgSO_4 , 0.02 CaCl_2 , 0.1 BAPTA, 15 glucose, 5 HEPES, 3 MgATP , and 0.1 NaGTP , pH 7.3, 290 mosmol/L. Spontaneous excitatory post-synaptic currents (sEPSCs) were recorded in the presence of 30 μM Bicuculline, 5 μM CGP55845 (Tocris Bioscience, Ellisville, MO) to inhibit GABAA and GABAB receptors respectively.

Data were acquired with pClamp 10.4 software (Molecular Devices) and analyzed offline with Clampfit 11.2 (Molecular Device), Excel, and GraphPad Prism 8.0.2 (GraphPad Software, USA).

2.10. Analysis of electrophysiologic data

To analyze the variation of the firing rate in the presence of acetate we plot the firing rate relative to the baseline of 5–10 min calculated as 100 %. DSI and DSE were examined by recording spontaneous inhibitory post-synaptic currents (sIPSCs) and spontaneous excitatory post-synaptic currents (sEPSCs) respectively ($V_c = -60$ mV) for 10 sec. Subsequently, a depolarization step of 5 sec duration at 0 mV was applied to

the recorded Hcrt-eGFP, followed by the sIPSCs recorded for 20 sec at -60 mV. We reported the percentage changes in frequency and amplitude from the baseline calculated in the 5 sec bin before and after the depolarization step.

2.11. Statistic

Statistical analysis was conducted using GraphPad Prism Software version 8.0.2. Tests were considered significant when $p < 0.05$. Data are presented as mean $\pm$ SEM. Sample sizes for experiments were determined based on those used in similar experiments previously reported in the literature. The data distribution was analyzed using the D'Agostino-Pearson test. One-sample t-tests were used to analyze comparisons of a single group relative to its baseline. For unpaired group comparisons, data were analyzed using a two-tailed Mann-Whitney *U* test. Paired group comparisons were analyzed using the Wilcoxon matched-pairs signed-rank test (two-tailed). When comparing three or more groups, data were analyzed using One-way ANOVA/Bonferroni, Kruskal–Wallis/Dunn's tests, or 2-way ANOVA with Sidak's post hoc.

3. Results

3.1. Acetate reduces food-intake less efficiently in ob/ob mice than in Wt mice

SCFA levels were measured by NMR-based analysis of hypothalamic brain extracts from adult Wt and ob/ob mice fed ad libitum. In line with the increment of acetate metabolism reported in the adipose tissue of obese mice [49,50], we observed a decrease of acetate concentrations in the hypothalamus of ob/ob mice compared with lean Wt control, although propionate concentrations remained unchanged in the same range, and butyrate was not detectable (Fig. 1A and 1B).

Caloric restriction reduces acetate levels [51], whereas acute intraperitoneal (i.p.) acetate injection reduces food-intake in lean mice [24]. To test if acetate (i.p. 500 mg/kg) produces an anorectic effect in obese ob/ob mice we measured food intake at 1, 2, and 4 h from acetate injection in ob/ob and Wt mice after overnight food deprivation.

We found a slight but non-significant tendency of ob/ob mice to eat more than Wt mice after vehicle injection (Fig. 1C). Acetate injection decreased food-intake in both Wt (Fig. 1D) and ob/ob mice (Fig. 1E). However, this effect lasted up to 4 h in lean Wt mice in comparison to 2 h in ob/ob mice (Fig. 1F), suggesting an increment of acetate degradation, or a reduction of its actions, in obese mice. Therefore, hypothalamic acetate concentrations were quantified by NMR-based analysis in vehicle- or acetate-treated mice after 1, 2 and 4 h from injection. An increase of acetate levels was found in the brain of Wt mice at 1 h and 2 h after acetate treatment, while no differences were observed in the obese mice at different time-point of acetate injection (Fig. 1G), indicating increased acetate degradation in ob/ob mice.

3.2. Acetate modulates c-FOS expression in orexin neurons, GPR43 expression and eCB levels in obese mice

Among the brain regions, the hypothalamus has the highest efficiency in acetate uptake [24]. Several mechanisms have been proposed to mediate the anorectic effect induced by acetate [24,25], but no data have been reported regarding the possible role of OX neurons. Consistent with previous observations reporting the enhancement of orexigenic activity in the brains of obese mice [32,33], here we observed increased c-FOS expression in OX-A neurons from ob/ob mice vs lean Wt mice (Fig. 2A and 2B). Injection of acetate (every 12 h for 3 consecutive days; 500 mg/kg i.p.[24]) reduced the percentage of c-FOS/OX-A immuno-coexpressing neurons in both Wt and ob/ob mice (Fig. 2A and 2B).

To investigate if acetate was able to affect OX-A neurons by acting at the selective SCFA receptors GPR43 [52] we performed an

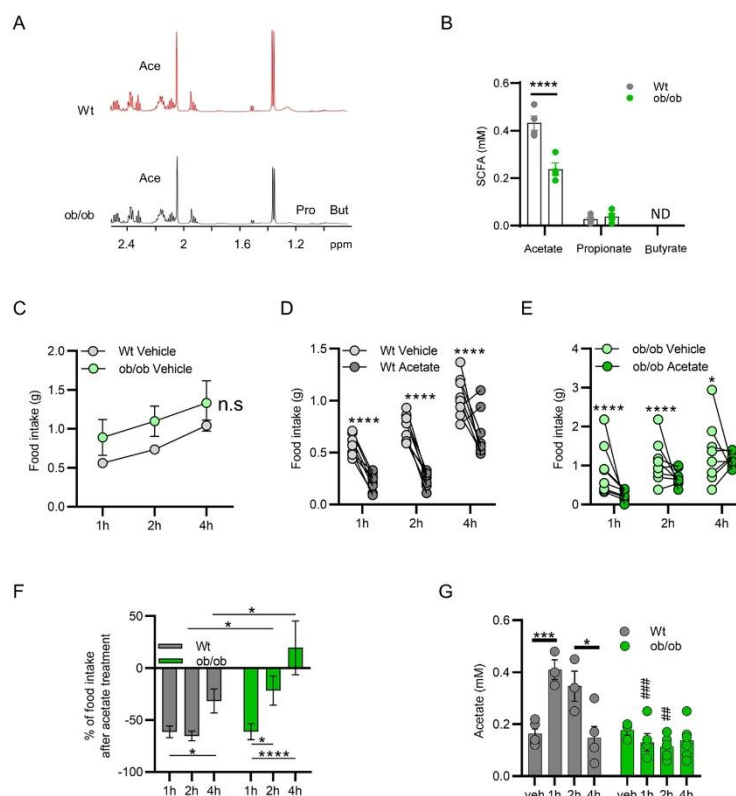


Fig. 1. Acetate is less effective at reducing food-intake in ob/ob mice compared to Wt mice. (A) 600-MHz NMR spectra of the high-field regions of the brain extract from Wt and ob/ob mice. The regions contain the acetate (Ace) signal; Pro, propionate and But, butyrate. (B) NMR quantitative analysis of SCFAs concentration in the hypothalamus of obese and Wt mice. ****, $p < 0.0001$, two-way ANOVA and Sidak's multiple comparisons test. (C) Data showing food-intake in Wt and ob/ob mice treated with vehicle. (D) Effect of acetate injection (i.p. 500 mg kg⁻¹) on food-intake in Wt mice. N = 8 mice, ****, $p < 0.0001$, Two-way ANOVA and Sidak's multiple comparisons tests. (E) Effect of acetate injection (i.p. 500 mg kg⁻¹) on food-intake in ob/ob mice. ****, $p < 0.0001$, two-way ANOVA and Sidak's multiple comparisons test. (F) Percent reduction of food-intake after acute acetate injection in Wt and ob/ob mice. *, $p < 0.05$, ****, $p < 0.0001$, two-way ANOVA and Sidak's multiple comparisons test. (G) NMR quantitative analysis of acetate concentration in the hypothalamus of obese and Wt mice in the vehicle and after 1 h, 2 h and 4 h post-injection. ****, ### $p < 0.0001$, ## $p < 0.01$ and *, $p < 0.05$, two-way ANOVA and Sidak's multiple comparisons test; ## = Wt, 1 h vs ob/ob, 1 h; # = Wt, 2 h vs ob/ob, 2 h.

immunohistochemical examination of the immunoreactivities of these two proteins in the hypothalamus of Wt and ob/ob mice, after vehicle or acetate injection. No colocalization was observed with GPR43 immunoreactivity in OX-A-immunoreactive cell bodies or processes, in both Wt and ob/ob mice (Fig. 2C) whereas expression of GPR43-immunoreactivity was mainly detected in the epithelia lining the 3rd ventricle, more intensely in ob/ob than in Wt mice (Fig. 2C). Acetate injection reduced both the intensity and the number of GPR43-positive cell profiles in both Wt and ob/ob mice (Fig. 2C).

Since the eCB 2-AG is both a potent modulator and a product of the activity of OX neurons [32,53], and SCFAs have been proposed to inhibit the expression of eCB biosynthetic enzymes [22], or of CB1 receptors [21], we quantified the levels of 2-AG in the hypothalamus of Wt, Wt + acetate, ob/ob and ob/ob + acetate treated mice by LC-APCI-MS. As expected, we observed an increase of 2-AG levels in ob/ob compared with Wt mice. Acetate treatment resulted in a reduction of 2-AG levels in ob/ob mice, whereas in Wt mice a trend towards an increase was observed (Fig. 2D).

3.3. Acetate reduces the firing of orexin neurons by increasing GABAergic transmission

To test if acetate directly modulates the firing rate of OX neurons, loose cell-attached recording was performed in Hcrt-eGFP neurons from lean mice. Acetate was tested at a concentration of 10.5 μ M, corresponding to the maximal concentration of acetate found in the brain after an i.p. injection of 500 mg/kg [24]. Following a 10 min acetate treatment, a decrease in the firing rate was observed (Fig. 3A, B).

To better understand the mechanism by which acetate reduces the firing of OX neurons, the amplitude and frequency of sIPSCs were measured by patch clamp recordings on Hcrt-eGFP cells in Wt mice, in sections treated with acetate (10.5 μ M) and acetate plus the GPR43 antagonist GLPG0974 (100 nM) [54]. In agreement with the decreased firing mentioned above, a significant increase in the frequency of sIPSCs was observed in acetate treated slices (Fig. 3C, D), whereas no differences were observed in the amplitude (Fig. 3E). This effect was prevented by GLPG0974.

Subsequently, we examined the effect of acetate on glutamatergic transmission at OX-A neurons. When comparing the frequency and

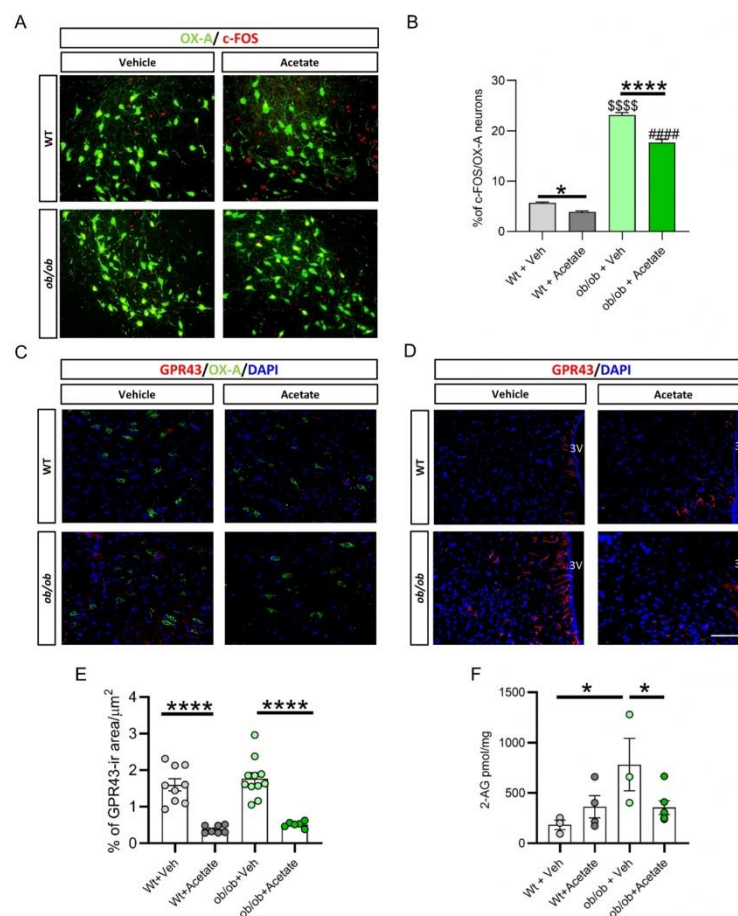


Fig. 2. Acetate modulates c-FOS expression in orexin neurons, GPR43 expression and eCB levels in obese mice. (A) c-FOS/OX-A double immunolabeling in the LH of Wt and ob/ob mice treated with vehicle or acetate. Hypothalamic leptin signal deficiency in ob/ob mice is accompanied by enhanced c-FOS expression in OX-A neurons. Acetate treatment (500 mg/kg i.p. for 1 h starting from ZT2) reduces c-FOS expression in OX-A neurons, both in Wt and ob/ob mice. Scale bar: 100 μm . (B) Bar graph showing the percentage of c-FOS/OX-A positive neurons in Wt and ob/ob mice after 1 h of injection with vehicle ($Wt_{veh} = 120 \pm 8$ cells) or acetate, calculated on the total of OX-A-positive neurons. Data are means $\pm$ SEM; $n = 150$ cells per group, $n = 3$ mice per group. * $p < 0.05$, **** $p < 0.0001$, two-way ANOVA and Sidak's multiple comparisons test. * = Vehicle vs acetate, \$ = Wt vehicle vs ob/ob vehicle, # = Wt acetate vs ob/ob acetate. (C) Representative coronal section of LH showing GPR43 and OX-A immunoreactivity in Wt and ob/ob mice. No colocalization was observed in OX-A-immunoreactive cell bodies or dendritic spines with GPR43 immunoreactivity. (D) Representative coronal sections of the similar rostrocaudal position of the hypothalamic nuclei showing GPR43 labelled fibres and puncta in Wt and ob/ob mice. Elevated GPR43-immunoreactive expression occurs in untreated Wt and ob/ob mice. (E) Bar graph showing the percentage of GPR43 immunoreactivity/ μm^2 from 100 μm^2 of an ROI area around the perifornical fornix as a reference point for vehicle- or acetate-injected Wt mice and vehicle- or acetate-treated ob/ob mice. **** $p < 0.0001$, two-way ANOVA and Sidak's multiple comparisons test. (F) Bar graph of hypothalamic 2-AG levels in Wt + Veh, Wt + acetate, ob/ob + Veh and ob/ob + acetate mouse groups. * $p < 0.05$, two-way ANOVA and Sidak's multiple comparisons test.

amplitude of sEPSCs no changes were observed in the control, acetate, and acetate + GLPG0974 groups (Fig. 3F, G, H).

3.4. Acetate increases depolarization-induced suppression of excitation onto orexinergic neurons

Because acetate showed the ability to modulate hypothalamic 2-AG levels, the eCB-mediated DSE of sEPSCs was examined in Hcrt-eGFP neurons from lean mice. A baseline of sEPSCs ($V_c = -60$ mV) was recorded for 15 sec, then a 5 sec depolarization step was applied at 0 mV, and finally, the sEPSC frequency was measured for 20 sec at -60 mV (Fig. 4A). We report the percent changes in frequency and amplitude

from baseline, calculated in the 5-sec bin before and after the depolarization step. Analysis of DSE in OX neurons showed that the sEPSC frequency variation in comparison to the baseline had the same trend in the three groups (Fig. 4B), whereas the decrease of sEPSC amplitude lasted longer in the acetate- and acetate + GLPG0974-treated slices (Fig. 4C). The induction of 2-AG production and release by our protocol of DSE was confirmed by using the CB1R antagonist AM251, which completely abolished the reduction in frequency of sIPSCs observed after the depolarizing step (Fig. 4D).

On the other hand, the data obtained from the DSI analysis was controversial since we observed a significant decrease in the frequency of sIPSCs, which lasted 15-sec in control and 20-sec in acetate and

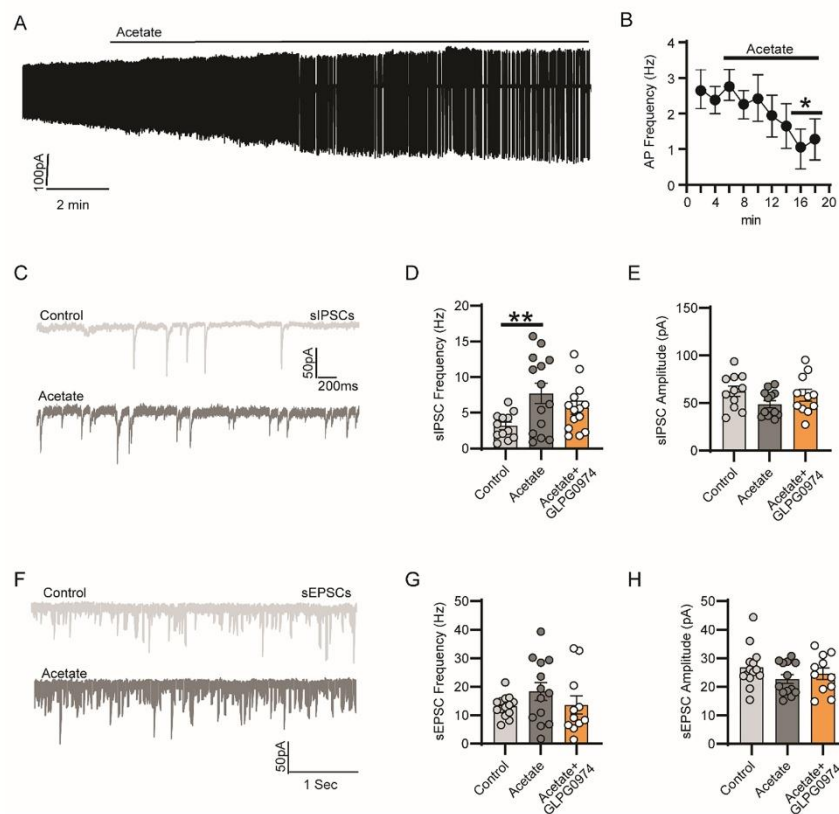


Fig. 3. Acetate decreases the activity of orexin neurons by enhancing GABAergic transmission. (A) Representative trace and scheme of cell-attached recording in Hcrt-eGFP neurons in the control condition and during the application of acetate 10.5 μM. (B) Time course of Hcrt-eGFP neuron action potential frequency before and during acetate treatment. * $p < 0.05$, two-tailed paired Wilcoxon's signed-rank test. $n = 9$ from 4 mice. Two-tailed paired Wilcoxon's signed-rank test. (C) Representative traces of sIPSCs were recorded in Hcrt-eGFP lean mouse slices in control and acetate (10.5 μM) groups. (D) Bar graph of sIPSC frequency recorded in untreated, acetate and acetate + GLPG0974 (100 nM), ** $p < 0.01$, Brown-Forsythe and Welch ANOVA test, 3 mice per group. (E) Bar graph of sIPSC amplitude recorded in control, acetate and acetate + GLPG0974 groups. (F) Representative traces of sEPSCs recorded in Hcrt-eGFP lean mouse slices in control and acetate 10.5 μM groups. (G) Bar graph of sEPSC frequency recorded in control, acetate and acetate + GLPG0974 groups (100 nM). (H) Bar graph of sEPSC amplitude recorded in control, acetate and acetate + GLPG0974 groups.

acetate + GLPG0974 (Fig. 5A, B). Conversely, the amplitude of sIPSC was enhanced after the 5-sec depolarization step in both acetate and acetate + GLPG0974 groups vs the control group (Fig. 5C). Finally, we tested if the CB1R inhibition was sufficient to block the ability of acetate to reduce the firing rate of OX-A neurons. We still detect a reduction of the firing rate after acetate treatment in the hypothalamic slice of Hcrt-eGFP mice pretreated with CB1R antagonist AM251 (4 μM) (Fig. 5D, E, F).

A parsimonious interpretation of these data, and those reported in the previous section, is that acetate reduces orexin neuron firing in lean mice via a mechanism that is mostly GPR43-dependent and CB1-independent.

3.5. Acetate and orexin modulate POMC mRNA levels in opposing manners

It has been suggested that the anorectic effects of acetate administration are mediated by a change in hypothalamic neuropeptides and in particular by an increase in POMC and a decrease in NPY and AgRP via an increase in malonyl-CoA concentrations [24]. However, POMC

neurons are also inhibited by OX neurons [55]. We observed here that injection of OX in mice negatively modulated *Pomc* mRNA levels, this effect being counteracted by SB334867, an orexin-1 receptor inhibitor (Fig. 6). Injection of acetate instead enhanced POMC mRNA levels, in agreement with Frost et al. [24], and co-injection with OX could only reduce the extent of this effect, possibly due to additive opposing effects (Fig. 6). These data, together with those described in the previous sections, suggest that acetate might enhance POMC neurons also by inhibiting OX signaling.

4. Discussion

SCFAs have demonstrated diverse effects, including anti-inflammatory, anti-obesity, and neuroprotective properties [56], but their precise mechanism of action at the cerebral level remains poorly understood. The present study was based on prior observations indicating that acetate diminishes food-intake and increases POMC-derived peptide release [24]. Our investigation focused on the effects of acetate supplementation on OX-A neurons, a cluster of cells situated in the LH crucial for energy homeostasis. Through a multidisciplinary approach,

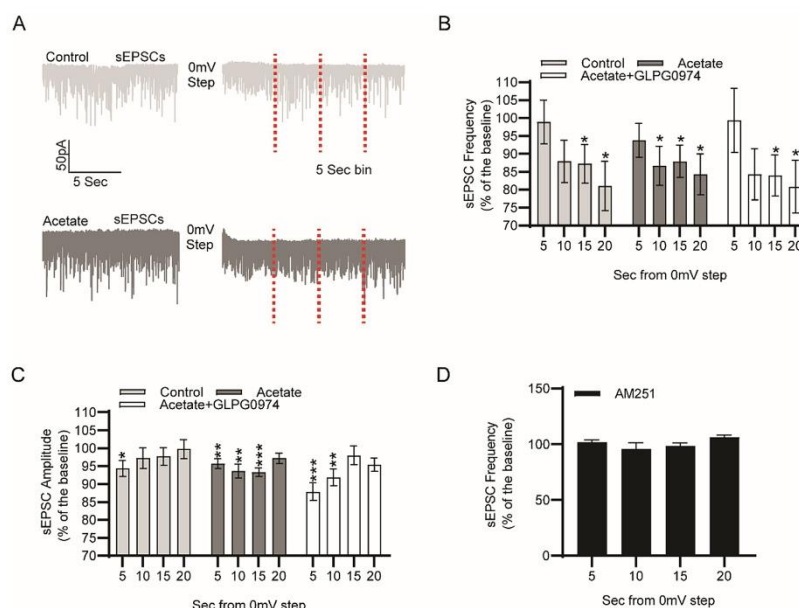


Fig. 4. Acetate enhances DSE onto OX-A neurons. (A) Representative traces of the sEPSC-DSE in untreated and acetate 10.5 μ M treated slices. After 10 sec of baseline at -60 mV, Hert-eGFP neurons were depolarized for 5 sec at 0 mV and then clamped at -60 mV for 20 sec. Vertical red lines indicate the 5 sec bins. (B) Percentage changes of sEPSC frequency relative to 100 % baseline in the control (12.8 ± 1.12 Hz at $t_{5\text{sec}}$), acetate (16.66 ± 2.6 Hz at $t_{5\text{sec}}$) and acetate + GLPG0974 (13.35 ± 2.9 Hz at $t_{5\text{sec}}$) groups. The comparison of each group at a given time point compared to a 100 % baseline was performed using one sample *t*-test. (C) Percentage changes of sEPSC amplitude relative to 100 % baseline in the control (25.33 ± 1.7 pA at $t_{5\text{sec}}$), acetate (21.68 ± 1.4 pA at $t_{5\text{sec}}$) and acetate + GLPG0974 (21.27 ± 1.5 pA at $t_{5\text{sec}}$) groups. The comparison of each group at a given time point compared to the baseline was performed using a one-sample *t*-test, and the intergroup analysis was performed using two-way ANOVA and Sidak's multiple comparisons test. $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$. (D) Percentage changes of sEPSC frequency relative to 100 % baseline (14.10 ± 2.1 Hz) in slices preincubated with AM251 (4 μ M).

our findings suggest that the anorexigenic impact of acetate is partially attributable to the reduction of OX-A neuronal activity in both Wt and ob/ob mice.

Obesity induces alterations in acetate metabolism. Despite prior reports indicating increased (or unchanged) concentrations of intestinal acetate in obese mice [13,27], our study reveals a diminished concentration of this molecule in the hypothalamus of obese mice. These data are consistent with an increase in acetate metabolism in adipose tissue in obesity [49,50]. Notably, while acetate injection induces an anorectic effect within the initial hour in both Wt and ob/ob mice, this effect diminishes in obese mice, likely due to accelerated acetate metabolism by adipose tissue [49,50]. Accordingly, while we observed an increment in the acetate concentration in the brain of Wt mice at 1 h and 2 h post-injection we failed to observe any variation of acetate concentration in the brains of ob/ob mice.

A limitation of our study arises from the fact that the protocol for measuring food-intake does not adhere to the circadian rhythm of mice. However, we opted for this approach in line with the literature [24]. Animals were subjected to caloric restriction for 12 h before testing, to enhance their motivation to eat during the test despite they are in resting phase. Nonetheless, the data obtained remain consistent with existing literature on the effects of acetate on food-intake and with published findings implicating orexin neurons in the regulation of the dopaminergic system and motivation in both lean [57,58] and in ob/ob mice [59].

Furthermore, we report here the influence of acetate on the orexinergic system, presenting a novel mechanism for the regulation of food-intake. Our findings support the notion that acetate reduces appetite by entering in the glutamate-glutamine cycle and supporting GABAergic neurotransmission directly onto OX neurons [24,60]. Our

electrophysiological recordings in hypothalamic brain slices from Hert-eGFP mice demonstrate that acetate treatment triggers an increase in sEPSC frequency via the GPR43 receptor without affecting glutamatergic neurotransmission. This effect is mediated by the network surrounding the OX cell since no co-immunolocalization was found between orexin neurons and the GPR43 receptor. On the other hand, GPR43 does not seem to be expressed in astrocytes, the other source of glutamine fundamental in the GABA synthesis [61]. Moreover, we have shown that chronic injection of acetate modulates the levels of GPR43 in the brain, which is indicative of receptor activation followed by desensitization. The expression of GPR43 and its precise role in enhancing GABA transmission remain to be fully determined.

We cannot exclude the possibility that acetate modulates neuronal activity of OX-A neurons via other mechanisms, such as by modulating K^+ conductance, as has been observed in the colon [62,63]. Notably, the increase in POMC reported in the presence of acetate may contribute to the decreased activity of OX neurons itself. Indeed, POMC neurons are known to electrically inhibit OX cells [55]. Conversely, acetate may owe part of its effect on POMC to its inhibition of OX signalling, since in an experiment with double injection of acetate and orexin into lean mice, we observed an increase in POMC mRNA, a sign that acetate can counteract orexin signalling, which alone causes POMC mRNA depletion (see also [33]). Finally, activation of vagal afferents and an increase in leptin levels have been reported to play a role in the acetate-mediated reduction in food-intake [64–66].

It has been proposed that acetate modulates the eCB system [22]. We observed a decline in 2-AG levels following acetate treatments in ob/ob mice, possibly attributable to elevated baseline 2-AG levels in obese mice compared to Wt, an effect that we previously showed to be due in part to higher orexin activity [33]. Our electrophysiological recordings

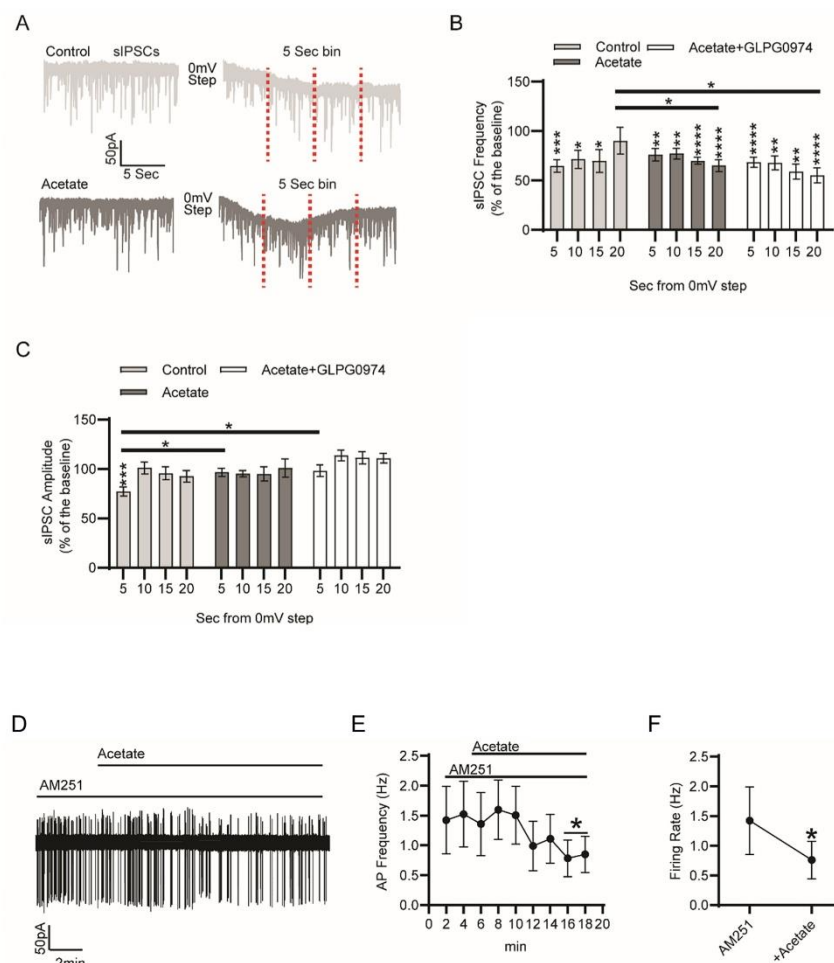


Fig. 5. Acetate modulates DSI onto OX-A neurons. (A) Representative traces of the sIPSC-DSI in control and acetate. After 10 sec of baseline at -60 mV, Hcrt-eGFP neurons were depolarized for 5 s at 0 mV and then clamped at -60 mV for 20 s. Vertical red lines indicate the 5-s bins. (B) Percentage changes of sIPSC frequency relative to 100 % baseline in the control (1.85 ± 0.2 Hz at t_{5sec}), acetate (6.1 ± 1.1 Hz at t_{5sec}) and acetate + GLPG0974 (3.9 ± 0.6 Hz at t_{5sec}) groups. The comparison of each group at a given time point compared to 100 % baseline was performed using One sample *t*-test, and the intergroup analysis was performed using Two-way ANOVA and Sidak's multiple comparisons test. (C) Percentage changes of sIPSC amplitude relative to 100 % baseline in the control (52.69 ± 7.2 pA at t_{5sec}), acetate (53.22 ± 8.1 pA at t_{5sec}) and acetate + GLPG0974 (52.13 ± 6.4 pA at t_{5sec}) groups. The comparison of each group at a given time point compared to the baseline was performed using a one-sample *t*-test, and the intergroup analysis was performed using two-way ANOVA and Sidak's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (D) Representative trace of cell-attached recording in Hcrt-eGFP neurons in the presence of AM251 before and after acetate 10.5 μ M. (E) Time course of Hcrt-eGFP neuron action potential frequency before and during acetate treatment. $^{*}p < 0.05$, two-tailed paired Wilcoxon's signed-rank test. (F) Mean $\pm$ SEM of the AP frequency of Hcrt-eGFP neurons in AM251 and after acetate treatment. $n = 6$ from 4 mice. $^{*}p < 0.05$, two-tailed paired Wilcoxon's signed-rank test.

of DSE/DSI suggest a cell-specific role for acetate in the two forms of synaptic plasticity in hypothalamic slices of Hcrt-eGFP Wt mice, previously (and here) shown to be 2-AG/CB1-mediated [32]. However, the eCB system does not appear to be the primary mechanism by which acetate diminishes the firing of OX neurons in lean mice, as CB1 receptor antagonism with AM251 did not prevent the reduction in firing rate of Hcrt-eGFP neurons in the presence of acetate, an effect that was, instead, fully prevented by a GPR43 antagonist. Therefore, we propose that acetate reduces orexin neuron firing in lean mice via a mechanism that is mostly GPR43-dependent and CB1-independent. Nevertheless, CB1-dependent DSE might also play a (not necessary) role, since its

duration, in terms of reduction of sEPSC amplitude, was enhanced by acetate (in a GPR43-independent manner), in agreement with the non-statistically significant elevation by this SCFA of 2-AG levels observed in hypothalamic slices from Wt mice. However, the fact that acetate significantly reduced 2-AG levels in hypothalamus from ob/ob mice suggests that, if anything, in these mice, we might have found a weaker DSE with the SCFA, which would have been inconsistent with – and/or insufficient to reverse – the observed decrease of orexin neuron activity. This hypothesis is also supported by the previously observed decrease of excitatory/glutamatergic terminals onto orexinergic neurons of ob/ob mice [32]. Regarding DSI, this form of plasticity is, instead, unlikely to

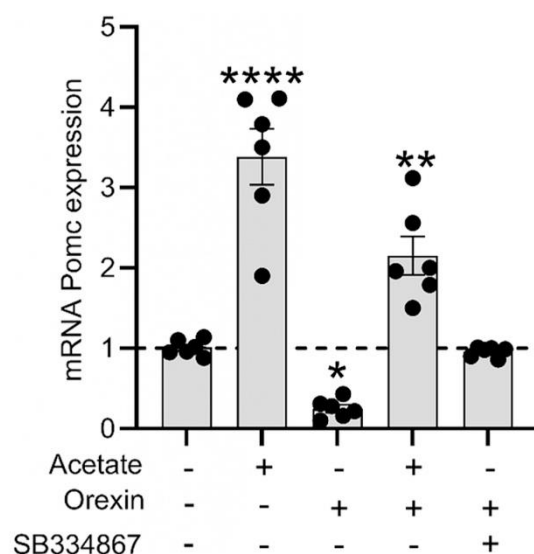


Fig. 6. Acetate and orexin modulate POMC mRNA levels. Reduced Pomc mRNA expression in the mouse arcuate nucleus (ARC) is triggered by i.p. injection of OX-A and prevented by co-treatment with SB334867, while acetate enhances *Pomc* mRNA expression. The bar graph of the mRNA levels is reported as normalized to the reference genes *Hprt* and β actin and each condition was scaled to the control value (no-treated mice) as baseline (dotted line). The mean of the quantitative cycles (Cq) for the Control was 22.41 (SD = 0.054). The Cq of the negative controls (NTC) were undetectable for up to 40 reaction cycles. ANOVA with Bonferroni post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Expression differences between Control vs OX-A, OX-A vs OX-A + SB334867 were significant as evaluated by the REST[®] software [77] (* $p < 0.05$).

participate in acetate-induced inhibition of orexin neuron firing in lean since the data on sIPSC frequency indicate an increase in DSI, while the data on sIPSC amplitude suggest a decrease of DSI under acetate treatment. However, we speculate that in ob/ob mice, where acetate reduced hypothalamic 2-AG levels, DSI would have been reduced, in agreement with the observed decrease of orexin neuron activity. Indeed, acetate may produce its inhibitory effect on orexinergic activity in ob/ob mice through reduced DSI, since: 1) this form of synaptic plasticity was shown to prevail on DSE and underlie, instead, increased basal orexinergic neuron activity in these as well as in diet-induced obese (DIO) mice [32], and 2) stimulation of GABAergic transmission onto these same neurons were shown here to underlie the effects of acetate in lean mice. It will be, therefore, of great importance to study the effect of acetate on orexinergic cells, and on the surrounding neural network, in obese mice in correlation with the eCB system using *Hcrt*-eGFP DIO mice.

OX-A signalling is enhanced in obesity [32,67], and alterations of OX/eCBs levels have been linked to alteration of memory performance [67]. It is intriguing to speculate that acetate, which reduces OX discharge, could theoretically enhance cognitive performance in obesity [67]. Indeed, dietary fibre deficiency has been demonstrated to alter SCFA levels and impair memory in mice [68]. Thus, the interaction between SCFAs and OX-A neurons might play a crucial role also in connecting gut dysbiosis to cognitive decline in obesity.

Interventions targeting the gut microbiota, such as dietary modifications and probiotic supplements, are areas of ongoing research for potential therapeutic strategies in the management of obesity and related metabolic disorders [69]. A high-fiber diet can mitigate weight gain by regulating food-intake and appetite while fostering the growth and activity of bacteria that produce SCFAs [70]. Specifically, bacteria

belonging to the genera *Coprococcus*, *Barnesiella*, *Ruminococcus*, and *Ruminococcaceae* NK4A21 demonstrated a positive correlation with circulating acetate levels, while *Lachnospirillum* and *Bacteroides* exhibited a negative correlation [70]. It is well-established that SCFA-producing bacteria like *Akkermansia* and *Bifidobacterium*, essential components of a healthy gut microbiota, are diminished in obesity [71,72]. Studies have shown that modifying the gut microbiome in ob/ob mice by selectively increasing *Bifidobacterium* improves gut permeability and reduces inflammation, both attributed to the actions of acetate on inflammation [73,74]. On the other hand, the supplementation with a probiotic containing *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*, in line with an increment in acetate levels, reduced body weight and food-intake in diet-induced obese mice [75,76].

In conclusion, based on data presented here and previously published, our study supports the concept that acetate and the probiotic supplementation of acetate-producing bacteria may represent novel anti-obesity strategies, and enlarges our perspectives on the mechanism of action of this SCFA.

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CRediT authorship contribution statement

Nicola Forte: Writing – original draft, Validation, Methodology, Formal analysis. **Brenda Marfella:** Methodology. **Alessandro Nicois:** Methodology. **Letizia Palomba:** Validation, Methodology, Conceptualization. **Debora Paris:** Methodology, Formal analysis. **Andrea Motta:** Validation, Resources. **Maria Pina Mollica:** Funding acquisition, Conceptualization. **Vincenzo Di Marzo:** Writing – review & editing, Funding acquisition, Conceptualization. **Luigia Cristino:** Writing – review & editing, Writing – original draft, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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3.3 Task III – “The Blue Light-Responsive Lateral Pathway of the Retinohypothalamic Tract Promotes Endocannabinoid-Driven Modulation of Orexin Neurons”

Light exposure plays a critical role in regulating the circadian rhythm, which is the body’s internal clock controlling physiological processes over a 24-hour cycle. This rhythm influences appetite by modulating neuroendocrine signals and metabolic pathways.

The hypothalamus integrates light cues received through the retina to synchronize feeding behavior with the day-night cycle. Typically, appetite peaks during the active phase (daytime for diurnal species, nighttime for nocturnal species). Disruption of circadian rhythm—such as through irregular light exposure—can lead to altered hunger signals and metabolic imbalance.

2-AG is a major endocannabinoid that activates CB1 receptors, promoting food intake and energy storage. Its levels fluctuate in a circadian manner, generally increasing before the active feeding phase. Light-driven circadian signals influence the enzymes that synthesize and degrade 2-AG, thereby modulating its orexigenic (appetite-stimulating) effect.

Orexin is a neuropeptide produced in the hypothalamus that stimulates wakefulness and feeding. Its secretion is tightly linked to circadian rhythm and light exposure. During the active phase, orexin levels rise, enhancing motivation to eat and reinforcing the orexigenic action of 2-AG through synergistic signaling in hypothalamic circuits.

Since blue light is a powerful environmental cue (Zeitgeber) for the synchronization of circadian rhythms, wakefulness, arousal, and homeostatic functions controlled by hypothalamic circuits, and both 2-AG and orexin exhibit circadian fluctuations, with their orexigenic properties being strongest during the active phase, in this task of my study we sought to evaluate if blue light eye stimulation in mice could trigger hypothalamic 2-AG levels and disinhibition of OXA neurons thus providing the anatomo-functional mechanism linking arousal with wakefulness and food-intake behaviour.

Intrinsically photosensitive retinal ganglion cells (ipRGCs), expressing melanopsin, are particularly sensitive to the blue component of the spectrum and send the classic projections to the suprachiasmatic nucleus (SCN) via the medial retinohypothalamic tract, but more recent evidence indicates the existence of alternative projections to lateral hypothalamic areas. Among these, the perifornical hypothalamic area (PFH) hosts orexin (OXA) neurons that show marked circadian oscillations in activity and are crucial for maintaining wakefulness, regulating arousal, food intake, and energy expenditure.

Within this anatomo-functional context, the demonstration that a lateral branch of the retinohypothalamic tract (LRHT) provides the PFH with glutamatergic retinal afferents co-expressing the vesicular glutamate transporter VGluT1 and the CB1 receptor defines a circuit in which photic signaling is intrinsically susceptible to endocannabinoid modulation

Experiments using presynaptic calcium probes (Fluo4-Dextran) show that 10 minutes of blue light increase recruitment of retinal afferents onto OXA neurons, whereas prolonged stimulation leads to a reduction in this signal, paralleling the bell-shaped time course of 2-AG levels. Application of the CB1 antagonist AM251 abolishes the prolonged suppressive phase of hypothalamic firing while preserving the initial activation, confirming that the late inhibitory component is mediated by CB1 activation on LRHT glutamatergic terminals. Taken together, these data support a model in which the ipRGC–LRHT–PFH–2-AG–CB1 axis constitutes a fine mechanism for translating blue light input into modulation of orexinergic activity and, by extension, of wakefulness, arousal, and energy homeostasis. This study aimed to comprehensively characterize the blue light–responsive lateral retinohypothalamic pathway which, through CB1-sensitive glutamatergic afferents to the PFH, promotes endocannabinoid-driven modulation of orexin neurons, combining anatomical tracing, time-resolved measurements of hypothalamic 2-AG, cFOS analysis, and electrophysiological assessment of CB1-dependent DSE at retinal terminals.

ORIGINAL ARTICLE OPEN ACCESS

The Blue Light-Responsive Lateral Pathway of the Retinohypothalamic Tract Promotes Endocannabinoid-Driven Modulation of Orexin Neurons

Nicola Forte¹  | Roberta Imperatore² | Brenda Marfella^{1,3} | Alessandro Nicois^{1,4} | Roberta Verde¹ | Letizia Palomba^{1,4} | Vincenzo Di Marzo^{1,5,6,7}  | Luigia Cristino¹ 

¹Institute of Biomolecular Chemistry, National Research Council, Pozzuoli, Italy | ²Department of Sciences and Technologies, University of Sannio, Benevento, Italy | ³Department of Biology, University of Naples Federico II, Naples, Italy | ⁴Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy | ⁵Canada Excellence Research Chair on the Microbiome-Endocannabinoidome Axis in Metabolic Health, Université Laval, Québec City, Québec, Canada | ⁶Heart and Lung Research Institute of Université Laval, Québec City, Québec, Canada | ⁷Institute for Nutrition and Functional Foods, Centre NUTRISS, Université Laval, Québec City, Québec, Canada

Correspondence: Vincenzo Di Marzo (vincenzo.dimarzo@criucpq.ulaval.ca) | Luigia Cristino (luigia.cristino@cnr.it)

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ABSTRACT

Circadian light influences brain functions in mammals. Photoreceptive non-image-forming stimuli are transduced into electrochemical signals by photosensitive retinal ganglion cells containing melanopsin, a selective blue light-responsive photopigment. The hypothalamus receives light-related information via the retinohypothalamic tract (RHT). Here, we demonstrate that, in the mouse, a lateral branch of the RHT (l-RHT) projects monosynaptically to orexin-A (OX-A) neurons in the perifornical hypothalamic area (PFH). Intravitreal injection of the anterograde tracer cholera toxin- β (CT β) filled most of the vesicular glutamate transporter (VGluT1)/cannabinoid receptor 1 (CB1R)-positive retinal-derived inputs projecting to the OX-A neurons. Monocular injection of Fluo4-Dextran, a fluorimetric sensor of calcium mobilization, yielded fast labeling of these inputs 10 min after eye exposure to blue light, concomitantly with the enhancement of hypothalamic 2-arachidonoylglycerol (2-AG) levels, and inhibition of OX-A neuronal firing, an effect prevented by in vivo administration of the CB1R antagonist AM251. Our findings provide anatomical and functional evidence of a selective retino-hypothalamic network responsive to blue light, whose control should be suitable for therapies to counteract sleep disorders, seasonal affective disorder, or even conditions like narcolepsy or anxiety.

Abbreviations: CB1R, cannabinoid receptor; 2-AG, 2-Arachidonoylglycerol; CT β , cholera toxin- β ; OX-A, orexin-A; PFH, perifornical hypothalamic area; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; VGluT1, vesicular glutamate transporter.

This work is dedicated to the loving memory of Prof. Denis Richard, deceased in December 2023.

Vincenzo Di Marzo and Luigia Cristino contributed equally to this work.

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1 | Introduction

Circadian shifts in environmental light frequencies represent the primary “Zeitgeber” by entraining homeostatic brain functions such as eating, sleep/wake cycles, arousal, and hormone production. The mammalian eye contains a non-image-forming system constituted by a small group of ipRGCs expressing melanopsin (Provencio et al. 2000, 2002; Hannibal et al. 2002; Berson et al. 2002; Warren et al. 2003), a photopigment maximally sensitive to the blue part of the light spectrum (460–480 nm). The ipRGCs synchronize biological clocks with the external light/dark cycle to regulate the circadian system (Foster et al. 2020). Most ipRGC projections innervate non-image-forming hypothalamic areas enrolled in circadian photoentrainment, pupillary light reflexes, and melatonin synthesis (Seabrook et al. 2017; Schmidt et al. 2011). These projections form the retinohypothalamic tract (RHT) (Hannibal 2002) and are mostly glutamatergic, despite a small inhibitory subset regulating the pupillary light reflex (Sonoda et al. 2020). The RHT has been extensively studied in several mammalian species, including mice, using the intravitreal injection of the anterograde-tracing CT β (Hannibal and Fahrenkrug 2004; Pickard and Silverman 1981; Johnson et al. 1988; Levine et al. 1991; Murakami et al. 1989; Murakami and Fuller 1990; Mikkelsen 1992; Tessonneaud et al. 1994; Hannibal et al. 1997, 2001). This approach has demonstrated that the RHT innervates the suprachiasmatic nucleus (SCN) bilaterally with approximately equal contralateral and ipsilateral dominance (Hannibal and Fahrenkrug 2004; Abrahamson and Moore 2001). The RHT, in turn, is formed by a medial and lateral retinofugal tract with the lateral part largely overwhelming the medial one into projections to the LH (Leak and Moore 1997), SCN (Welsh et al. 2010), retrochiasmatic and anterior hypothalamic areas (Berson et al. 2002; Hattar et al. 2002; Gooley et al. 2001). The lateral projections (l-RHT) terminate mainly in the ventral zone of the LH anterior group, including preoptic, lateral, and subparaventricular hypothalamic retinorecipient regions (Hannibal and Fahrenkrug 2004; Hattar et al. 2002; Gooley et al. 2003). Although the medial RHT regulates light entrainment of circadian rhythms to the solar cycle (Hannibal 2002), the function of the l-RHT is still unknown despite its relevance to the hypothalamic regulation of circadian-related functions. Here, we focused our study on a putative target of the l-RHT, a subset of hypothalamic neurons producing Orexin-A (OX-A) (Diaz et al. 2023), also called hypocretin-1. OX-A is a 33-amino-acid peptide that regulates circadian functions such as arousal, feeding, and the sleep–wake cycle. In nocturnal animals like rodents, a melanopsin-induced activation of OX-A neurons has been described in association with sleep-to-wake transition (Tsunematsu 2021; Deboer et al. 2004; Zhang et al. 2004; Azeez et al. 2018; Ventzke et al. 2019). These data are in line with the circadian changes of cerebrospinal and hypothalamic OX-A levels, which are increased in the awake phase (i.e., ZT13–24) and decreased in the resting phase between ZT8 and ZT12, immediately before the onset of the awake phase (Yoshida et al. 2001; Fujiki et al. 2001; Desarnaud et al. 2004).

A circadian daytime-induced change of the endocannabinoid 2-AG levels has been described in the rodent hypothalamus (Valenti et al. 2004). Notably, changes in OX-A levels were

also associated with fast non-genomic 2-AG-driven control of orexinergic neurons at CB1R receptors (Cristino et al. 2013). In turn, OX-A signaling regulates 2-AG synthesis via OX-1R receptors, promoting a close relationship between the circadian changes of OX-A and 2-AG levels in regulating sleep timing and caloric intake (Cristino et al. 2013; Hanlon et al. 2015, 2016; Cedernaes et al. 2016; Forte et al. 2021). For instance, (i) intra-LH administration of 2-AG increases REM sleep, (ii) the CB1R agonist (CP55940) attenuates light-induced clock-phase advance (Sanford et al. 2008), and (iii) the cannabinoid Δ^9 -THC, the primary psychoactive agent of marijuana and hashish, tends to generate a distorted sense of time by signaling at hypothalamic CB1R (Sewell et al. 2013; Tinklenberg et al. 1976). However, anatomical evidence of an ipRGCs retinofugal projections to OX-A neurons, as well as the functional evidence of a blue light-mediated change of OX-A neuronal activity and 2-AG levels in the hypothalamic nuclei, are still missing.

2 | Material and Methods

2.1 | Animals and Drugs

The study was conducted according to the ARRIVE Guidelines to improve bioscience research reporting using laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used. The experiments were performed by the European Union animal welfare guidelines [European Communities Council Directive of September 22, 2010 (2010/63/EU)] and the Italian Decree no. 26/2014, authorization no. 589/2018-PR. Eighty-seven C57BL/6J male mice (Charles River Laboratories Int.; Wilmington, Massachusetts, USA; 2–3 months old, ~25 g body weight) were housed in standard cages receiving chow and tap water *ad libitum* under controlled illumination (12 h light/dark cycle; light on at 6:00 am, ZT0) and environmental conditions (ambient temperature 20°C–22°C, humidity ~50%). As orexin levels exhibit robust diurnal fluctuations, gradually increasing between ZT13–24 (dark period) and decreasing during ZT0–12 (light period), all the experiments were performed between ZT6 and ZT12, when orexin neurons are less active (Yoshida et al. 2001; Fujiki et al. 2001; Desarnaud et al. 2004). Before the blue light exposure, mice were habituated to total darkness for 1 h starting from ZT5 to maximize this specific wavelength's effect on the ipRGCs stimulation.

2.2 | Intravitreal Injection of Tracer Cholera Toxin Subunit B or Fluo-4-Dextran, and Eye Stimulation

An intraocular injection was performed into the vitreous of the left eye of adult C57BL/6J mice anaesthetized with a mixture of ketamine (0.08 mg/g body weight; Sigma, cat. no. K2753) and xylazine (0.01 mg/g body weight; Sigma; cat. no. X1251), and topical treatment with proparacaine hydrochloride 0.5% (Akorn, Buffalo Grove, IL, USA) maintained under 12 h:12 h dark/light cycle. Intravitreal injection of anterograde tracer cholera toxin- β (CT β , List Biological cat. no., 104, [RRID:AB_2313636](#), Campbell, CA, USA), or Fluo4-Dextran (Fluo-4, Life Technology, cat. no. F14201) was performed in two different groups of mice (no. 6

mice/group) maintained under 12h:12h dark/light cycle as described by Prichard et al. (2002). Briefly, a small hole was made in the temporal sclera margin of the eye with a sterile 26-gauge needle, and 2.0 μ L of CT β dissolved in saline with 0.1% Evans Blue (Sigma) or 2.0 μ L of Fluo4-Dextrane were injected into the vitreous by holding the needle in place for 1 min after administration to prevent tracer's reflux. 7 days after Fluo4-Dex injection, the mice were kept in total darkness for 1 h before being stimulated with a blue light pulse (LP, 800 Lux) (Huang et al. 2023; Bilu et al. 2019) at constant irradiance for 10 or 20 min and then rapidly euthanised and perfused transcardially with 4% PFA.

2.3 | Immunohistochemistry

Mice were deeply anesthetized with a mixture of ketamine and medetomidine (100 mg/kg + 1 mg/kg i.p.) and perfused transcardially with 4% (wt/vol) paraformaldehyde (SERVA, cat no. 3162802)/0.1 M phosphate buffer (PB), pH 7.4. The brains were extracted and cryopreserved by immersion in 30% (wt/vol) sucrose/0.1 M PB until sinking and then cut with a Leica CM3050S cryostat in 10 μ m-thick serial coronal sections collected in alternate series to be processed for immunofluorescence. Sections were incubated for 1 h at room temperature in PB containing 0.3% Triton X-100 (Sigma, cat no. X100) and 5% normal donkey serum (blocking buffer, Abcam, cat. no. ab7475), and then incubated overnight at 4°C with the following mixture of primary antibodies diluted in donkey serum: mouse anti-CT β (Abcam, cat. no. ab62429; 1:500), goat anti-OX-A (Santa Cruz, cat. no. SC8070; 1:200), guinea pig anti-VGluT1 (Synaptic System, cat. no. 135304; 1:500), rabbit anti-CB1R antibody (anti-C terminus 461–472, Abcam, cat. no. ab23703; 1:300), rabbit anti-c-Fos (Abcam, cat. no. ab190289; 1:200). Immunofluorescence was revealed by specific Alexa-488, or –546, or –350 secondary donkey anti-IgGs (Invitrogen, ThermoFisher Scientific, France, cat. no. A10035, A21081, A17655, A21207) incubated 2 h at room temperature at 1:250–1:100 dilution range. Sections were coverslipped with Aqueatex mounting medium (Merck, Darmstadt, Germany). Controls of the specificity of immunolabeling in multiple fluorescence experiments were performed by omission of primary and secondary antibodies or by preabsorption of primary antibodies with the respective blocking peptides. The immunostained sections were observed with a confocal microscopy Nikon Eclipse Ti2 (Nikon, Florence, Italy) equipped with an x-y-z motorized stage, a digital camera DS-Qi2 (Nikon, Florence, Italy), and the acquisition and image analysis software NIS-Elements C (Nikon, Florence, Italy). Digital images were acquired using the $\times 20$ – $\times 40$ objectives and serial Z-stacks were collected throughout the area of interest ($n \leq 20$ planes with an increment of 0.5 μ m). Images were deconvolved using the imaging deconvolution software by application of $n=10$ iterations. Serial Z-plane images were collapsed into a single maximum projection image. Micrographs were saved in TIFF format and adjusted for light and contrast before being assembled on plates using Adobe Photoshop 6.01 (Adobe Systems, San Jose, CA).

2.4 | Immunohistochemical Analysis

Assessment of immunoreactivity was performed throughout the anterior–posterior extension of the hypothalamic nucleus, in a series of 10 μ m coronal sections spaced 30 μ m apart (1:3 as

the frequency of section sampling to cell count, starting from Bregma -1.34 mm up to Bregma -2.18 mm), through 0.85 mm³ rostrocaudal extension from the 3rd ventricle. For the quantification of single or multiple immunoreactivity and cell counting, an unbiased physical dissector-based protocol was applied by an operator blind to the experimental groups, according to Forte et al. (2021).

2.5 | Lipid Extraction and 2-AG Measurement

PFH of the hypothalami from the brains of mice under the same experimental conditions were accurately dissected and pooled ($n=12$ /group) to be analyzed using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry. The 2-AG was extracted from the tissues and then purified and quantified as previously described. First, tissues were pooled and homogenized in 5 vol chloroform/methanol/Tris–HCl 50 mM pH 7.5 (2:1:1 by volume) containing 50 pmol of d5-2-arachidonoylglycerol (d5-2-AG) as an internal deuterate standard. Homogenates were centrifuged at 13 000 g for 16 min (4°C), and the aqueous phase plus debris were collected and extracted four times with 1 vol of chloroform. The lipid-containing organic phases were dried and pre-purified by open-bed chromatography on silica columns eluted with increasing concentrations of methanol in chloroform. Fractions for 2-AG measurement were obtained by eluting the columns with 9:1 (by volume) chloroform/methanol and then analyzed by liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry (LC-APCI-MS). LC-APCI16 MS analyses were conducted in the selected ion monitoring mode, using m/z values of 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG). Values are expressed as pmol per mg of wet tissue extracted.

2.6 | Electrophysiology

Male C57BL/6J mice, aged 2–3 months, were used throughout all experiments. Mice were anesthetized under urethane (1.2 g/kg, i.p.), oxygen was administered to the animal through a mask, and body temperature was held at 37°C through a thermostatic blanket. During experiments, *lacrigel* was used to prevent corneal dehydration. A bilateral craniotomy of approximately 1 mm was made on the LH. The following stereotaxic coordinates were used to target the LH: anterior/posterior -1 mm from Bregma, lateral ± 1 mm, and dorsal/ventral between -4.3 and -5.3 mm. Single-unit recordings from unidentified neurons in the LH were acquired using a microelectrode filled with K-acetate 0.5 M, 6–10 μ m at the tip. The recordings were performed using a model 410 amplifier (Brownlee Precision Co) and power 1401 mkII (CED), band-pass (200–3000 Hz). The recording sessions were conducted in the dark. The eye contralateral to the recording site was stimulated using a blue light emitted from LEDD1B (Thor Labs) coupled with a lens (Toptica Photonics, Fiberout_011555). The light power, measured with a power meter 3 cm away from the light source, was approximately 4 mW/mm². At the end of the recording session, animals were perfused with 4% PFA to confirm the correct position of the recording site. All data were analyzed using Spike2 (CED).

Spontaneous neuronal activity in the dark was recorded for 5 min in the PFH, followed by a 10 min of blue light pulse. Subsequently, additional 5 min of recording in the dark were performed. In a subset of experiments, the CB1R inverse agonist AM251 (Tocris cat no. 1117, 1 mg/kg) was administered intraperitoneally (i.p.) 1 h before the start of the recording session.

2.7 | Statistical Analysis

Data were analyzed using GraphPad Prism (RRID:SCR_002798). No sample size was calculated a priori, but the sample size was based on previous studies of a similar nature (Forte et al. 2021). No exclusion criteria were pre-determined, and no blinding was performed. Outliers were detected using the Rout method with $Q = 1\%$. Data were reported as bar or box plots. The box plot elements were center line, median (Q2); square symbol, mean; box limits, 25th (Q1), and 75th (Q3) percentiles; the outermost data points determined whisker length. Normal distribution was assessed using the Shapiro–Wilk normality test. The electrophysiological experiments were analyzed using a one-sample *T*-Test and a two-way ANOVA test. For the neuroanatomical and immunohistochemical experiments, One-way ANOVA/Bonferroni tests or Kruskal–Wallis/Dunn's tests were used to analyze more than two experimental groups with normally or non-normally distributed data, respectively. Data are expressed as mean $\pm$ SEM.

3 | Results

3.1 | Retinal VGlut1/CB1R-Positive Projections Innervate the Hypothalamic PFH

Previous studies have identified RHT projections to the LH in the rat brain by using the anterograde-tracer CT β (Hannibal and Fahrenkrug 2004; Levine et al. 1991; Hattar et al. 2002; Gooley et al. 2003). Other studies demonstrated the high expression of CB1R at the pre-synaptic level in the LH (Cristino et al. 2013; Jo et al. 2005). However, whether CB1Rs are expressed at retinal-derived hypothalamic inputs is still unknown. To trace the retinal projection to PFH in the LH we used intravitreal injection of CT β , which does not travel trans-synaptically (Coolen et al. 1999; Mehlman et al. 2021). CT β was chosen for its superior uptake, sensitivity, rapid transport, and frequent use in the analysis of the retinocollicular tract (Angelucci et al. 1996). Analyzing the VGlut1 and CB1R immunofluorescence on diencephalic sections from CT β -injected brains, we observed a vast majority of retinohypothalamic CT β -labeled inputs in the PFH (Figure 1A–D, A₁–D₁), most of which expressed VGlut1/CB1R immunoreactivity (Figure 1E).

3.2 | Eye Blue Light Stimulation Regulates Hypothalamic Endocannabinoid 2-AG Levels and c-Fos Expression in OX-A Neurons

A circadian daytime-induced change in endocannabinoid levels has been described in rat brain and human plasma (Valenti et al. 2004; Hanlon et al. 2015). In this study, by exploiting

mass spectrometry, we report a blue light-dependent change of endocannabinoid 2-AG levels in the PFH. Specifically, we observed an almost 10-fold increase of 2-AG levels (20.50 ± 2.309 pmol/mg) after 10 min of exposure to blue light, compared to the control group (ins levels; Figure 1F). On the other hand, 20 min of blue light exposure induced a significant reduction of 2-AG levels in comparison to the levels observed after 10 min of blue light exposure (5.6 ± 3.7 pmol/mg) (Figure 1F).

Subsequently, we observed a significant increase in c-Fos immunoreactivity in OX-A neurons within the PFH following 10 min of blue light exposure ($82.63\% \pm 2.7\%$ mean $\pm$ SEM), compared to the no-light pulse (No-LP) condition ($32.09\% \pm 1.9\%$ mean $\pm$ SEM; Figure 2A,B, A₁–B₁, D, neurons). In contrast, no significant difference was detected when the stimulation was extended to 20 min ($34.14\% \pm 2.4\%$ mean $\pm$ SEM; Figure 2C–C₁, 2D). Furthermore, no changes in c-Fos expression were observed in the LH or DMH after either 10 or 20 min of light stimulation (Figure 2E,F). No variation in the number of OX-A immunoreactive neurons was detected in No LP, 10 min LP, and 20 min LP in the PFH ($n = 80 \pm 5$ neurons, $n = 85 \pm 7$ and $n = 78 \pm 8$), LH ($n = 4240 \pm 125$; 4180 ± 220 ; 4030 ± 185) and DMH ($n = 90 \pm 6$ neurons, $n = 87 \pm 8$ and $n = 93 \pm 4$; Figure S1).

3.3 | Eye Blue Light Stimulation Activates Retinal Projections to OX-A Neurons

We hypothesized that c-Fos activation in OX-A neurons could be induced by the excitatory drive resulting in glutamate release from the VGlut1-positive ipRGCs projection via the l-RHT since these cells are specifically excited by blue light (Provencio et al. 2000, 2002; Hannibal et al. 2002; Berson et al. 2002; Warren et al. 2003). To support this hypothesis, we performed a mono-ocular intravitreal injection of Fluo4-Dextran (Fluo4-Dex), a conjugated low-affinity calcium indicator suitable to label pre-synaptic fibers and quantify changes in pre-synaptic Ca²⁺ flux, both in brain slices and in vivo (Kreitzer and Regehr 2001).

Quantification of Fluo4-Dex-positive inputs contacting OX-A neurons was performed in mice after 10 or 20 min of retinal stimulation with a blue light pulse. Accordingly, in addition to the 2-AG level (Figure 1F) and c-Fos expression (Figure 2D) pattern, we observed a change of Fluo4-Dex fluorescence intensity in a time-dependent response to blue light exposure (Figure 3A–D).

Specifically, the RHT fibers projecting to OX-A neurons exhibited an increase in Fluo4-Dex optical density 10 min after LP (O.D. = 2.315 ± 0.009) as compared to mice under No LP (O.D. = 1.631 ± 0.006), or after 20 min exposure to LP (O.D. = 1.477 ± 0.005) (Figure 3D). Furthermore, an increase in the number of Fluo4-Dex-positive inputs to OX-A neurons was observed in mice after 10 min of LP (39.17 ± 1.8) compared to mice under No LP (10.91 ± 0.7), or after 20 min LP (16.11 ± 1.1) (Figure 3A–C, E).

Finally, we found that the large majority of these inputs also carried CB1R and underwent a similar time-lapse-dependent change

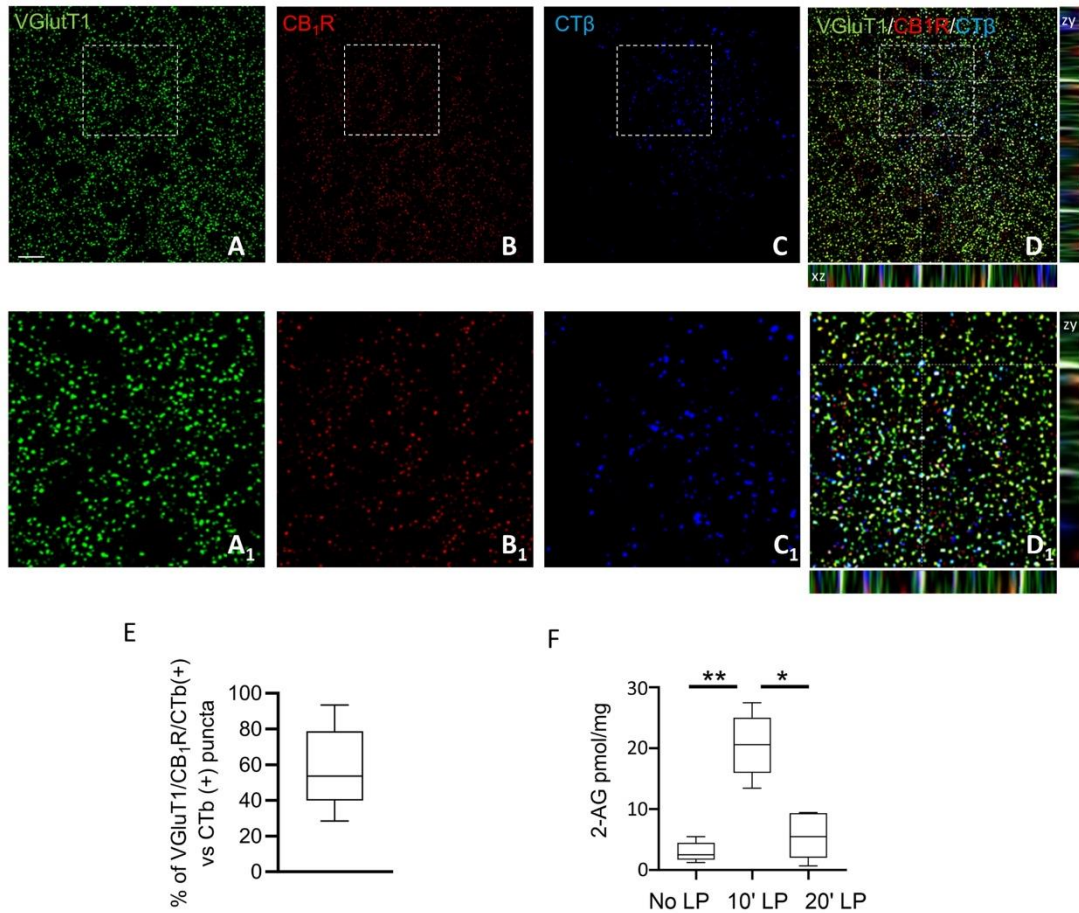


FIGURE 1 | Intravitreal CT β loading of VGluT1/CB1-positive inputs to the lateral hypothalamus and blue light-mediated regulation of 2-AG levels. (A–C) Confocal microscopy showing retinal fibers labeled with CT β (blue), expressing vesicular glutamate transporter VGluT1 (green) and CB $_1$ R (red). Scale bar: 20 μ m. (D) High-power fluorescent micrographs of orthogonal stacks; dotted lines and crosshairs are used to show 3D coordinates and define the area of interest. (A $_1$ –D $_1$) Magnification of the details highlighted in the squares reported in A–D. (E) Bar graph showing the percentage of VGLUT1/CB1R/CT β –immunoreactive puncta despite to the total number (100%) of CT β –labeled puncta, $n = 30$ sections/mice; $n = 3$ mice. (F) LC–MS quantification of 2-AG levels (pmol/mg) in the PFH of mice under different conditions: No LP, 10 min blue light pulse (10' LP), or 20 min blue light pulse (20' LP) exposure. $n = 10$ mice per group. Kruskal–Wallis Test and Dunn's post hoc test, $DF = 2$, Kruskal–Wallis statistic = 9.893, 30.7, $*p = 0.042$, $**p = 0.0053$. Mean $\pm$ SEM.

of Fluo-4-Dex fluorescence at 10 min of LP (43.67 ± 1.8), as compared to No LP (12.62 ± 0.8) or 20 min LP (17.61 ± 1.3) (Figure 3F).

3.4 | Eye Blue Light Stimulation Modulates the Neuronal Firing in the Lateral Hypothalamus

Previous studies based on tract-tracing approaches have demonstrated I-RHT is formed by ipRGCs projections to the LH (Leak and Moore 1997; Hattar et al. 2006; Delwig et al. 2016). Our data dissected the anatomical features of this branch by identifying OX-A neurons of the PFH as targets (Figure 3E). Furthermore, by exploiting the calcium-sensor fluorescent dye Fluo4-Dextran, we unraveled a time-dependent blue light-induced calcium flux at the

Fluo-4-Dex/CB1-positive projections to OX-A neurons (Figure 3F), in concomitance with c-Fos activation of the targeted OX-A neurons (Figure 2D) and the enhancement of 2-AG levels (Figure 1F).

To investigate the functional role of 2-AG in this circuit, we conducted an *in vivo* single-unit recording of unidentified neurons in the LH of mice under urethane anesthesia. Baseline firing activity was recorded for 5 min in darkness, followed by 10 min of light pulse (LP) (Figure 4A). Among the $n = 15$ neurons recorded in $n = 10$ mice, more than 50% exhibited a six-fold increase in firing rate in response to blue light stimulation (Figure 4B). However, all the recorded cells showed significant inhibition of their firing rate after 1 min of stimulation, a suppression that persisted even after the blue light was turned off (Figure 4C). Based

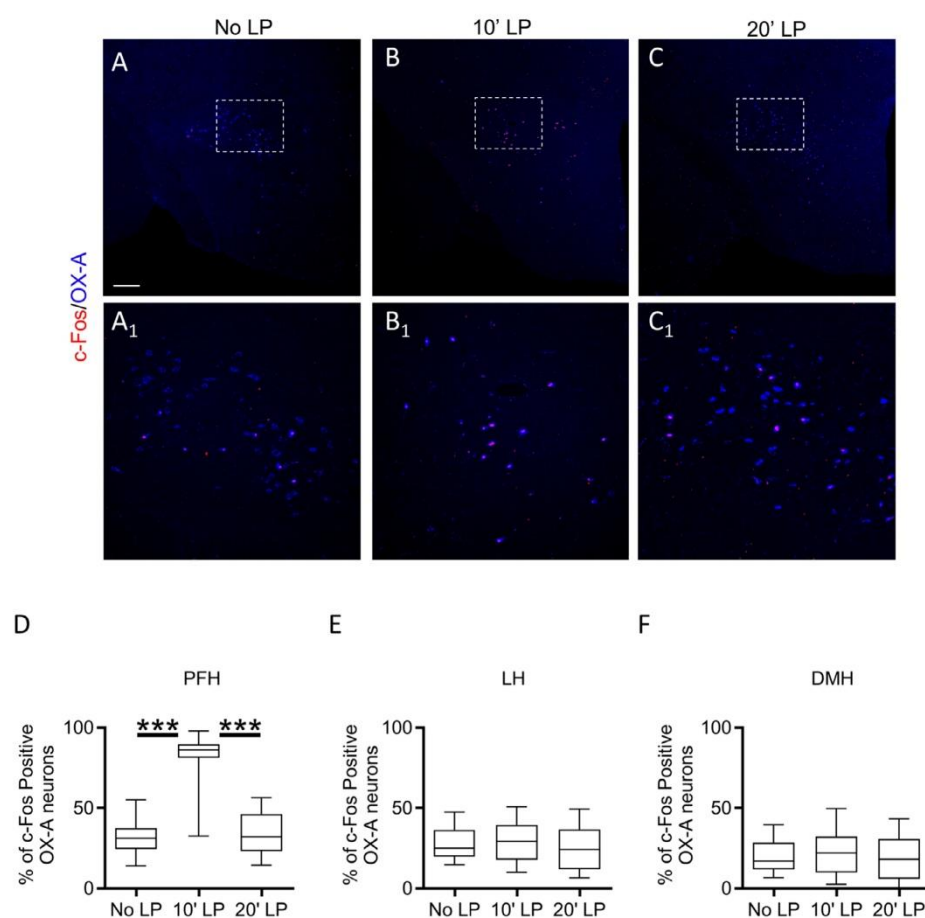


FIGURE 2 | Blue light exposure increases c-Fos expression in OX-A neurons in the PFH. (A–C) Confocal microscopy showing colocalization of c-Fos (green) and OX-A (red) immunoreactivity in the PFH of mice under No LP, 10' LP or 20' LP blue light pulse exposure. Scale bar: 300 μ m. (A₁–C₁) Magnification of the details highlighted in the squares depicted in A–C. (D–F) Percentage of c-Fos/OX-A-immunoreactive neurons in the PFH, LH, or DMH of mice under No LP, 10' LP, or 20' LP blue light pulse exposure. $n = 30$ sections/mouse; $n = 6$ mice/group. Kruskal–Wallis Test and Dunn's post hoc test, **** $p < 0.0001$, $DF = 2$, Kruskal–Wallis statistic = 48.56. Mean $\pm$ SEM.

on our previous observation of CB1R expression at VGLUT1/CT β -positive RHT fibers in the PFH (Figure 1E), as well as the observed increase of 2-AG levels following blue light stimulation (Figure 1F), we repeated the *in vivo* single-unit recording after *i.p.* injection of AM251, a selective CB1R antagonist (4 mg/kg) (Figure 4D). Despite an initial increase in firing rate during the first minute of recording under eye blue light exposure, AM251 administration was able to prevent the subsequent suppression of firing rate. (Figure 4E, $n = 12$ cells in 8 mice). Consistent with this observation, the neuronal activity in the PFH of mice treated with AM251 was higher than in the untreated control group (Figure 4F,G).

4 | Discussion

The ipRGCs projecting to the LH through the l-RHT are primarily located in the upper quadrant of the retina and represent

a homogeneous subset of type III ganglion cells (Canteras et al. 2011). These cells play a key role in mediating cognitive, physical, and behavioral functions influenced by blue light wavelengths, although they are not involved in the image-forming processes (Stuber and Wise 2016). Multiple studies indicate that both natural and artificial light can affect behavior, with blue light specifically regulating arousal (Vandewalle et al. 2009; Liu et al. 2020). Consequently, blue light exposure is emerging as a potential non-pharmacological treatment for promoting structural and functional recovery following mild traumatic brain injury (Srisurapanont et al. 2021; Bajaj et al. 2017), or psychophysiological disorders related to seasonality, such as Seasonal Affective Disorder (S.A.D) (Strong et al. 2009). However, the mechanism by which blue light regulates brain activity is not fully understood.

Previous studies have demonstrated that exposure to blue light increases c-Fos expression in orexin/hypocretin neurons (Liu

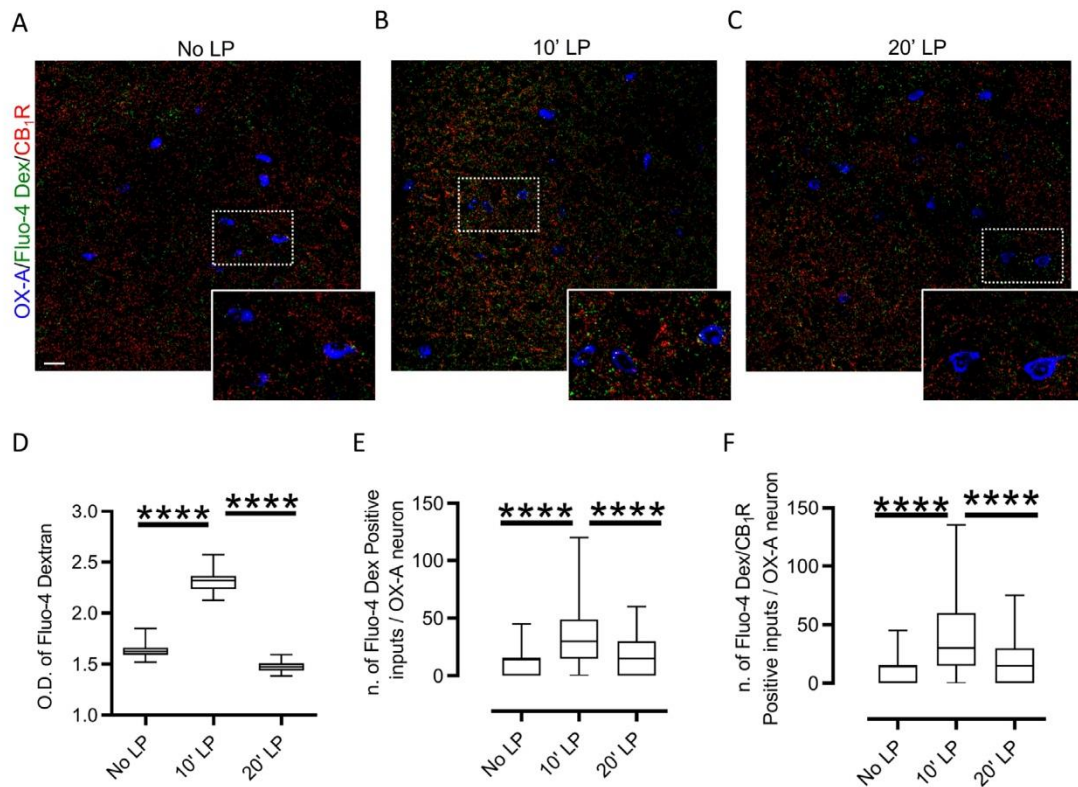


FIGURE 3 | Blue light exposure activates Fluo4-Dex/CB1R-positive inputs to OX-A neurons. (A–C) Confocal microscopy showing Fluo4-Dx (green) and CB1R (red) colocalization in OX-A neurons (blue) of the PFH. High magnification of the boxed areas is depicted at high magnification in each respective inset. Scale bar: 20 μ m. (D) Optical density of Fluo4-Dx inputs to OX-A neurons in the PFH of mice under No LP, 10' LP or 20' LP blue light pulse exposure; $n = 30$ sections/mouse; $n = 6$ mice/group; Kruskal–Wallis Test and Dunn's post hoc test, Kruskal–Wallis statistic = 235.5, $DF = 2$, **** $p < 0.0001$; data are mean $\pm$ SEM. (E) Number of Fluo4-Dx-positive inputs to OX-A neurons in the PFH of mice under No LP, 10' LP or 20' LP blue light pulse exposure; $n = 30$ sections/mouse; $n = 6$ mice/group; $DF = 2$, Kruskal–Wallis Test and Dunn's post hoc test, Kruskal–Wallis statistic = 186.2, **** $p < 0.0001$; data are mean $\pm$ SEM. (F) Number of Fluo4-Dx/CB1R-positive inputs to OX-A neurons in the PFH of mice under No LP, 10' LP, or 20' LP blue light pulse exposure; $n = 30$ sections/mouse; $n = 6$ mice/group. Kruskal–Wallis Test and Dunn's post hoc test, Kruskal–Wallis statistic = 199.4, $DF = 2$, **** $p < 0.0001$; data are Mean $\pm$ SEM.

et al. 2020; Adidharma et al. 2012), one of the two main cellular subgroups in the LH regulating light-dependent activities like sleep/wake and arousal (Stuber and Wise 2016). Consistent with these findings, our current observations indicate that short-time lapse stimulation of ipRGCs through this selective wavelength triggers c-Fos expression in OX-A neurons mainly in the PFH. This selective activation was confirmed by the increase in the optical density and in the number of Fluo4-Dex-positive puncta in OX-A neurons.

In this study, we examined the expression of CB1Rs among the RHT fibers projecting to OX-A neurons in the PFH, which are affected by blue light stimulation. Our findings indicate that RHT fibers innervating OX-A neurons in the PFH express both CB1R and VGluT1, and that blue light stimulation of these fibers induces a transient increase in 2-AG levels, likely linked to the OX-A neuronal activity as demonstrated by the concomitance of c-Fos induction of expression in most of OX-A neurons innervated by Fluo4-Dex-positive fibers.

In nocturnal rodents, orexin levels exhibit an increase during the dark period, coinciding with their active phase (i.e., ZT13–24). Conversely, orexin levels decrease during the light period, reaching their lowest levels between ZT6 and ZT12 (Yoshida et al. 2001; Fujiki et al. 2001; Desarnaud et al. 2004). We conducted our experiment between ZT6 and ZT12, when orexinergic neurons are less active (Lee et al. 2005; Marston et al. 2008), to maximize the effects of our stimulation.

Notably, the reduction in 2-AG after 20 min of blue light pulse despite the higher 2-AG levels observed at 10 min (i.e., at 10 min LP), may reflect a shift of the neuronal activity from being depolarized to becoming hyperpolarized (20 min LP) according to change of the firing activity and of fluorescence intensity of the Fluo4-Dex-positive inputs to OX-A neurons.

Blue light stimulation of the eye with 10 min LP triggers a transient increase in the neuronal discharge of LH neurons, followed by a long-lasting suppression of their activity at 20 min LP. This

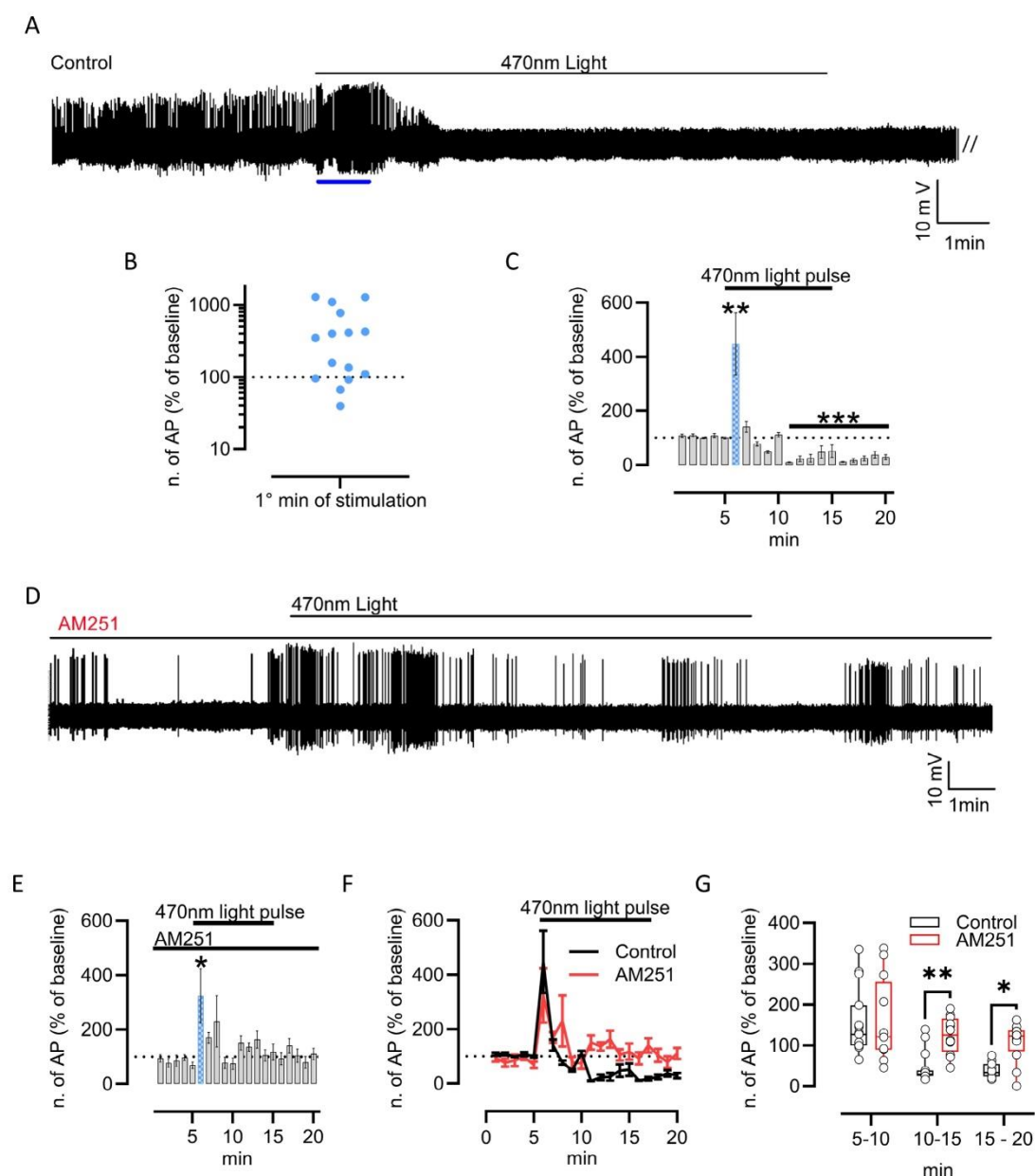


FIGURE 4 | Legend on next page.

second phase was reversed by the selective CB1R antagonist AM251, suggesting that this occurs through retrograde 2-AG signaling at pre-synaptic VGlut1/CB1Rs inputs, followed by inhibition of glutamate release by depolarization-induced suppression of excitation (DSE). Interestingly, it has been shown that 2-AG-mediated LTD requires pre-synaptic activity in target afferents, independently of the properties of these afferents to trigger or not a 2-AG release (Heifets and Castillo 2009). Therefore,

while the primary source of excitation in our experiment originates from the RHT fibers activated by blue light eye exposure, the subsequent increase of 2-AG levels could induce LTD in the nearest excitatory fibers not directly activated by the blue light stimulation. This 2-AG-mediated paracrine effect may lead to an overall CB1R-mediated decrease in excitatory synaptic activity, ultimately resulting in a complete cessation of electrical activity in the recorded cells.

FIGURE 4 | Eye Blue light exposure modulates hypothalamic neuronal activity via CB1R. (A) Representative trace and experimental design. After 5 min of baseline recording, 470 nm blue light stimulation was applied to the eyes for 10 min before recording for another 5 min in the dark condition. The blue line indicates the first minute of recording during eye stimulation. (B) Number of action potentials (AP) recorded during the first minute of stimulation expressed as a percentage of the baseline (dotted line). (C) Number of action potentials recorded during stimulation is expressed as a percentage of the baseline. $n = 15$ neurons from $n = 10$ mice, $*p < 0.05$ and $**p < 0.01$, at t_{6min} = two-tailed one-sample T -Test, $t = 3.044$, $df = 14$, $p = 0.0088$; at t_{11min} = two-tailed one-sample T -Test, $t = 48.53$, $df = 14$, $p < 0.001$. The blue bar underlines the percentage of the action potential in the first minute of recording during light stimulation. (D) Representative trace and experimental design. (E) Number of action potentials recorded during stimulation expressed as a percentage of the baseline; $n = 12$ neurons from 8 mice, t_{6min} = two-tailed one-sample T -Test, $t = 2.246$, $df = 11$, $p = 0.0462$. The blue bar represents the first minute of recording during light stimulation. (F) AP frequency trend in control (black line) and AM251-treated mice (red line). (G) Box plot comparing AP frequency over time between control (black box plot) and AM251-treated mice (red box plot). Control, $n = 15$ neurons from 10 mice; AM251, $n = 12$ from 8 mice. 2-way ANOVA with Sidak post hoc test. In particular, the ANOVA: Time F (DFn 2, DFd 75) = 14.54 $p < 0.0001$, Treatment F (DFn 1, DFd 75) = 13.87 $p = 0.0004$, Interaction F (DFn 2, 75) = 3.092 $p = 0.0512$; Sidak's multiple comparisons test = $**p < 0.01$ and $*p < 0.05$. t_{10-15} : $p = 0.0033$; t_{15-20} : $p = 0.0139$.

Conversely, in the presence of AM251, we observed a transient increase in the action potential discharge, suggesting the involvement of additional homeostatic plasticity mechanisms. Despite an initial increase in firing rate during the first minute of recording following eye blue light exposure, AM251 administration was able to prevent the firing rate suppression possibly because in the healthy brain, neuronal excitability and synaptic strength are finely regulated to maintain network activity within physiological limits (Lignani et al. 2020). This regulation may explain why, despite the initial increase in firing frequency, the effect was not sustained over time but diminished by returning to baseline levels. Another factor to consider when interpreting electrophysiological results is the possibility that the observed phenomena may reflect a depolarization block. This condition involves an initial increase in action potential frequency, followed by a gradual decrease in amplitude, ultimately resulting in the cessation of spiking. This pattern is commonly linked to strong depolarization, often driven by calcium channel activation and a diminished sodium ion driving force.

Our previous findings also support this aforementioned mechanism of 2-AG-mediated silencing of neuronal activity, as we observed a higher number of VGluT1/CB1R-positive puncta per OX-A neuron, as compared to the CB1R/VGAT-positive ones (Cristino et al. 2013).

Our study was conducted in *Mus musculus*, a nocturnal animal. Therefore, our findings should ideally be compared to diurnal rodent models such as *Arvicanthis niloticus*, which are also used to study circadian rhythm-related mechanisms. Several studies have highlighted differences in the activity of orexin neurons between diurnal and nocturnal species, indicating that further experiments are required to explore this aspect (Azeez et al. 2018; Martínez et al. 2002; Ikeno and Yan 2018).

To further deepen the experimental results shown in this work, we propose conducting juxtacellular recording-labeling and immunohistochemical identification of orexin neurons to high-light potential features in their firing responses to eye blue light stimulation. On the other hand, although we did not proceed with the identification of the recorded neurons in the LH in this study, we observed a surprising homogeneity in the pattern of responses to eye stimulation.

Therefore, we considered that the anatomical and functional results obtained could reasonably allow us to discuss the effect of the eye blue light stimulation on the selective activity of the ipRGC-derived fibers forming the I-RHT and projecting to OX-A neurons.

Nonetheless, the results of these experiments are consistent with the change of 2-AG levels and Fluo-4-dextran optical density, suggesting that the phenomena observed under blind conditions during electrophysiological recordings could also reasonably occur in OX-A neurons within the PFH, as indicated by c-Fos activity.

Overall, our findings lay the groundwork for future research to clarify other aspects of the impact of blue light on the brain, including its role in regulating the sleep/wake cycle, arousal, and its effects on food intake and mood.

Future research should investigate whether and how blue light-induced activation of the RHT influences metabolic pathways and appetite regulation. This could provide valuable insights into light-mediated metabolic adaptations, particularly in shift workers and individuals exposed to artificial lighting at night. Indeed, growing evidence suggests that excessive or inappropriate night-time exposure to blue light may have detrimental effects on cognitive and physiological functions. Our findings support the importance of orexinergic and endocannabinoid signaling in antidepressive non-pharmacological therapies, although further studies need to define the interplay of the two systems at the molecular level.

Author Contributions

Nicola Forte: data curation, investigation, methodology, writing – original draft, formal analysis. **Roberta Imperatore:** data curation, formal analysis, investigation, methodology, writing – original draft. **Brenda Marfella:** methodology, formal analysis. **Alessandro Nicois:** methodology, formal analysis. **Roberta Verde:** methodology, formal analysis. **Letizia Palomba:** methodology, formal analysis. **Vincenzo Di Marzo:** funding acquisition, writing – review and editing, supervision. **Luigia Cristino:** conceptualization, funding acquisition, project administration, writing – review and editing, supervision, resources.

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Disclosure

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Some of the authors were on the editorial board member of *Frontiers* at the time of submission. This had no impact on the peer review process, and the final decision on other papers.

Ethics Statement

Experiments were performed following the European Union animal welfare guidelines [European Communities Council Directive of September 22, 2010 (2010/63/EU)] and the Italian Decree no. 26/2014.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

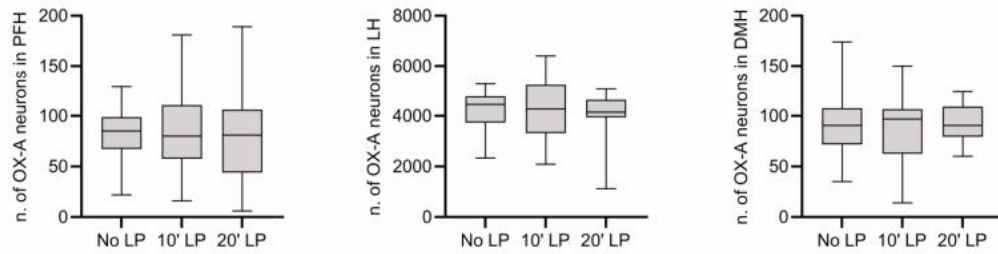
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Supplementary Data



Supplementary Figure. Box plot showing the number of OX-A neurons counted in the PFH, LH, and DMH respectively.

4. Discussion

The studies collected in this thesis converge on the notion that the endocannabinoidome operates as a highly plastic, integrative system that links peripheral metabolic and sensory signals to central mechanisms of neuronal excitability, synaptic plasticity, and behavior. Rather than behaving as a narrowly defined neuromodulatory pathway, the endocannabinoidome emerges as a distributed molecular platform in which classical endocannabinoids, endocannabinoid-like mediators, and their diverse receptors form a shared biochemical language through which the brain decodes and integrates information coming from the gut microbiota, the dietary environment and photic cues. Across the three experimental Tasks, this language is instantiated through context-dependent changes in 2-AG levels, dynamic redistribution of CB1 receptors on excitatory and inhibitory terminals, engagement of nuclear receptors such as PPAR α , and crosstalk with other neuromodulatory systems.

A first major axis delineated by the thesis concerns the interplay between microbiota-derived metabolites, dietary lipids, and hypothalamic–mesolimbic circuits of energy balance and reward (Tasks I and II). The case of the olive oil studies expands this view by showing that a specific qualitative feature of the diet—the presence of oleic-acid-derived lipoamino acids OIGly and OIAla—can shape both reward processing and obesity risk via the endocannabinoidome (Task I). Here, OIGly and OIAla act as endogenous brakes on palatable-food-induced reward, selectively abolishing conditioned place preference without inducing nonspecific anorexia under standard feeding conditions. At the neuronal level, these mediators reduce the firing rate of VTA dopaminergic neurons through a PPAR α -dependent mechanism, directly dampening the mesolimbic output that encodes the incentive salience of highly palatable food. At the systemic level, chronic administration of OIGly/OIAla, as well as feeding with an isocaloric high-olive oil diet, attenuates weight gain and cumulative caloric intake under a lard-rich obesogenic regimen, effects which are again dependent on PPAR α activation. Lipidomic and metataxonomic analyses further show that an olive-oil-based diet increases OIGly (and, to a lesser extent, OIAla) in multiple brain regions and the colon, while promoting a more favorable microbiota configuration and partially restoring diversity lost under lard feeding. Together with the acetate data from the task II, these results strongly support the concept that dietary

components, by modulating the eCBome and microbiota, can recalibrate both hypothalamic energy-sensing pathways and mesolimbic reward circuits. The study of acetate (Task II), a short-chain fatty acid produced by gut microbial fermentation, demonstrates that this metabolite is not merely a peripheral energy substrate but also a central signal capable of reshaping the activity of orexin/hypocretin neurons within the lateral hypothalamus and perifornical area. By engaging periventricular GPR43-expressing microcircuits, acetate enhances GABAergic drive onto orexin neurons, reduces their cFOS activation, normalizes an overactive hypothalamic 2-AG tone in obese animals, and concomitantly promotes POMC expression in the arcuate nucleus, thereby tipping the balance toward an anorexigenic state. These findings identify a microbiota–acetate–endocannabinoidome axis as a concrete route through which the gut can exert top-down control on hypothalamic hubs coordinating arousal and feeding and highlight orexin neurons as a critical point of convergence for microbial and lipid signals.

The third part concerns the integration of photic information with hypothalamic regulation of arousal and metabolic state, again through the lens of the endocannabinoidome (Task III). By identifying and functionally characterizing a blue light–responsive lateral retinohypothalamic pathway projecting from melanopsin-expressing ipRGCs to the perifornical hypothalamus, the thesis uncovers a circuit that operates in parallel to the canonical SCN projection but is specifically wired to orexin neurons. Retinal afferents in this pathway are glutamatergic, express VGluT1 and CB1, and form synaptic contacts with OXA neurons, establishing an anatomical substrate for endocannabinoid modulation of light-driven excitatory input. Blue light exposure elicits a rapid, transient increase in hypothalamic 2-AG levels, alongside an early phase of heightened OXA neuronal activity followed by a CB1-dependent suppression of firing and a reduction in presynaptic calcium signals at retinal terminals. Pharmacological blockade of CB1 abolishes the late inhibitory phase while sparing the initial activation, demonstrating that the endocannabinoid component is specifically responsible for implementing a feedback brake on sustained glutamatergic drive. In line with this, the overall time course of 2-AG and of electrophysiological responses follows a bell-shaped profile, consistent with a transient engagement of the endocannabinoid system to prevent overactivation of orexin circuits during prolonged blue light stimulation. These findings extend the concept of the endocannabinoidome

as a state-dependent modulator of excitability to the domain of photic entrainment, suggesting that it contributes to fine-tuning the impact of environmental light on arousal and, indirectly, on energy-related behaviors.

When considered together, these tasks outline a coherent picture in which endogenous lipid mediators and their receptors form a plastic, context-sensitive signaling system at the intersection of peripheral physiology and central network dynamics. In metabolic contexts, the endocannabinoidome translates microbiota- and diet-derived signals into adjustments of hypothalamic and mesolimbic circuits that govern appetite, reward, and body weight. In the photic domain, it tempers the impact of blue light on orexin neurons, preventing excessive excitation of arousal systems. Across these diverse scenarios, a recurring organizational motif emerges: a peripheral or environmental perturbation induces local changes in endocannabinoid tone and receptor distribution, which in turn recalibrate the excitation–inhibition balance and activity patterns of defined neuronal populations, ultimately leading to measurable alterations in behavior. This recurrent motif lends strong support to the guiding hypothesis of the thesis, namely that the endocannabinoids and endocannabinoid-like molecules shared a common molecular language through which the central nervous system integrates metabolic and sensory information to adapt its functional state to internal and external demands.

5. Conclusions

The work presented in this thesis provides experimental support for a unitary view of the endocannabinoidome as an integrative and dynamic system that governs the interplay between peripheral signals and central neuronal networks in multiple, apparently disparate, physiological and pathological conditions. In the domain of energy homeostasis and feeding, the data demonstrate that microbiota-derived acetate and olive oil–derived lipoamino acids converge on hypothalamic and mesolimbic targets, respectively, to modulate orexin neuron activity, endocannabinoid tone, dopaminergic firing, and gut microbiota structure, thereby influencing both appetite and vulnerability to diet-induced obesity. Finally, in the photic regulation of arousal, the thesis uncovers a blue light–responsive lateral retinohypothalamic pathway that

uses 2-AG–CB1-dependent feedback to modulate glutamatergic input onto orexin neurons, revealing a novel interface between environmental light, the endocannabinoidome, and hypothalamic control of wakefulness and energy balance.

Taken together, these findings substantiate the notion that endogenous lipid mediators and their receptors form a shared molecular framework through which the brain integrates metabolic and photic cues to adjust neuronal excitability, synaptic plasticity, and behavior. By articulating this framework across five distinct but conceptually aligned Task-based studies, the thesis not only refines the understanding of the endocannabinoidome's role in specific circuits and disease models but also opens translational perspectives in which nutritional, pharmacological, and chronobiological interventions targeting this system may be rationally combined to promote brain homeostasis and resilience.

6. Introduction - Ongoing Research

As a completion to the present dissertation, I have included a set of still unpublished experimental data, which are currently the object of a manuscript in preparation on which I will appear as first author. These results, presented in preliminary form, have been incorporated because they are closely consistent with the guiding theme of the thesis, namely the role of the endocannabinoidome, and in particular of the CB2 receptor and the 2-AGP/LPA1R axis – in maintaining the homeostasis of neuronal networks and glial cells under metabolic stress.

6.1 Task IV – “Studies of 2-Arachidonoyl-sn-glycerol-3-phosphate (2-AGP), a Lysophosphatidic Acid, Mediates High-Fat Diet–Induced Working Memory Impairment via Tau Hyperphosphorylation and Microglial Dysfunction”

Understanding why overeating in obesity can create self-reinforcing cycles requires investigation beyond basic physiological processes regulating appetite and eating behavior. Many influences that might seem secondary are in fact tightly linked to these processes, helping to sustain and strengthen the cycle over time.

From this perspective, investigating the molecular and cellular mechanisms underlying alterations in executive functions, such as working memory (WM), is of primary importance, given that impairments in memory are demonstrated to contribute to overeating (Higgs et al., 2005; Higgs et al., 2008; Seitz et al., 2021)¹⁻³, and memory deficits are observed in both obese animal models and humans (Chen et al., 2024; Butler et al., 2025; Coppin et al., 2014)⁴⁻⁶. Moreover, the memory deficits lend further support to the hypothesis that obesity represents a significant risk factor for the development of neurodegenerative disorders, including Alzheimer's disease (Neto et al., 2023; Koriath et al., 2025; Tabassum et al., 2020)⁷⁻⁹

Studies have shown that working memory dysfunction results from multiple converging mechanisms, including dysregulation of the dopaminergic system (Herzog et al., 2024)¹⁰,

inflammatory processes (Yang et al., 2020; Shields et al., 2020)^{11,12}, alteration in gut microbiota composition (Liu et al., 2025; Arnoriaga-Rodríguez et al., 2020)^{13,14}, structural and functional changes in the hippocampus (Tran et al., 2025)¹⁵, as well as alterations in hypothalamic circuits (Burdakov et al., 2020)¹⁶.

Among the hypothalamic neuron-derived metabolic regulators linking energy homeostasis to memory function, the neuropeptide OX-A has emerged as a pivotal modulator (Burdakov et al., 2025)¹⁷. OX-A is a 33-amino acid peptide synthesised in the lateral hypothalamus (LH) that regulates circadian functions like arousal, feeding, and the sleep–wake cycle (Sakurai et al., 2007)¹⁸. Both OX1R and OX2R receptors are broadly expressed within the hippocampus (Kostansek et al., 2023)¹⁹. Impaired OX-A signalling within the hippocampal dentate gyrus circuit compromises neurogenesis and ultimately episodic memory under conditions of obesity (Forte et al., 2021)²⁰. Moreover, I highlighted the role of orexin in the pathogenesis of Alzheimer's disease (Forte et al., 2022; Yin et al., 2023; Ragsdale et al., 2025)²¹⁻²³.

OX-A controls the production of the endocannabinoid 2-AG through OX1R receptors (Fernández-Rilo et al., 2023)²⁵. In turn, 2-AG can be converted into 2-arachidonoyl-sn-glycerol-3-phosphate (2-AGP), a lysophosphatidic acid (Forte et al., 2022; Fernández-Rilo et al., 2023; Nakame et al., 2002)^{21,25,26}, forming what can be called the OX-A/2-AG/2-AGP signalling axis. We demonstrated that in the hypothalamus, 2-AGP, via the LPA1 receptor (LPA1R), modulates the proline-rich tyrosine kinase 2 – glycogen synthase kinase 3 beta (Pyk2-GSK3 β) pathway and induces Tau hyperphosphorylation at the residue T231 (pT231-tau)(Forte et al., 2022; Fernández-Rilo et al., 2023)^{21,25}. This mechanism rewires synaptic connections, thereby modulating feeding circuits (Fernández-Rilo et al., 2023)²⁵. Although the pT231-Tau production is not inherently neurotoxic (Forte et al., 2022)²¹, it is recognized as an early cerebrospinal fluid (CSF) biomarker for Alzheimer's disease (Hempel et al., 2001; Buerger et al., 2002; Buerger et al., 2002)²⁷⁻²⁹.

Paralleling these mechanisms, HFD-induced obesity profoundly compromises brain homeostasis by promoting neuroinflammation and synaptic dysfunction. Among central immune cells, microglia play a pivotal role as sensors of metabolic stress and orchestrators of neuroimmune responses. To this end, a genetically modified mouse model was employed. Heterozygous CX3CR1^{GFP/+} knock-in mice, in which one CX3CR1 allele is replaced by a GFP reporter while the second allele remains functional, were used to visualize CX3CR1-expressing cells without disrupting receptor function. HFD has been shown to drive

microglia toward a dysfunctional phenotype characterized by impaired phagocytosis, cholesterol ester accumulation, lipid droplet formation, and acquisition of a pro-inflammatory profile collectively defined as Lipid Droplet-Accumulating Microglia (LDAM). This change is particularly relevant in hippocampal regions, such as the DG, where microglia contribute to synaptic remodelling, the maintenance of the excitation–inhibition balance, and the support of adult neurogenesis, all processes crucial for learning and memory.

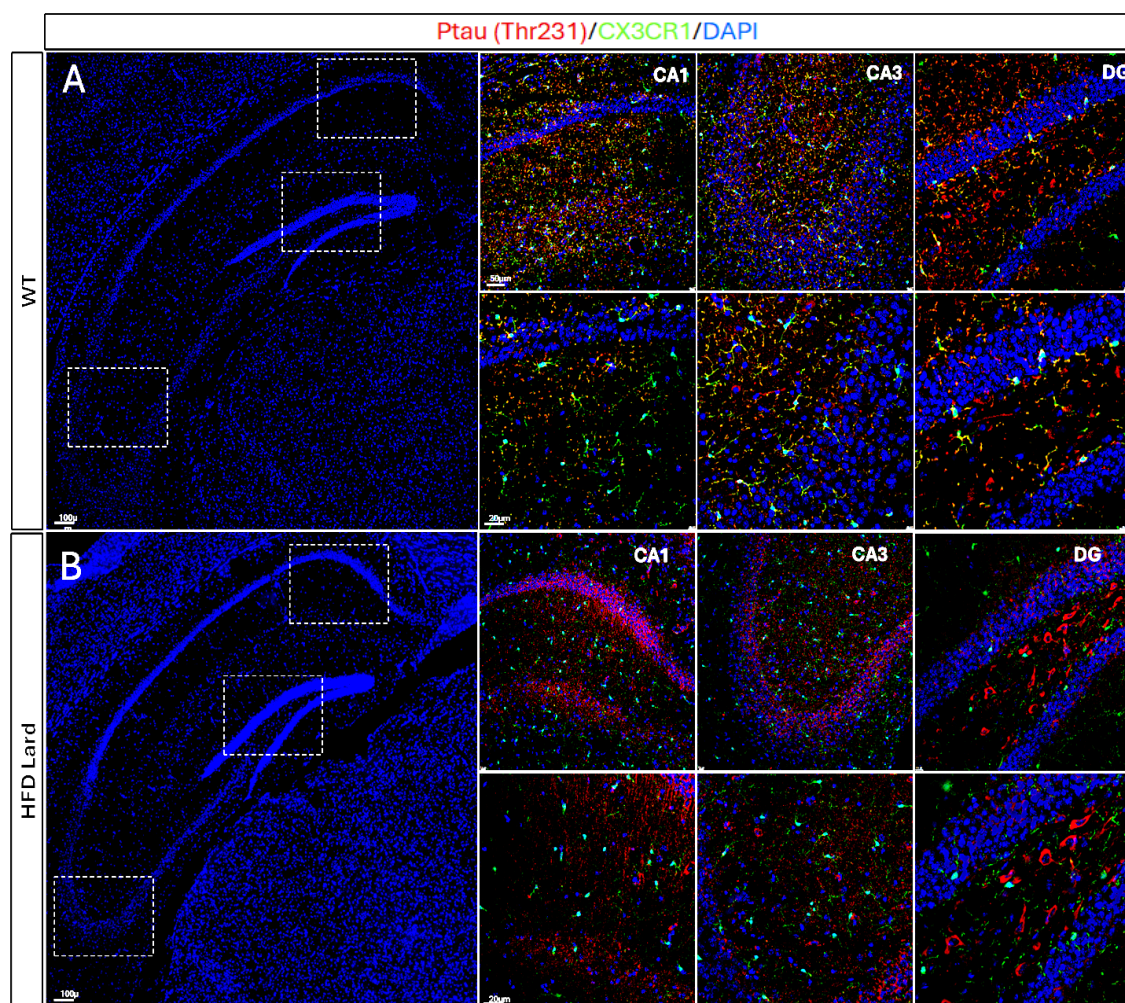
Within the endocannabinoid system, the CB2 is predominantly expressed in microglia and has been implicated in the regulation of neuroinflammation and immune responses, but its role in obesity-induced microglial and synaptic dysfunction remains largely unexplored. Emerging evidence suggests that metabolic challenges such as HFD may alter CB2 expression and signaling, potentially compromising the ability of microglia to efficiently handle lipid overload and to maintain synaptic integrity. Recent findings indicate that LPA1R can directly promote the acquisition of a pro-inflammatory microglial phenotype (Kwon et al., 2018; Plastira et al., 2020; Gaire et al., 2019)³⁰⁻³². LPA1 signaling has been demonstrated to trigger microglial activation in LPS-induced septic mouse brains as well as in LPS-stimulated rat primary microglia (Kwon et al., 2018)³⁰. This is particularly relevant in obesity, where gut microbiota dysbiosis elevates circulating LPS levels, priming microglia toward a chronically activated, pro-inflammatory state (Forte et al., 2020)³³. Notably, LPA1R knockout mice are resistant to diet-induced obesity, highlighting the receptor's pivotal role in linking metabolic stress to neuroinflammation (Dusauley et al., 2009)³⁴.

Based on this evidence, here I hypothesize that in obesity, increased LPA1R activation via the OX-A/2-AG/2-AGP signaling axis acts as a dual pathogenic driver: on one hand, by promoting Tau hyperphosphorylation, and on the other, by maintaining a chronic pro-inflammatory microglial state that further reinforces Tau phosphorylation through GSK3 β activation (Li et al., 2003; Green et al., 2012)^{35,36}. Concurrently, the loss of CB2 expression in DG microglia compromises the receptor's neuroprotective functions in controlling lipid metabolism, maintaining phagocytic competence, and dampening inflammatory responses. This integrated framework identifies CB2 activation as a potential strategy to counteract both the neuroinflammatory and metabolic aspects of HFD-induced cognitive decline.

6.2 Results

6.2.1 Obesity induces Tau hyperphosphorylation at the T231 residue in CA3-CA1 areas of the hippocampus.

Building on our initial observation that Tau protein is highly phosphorylated at residue T231 in the hypothalamus of obese mice, we next asked whether this modification also occurs in the hippocampus, a region critically involved in working memory. Analysis of hippocampal sections revealed a pronounced increase in phosphorylated Tau immunoreactivity in obese mice compared with controls. This increase was particularly evident in the CA3–CA1 subfields, whereas the dentate gyrus (DG) displayed comparatively lower levels of immunoreactivity [Fig. 1].



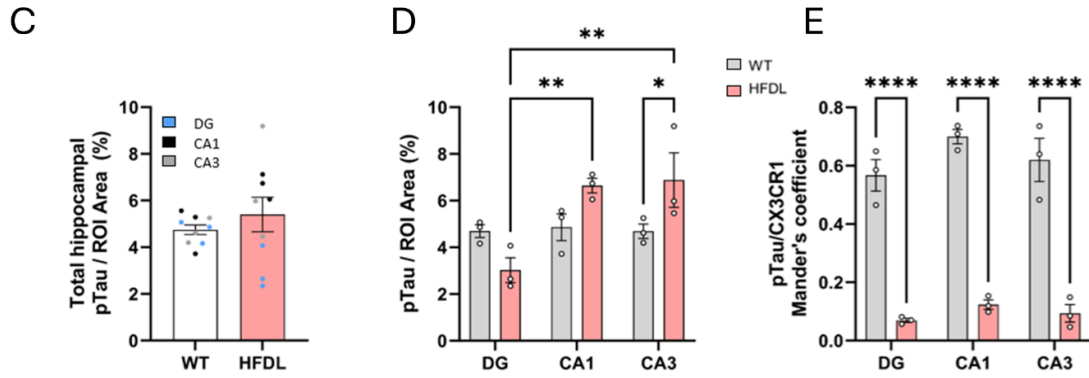


Figure 1. High-fat diet increases Tau phosphorylation in the CA3-CA1 hippocampal area. (A-B) Confocal images of immunolabeling of the hippocampus of lean and HFD CX3CR1 mice. (C) Bar graph showing the percentage of pTau in all the examined areas (DG, CA3, CA1). (D) Bar graph showing the percentage of pTau subdivided per area, Two-way ANOVA test. (E) Manders' coefficient of the pTau/CX3CR1 signal in DG, CA1 and CA3 areas. Two-way ANOVA test.

6.2.2 Inhibition of LPA1R via AM095 improve memory capacity and hippocampal plasticity in obese mice.

The spatial and working memory capacity refers to the amount of information that can be encoded, maintained, and retrieved in the brain within a specific time interval. It critically depends on the integrity of hippocampal circuits and has been here investigated in mice using the 6-IOT and 6 DOT tests. The 6-IOT (Six-Item Object Test) primarily assesses working memory and recognition memory based on object identity and/or location by using six distinct objects placed in an arena which are firstly explored by mice (Sample phase). After a short delay, one object is moved (object location) or replaced (object recognition). Preference for the novel/displaced object indicates memory performance in the cognitive domain of object recognition and location memory (visual/spatial object-based).

6-DOT focuses on spatial navigation and short-term memory and has been applied according to the protocol from (De Risi et al., 2025)³⁷ through the following steps:

- Presenting six distinct objects in an arena,

- Allowing mice short-term exploration,
- Measuring how many unique objects are inspected before repeat visits in a single trial,
- Using the count of unique explorations and revisit errors as metrics of working memory capacity.

These tests rely on the animal's ability to encode, maintain, and update information about objects and their spatial arrangement over short periods and our results didn't find difference from the 6-IOT performance in obese mice *vs* lean mice but revealed a critical impairment from the 6-DOT score in obese *vs* lean mice that was recovered in obese (both HFD or ob/ob) mice and not in lean mice after treatment with AM095, a selective LPA1R antagonist (mouse). Noteworthy, AM095 treatment worsened the 6-DOT score in lean mice suggesting a critical role of 2-AGP and LPA1R molecular pathway in the regulation of spatial and working memory in mice (Fig. 2)

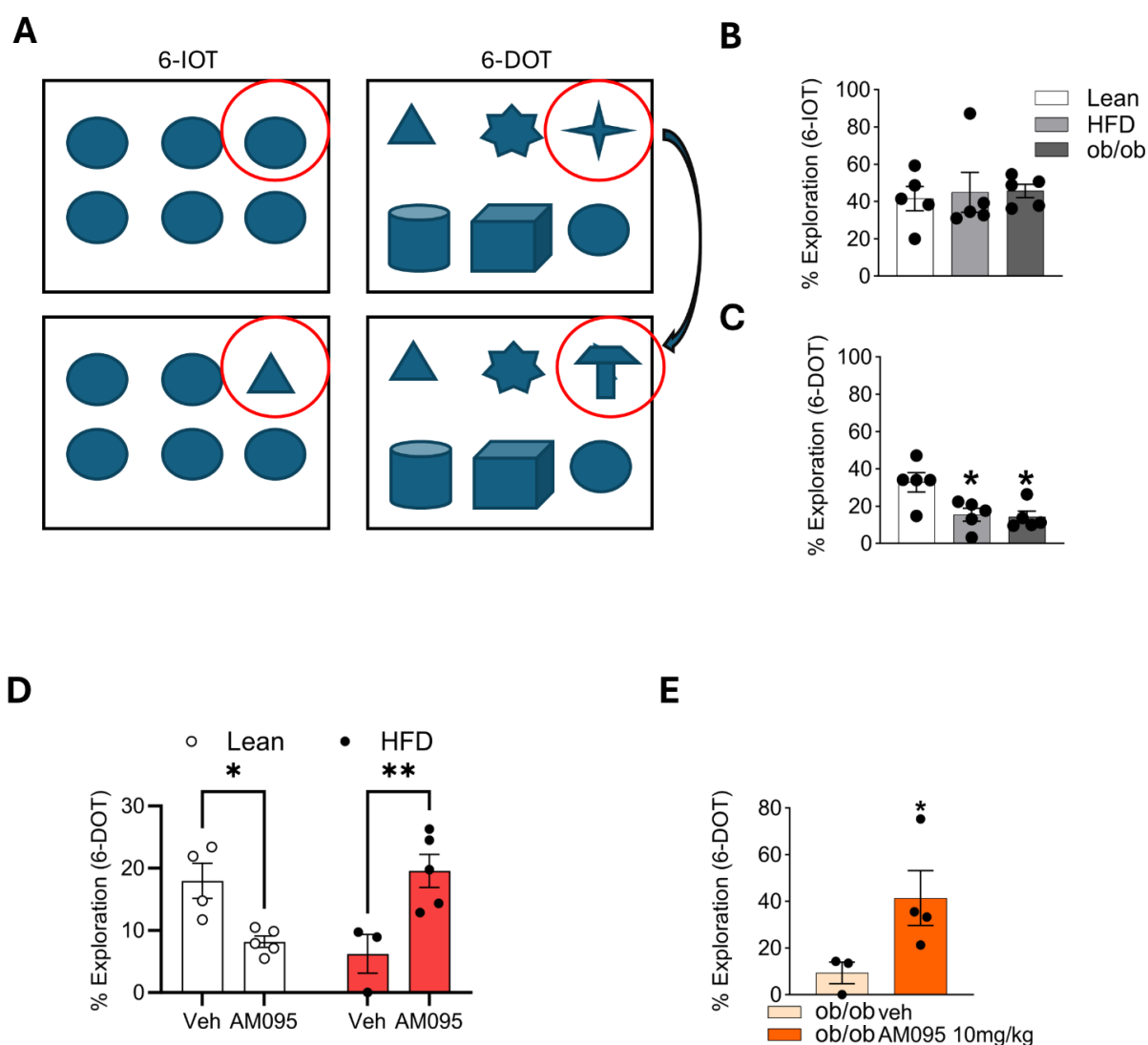


Figure 2. LPA1R agonist AM095 rescues impaired working memory capacity induced by a high-fat diet. (A) Schematic representation of the study and test phase of the object memory span task. During the test phase, only one object was substituted with a different one in both conditions – six identical objects (6-IOT) for the situation at low memory load and six different objects (6-DOT) in the case of high memory load. (B) Percentage of exploration of the new object 6-IOT. (C) Percentage of exploration of the new object in a 6-DOT. One-way ANOVA. (D) Percentage of exploration of the new object in a 6-DOT in mice treated with or without AM095 (10mg/Kg). (E) Percentage of exploration of the new object in a 6-DOT in mice treated with or without AM096 (10mg/Kg). T-Test.

6.2.3 HFD induces lipid droplet-rich microglia and reduces CB2 and synaptic markers in the DG

Histochemical analyses of hippocampal sections from HFD and STD mice revealed profound changes in microglial morphology and lipid content. Using IBA1 or CX3CR1-eGFP to label microglia and BODIPY staining to detect neutral lipid droplets, microglia in HFD mice displayed a foamy phenotype with prominent lipid droplet accumulation, particularly evident in the DG, consistent with the emergence of an LDAM-like state. Quantification of BODIPY-positive area per single microglial cell profile showed a significant increase in lipid load in HFD mice compared with STD animals, confirming that HFD promotes lipid storage in hippocampal microglia.

In the same brain region, microglial CB2 immunoreactivity was markedly reduced in HFD mice. Measurement of CB2-positive area normalized to individual microglial profiles indicated a significant down-regulation of CB2 expression in DG microglia from obese animals relative to STD controls, while CA1 and CA3 regions showed less pronounced changes. A skeleton analysis further revealed that microglia in HFD displayed altered branching patterns, consistent with a shift toward a more activated and less “surveillant” morphology. In parallel, immunostaining for PSD95 revealed a decrease in synaptic density in the DG of HFD mice, suggesting that microglial lipid overload and CB2 loss are associated with compromised synaptic remodeling and a potential vulnerability of hippocampal circuits supporting mnemonic functions [Fig.3]

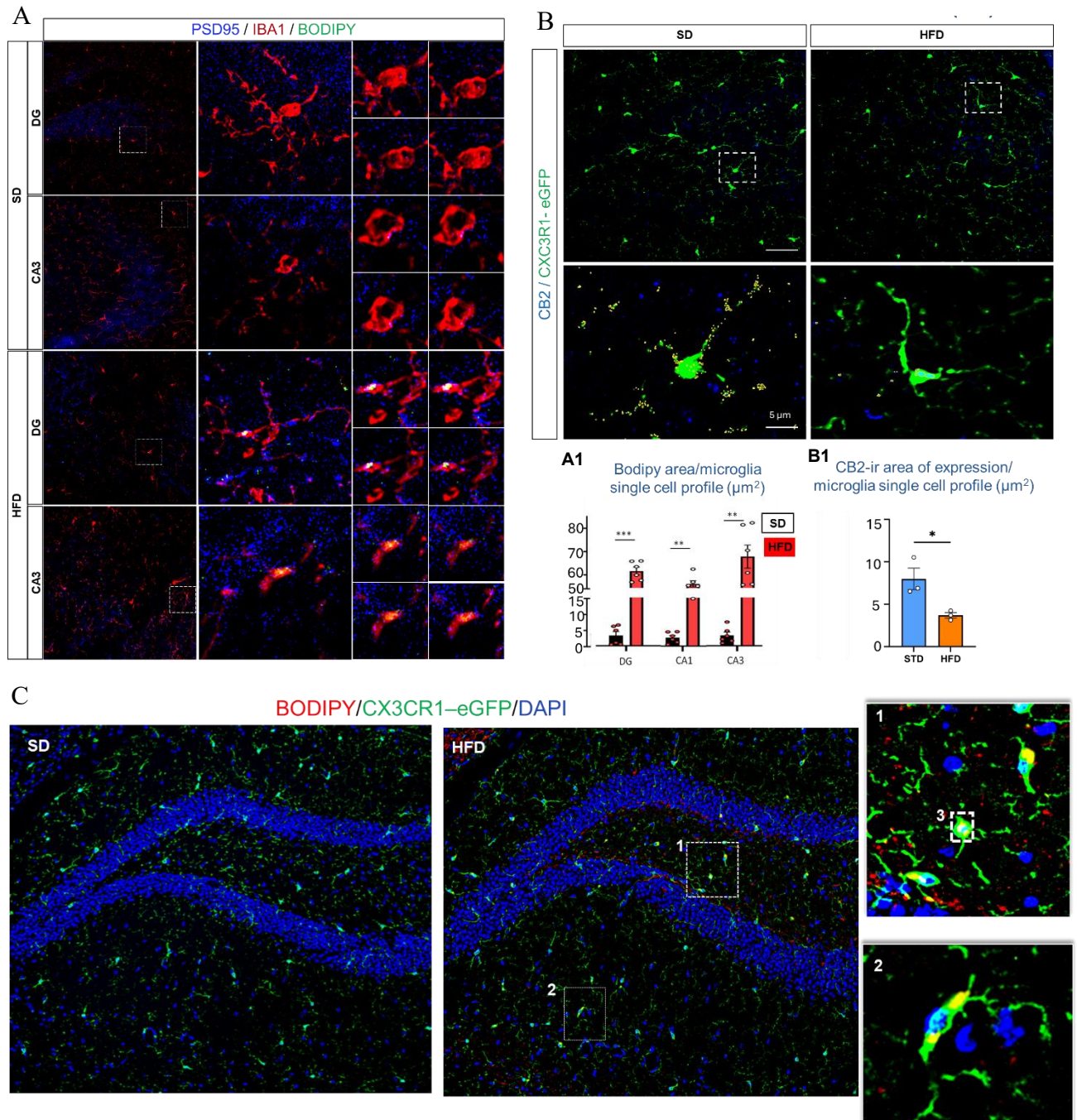


Figure 3. Lipids droplets accumulation and CB2- expression in microglia. (A) Representative micrographs showing microglial morphology at different magnifications in distinct hippocampal regions (dentate gyrus, DG, and CA3) of mice fed a standard diet (SD) or a high-fat diet (HFD). Microglia display a surveillant phenotype under SD

conditions and an activated/amoeboid phenotype following HFD. Microglial cells are labeled with IBA1 (red), post-synaptic density protein PSD95 is shown in blue, and intracellular lipid droplets are detected with BODIPY (green). **(A1)** Bar graph of BODIPY-positive area per microglial single-cell profile (μm^2). **(B)** Representative images of CB2 receptor expression in microglial cells from SD- and HFD-fed mice. Microglia are shown in green, CB2 expression in blue, and CB2 immunoreactivity colocalizing with microglia is highlighted in yellow. **(B1)** Bar graph of CB2-immunoreactive area per microglial single-cell profile (μm^2). **(C)** Representative images of the DG region from CX3CR1-eGFP mice fed SD or HFD. Microglia are shown in green, lipid droplets in red, and nuclei in blue. Micrographs 3 and 4 show higher-magnification views of lipid droplet accumulation from micrographs 1 and 2, respectively.

6.2.4 High-fat diet reshapes hippocampal synaptic transmission and plasticity

Adult male C57BL/6J mice maintained on HFD for eight weeks develop obesity and show marked alterations in hippocampal synaptic transmission, particularly in the dentate gyrus. Recordings of eEPSCs from granule cells revealed that HFD significantly reduces eEPSC amplitude compared with STD-fed controls, indicating a decrease in synaptic strength at perforant path–DG synapses, while synaptic depression during sustained 80 Hz stimulation remains unchanged. Analysis of the cumulative amplitude profile during 2-s trains showed a reduction in the estimated readily releasable pool (RRP_{syn}) at excitatory synapses, accompanied by an increase in release probability, suggesting that HFD primarily impairs the size of the vesicle pool immediately available for release rather than the kinetics of vesicle replenishment.

A similar analysis at inhibitory synapses showed that HFD also reduces the amplitude of eIPSCs, with a reorganization of the paired-pulse ratio consistent with changes in RRP and release probability at GABAergic terminals. The overall weakening of GABAergic transmission is interpreted as a homeostatic adaptation aimed at preserving the overall excitability of the DG network in the face of reduced excitatory input, rather than as a mere parallel deficit. In vivo LTP recordings in the medial perforant path–DG pathway showed that HFD, as well as the genetic model of obesity ob/ob, compromise the induction and/or

maintenance of LTP compared with lean controls, whereas this alteration was not fully reproduced in ex vivo slices from HFD animals, suggesting that circulating or paracrine inflammatory mediators contribute critically to the in vivo LTP deficit.

To directly investigate the role of inflammation and CB2 signaling in this context, hippocampal slices were exposed to LPS to mimic the inflammatory state associated with HFD. Under these conditions, LTP at medial perforant path–DG synapses was markedly reduced; however, treatment with the selective CB2 agonist JWH-133 restored LTP to values comparable to those of control slices not treated with LPS. Representative traces and time-courses of fEPSP slopes showed that JWH-133 effectively reverses LTP suppression induced by LPS without altering basal synaptic transmission, supporting a specific role of CB2 activation in rescuing long-term synaptic plasticity under conditions of elevated inflammatory tone. In line with this, quantification of LTP amplitude in the different groups indicated that, while LPS alone significantly reduces potentiation, co-treatment with JWH-133 brings LTP back toward control levels in both males and females.

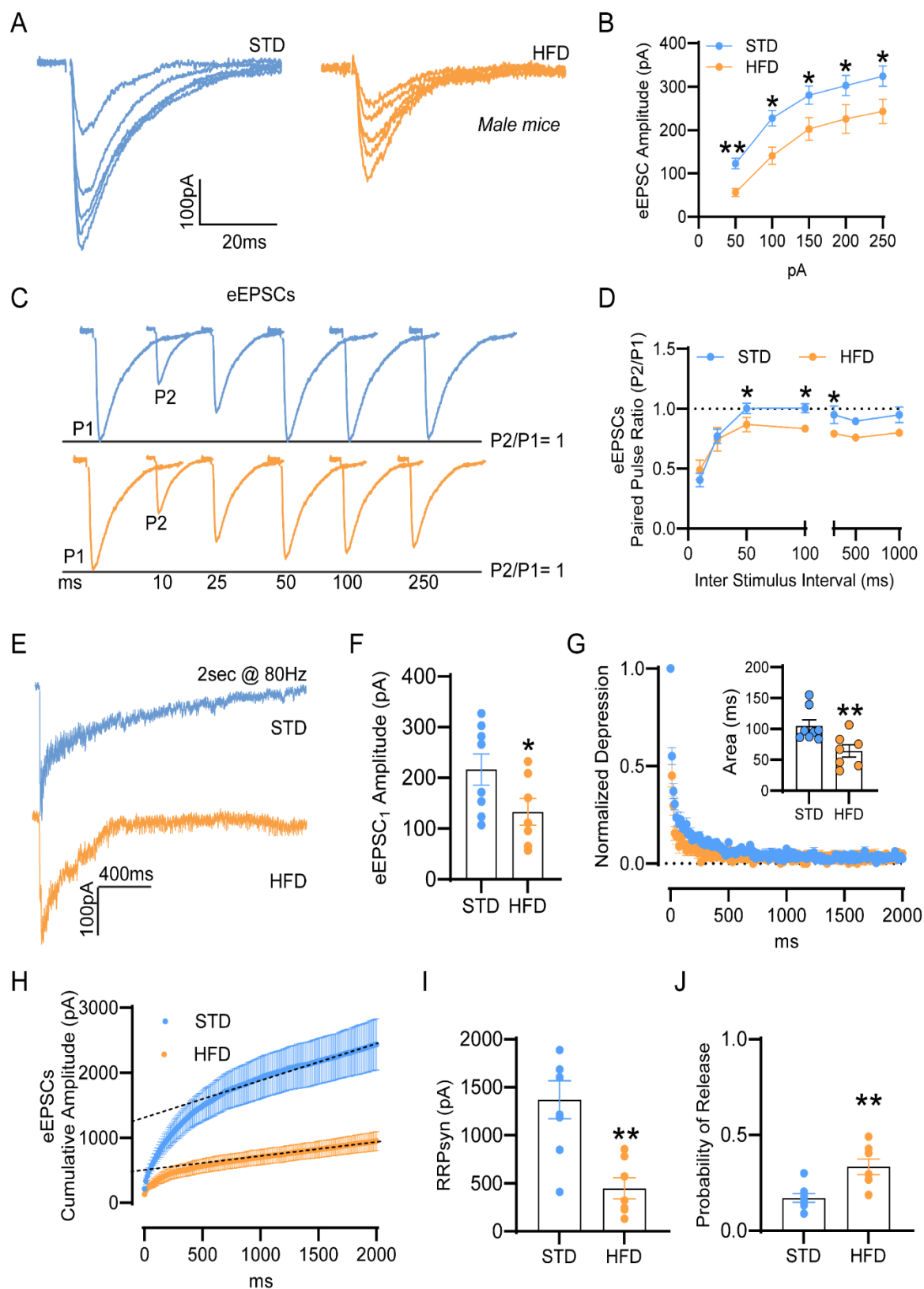


Figure 4. In male C57BL/6J mice, a high-fat diet decreases the amplitude of eEPSCs and modifies the paired-pulse ratio by reducing the size of the readily releasable pool

and enhancing the probability of neurotransmitter release. (A) Representative eEPSC traces from granule cells (GCs) of STD (blue) and HFD (orange) mice evoked by excitatory input (50–250 μ A, 100 μ s). **(B)** Input/output curves of eEPSC amplitude versus stimulation intensity. **(C)** Representative paired-pulse eEPSCs at interstimulus intervals (10–1000 ms). **(D)** Mean paired-pulse ratio (PPR $\pm$ SEM) as a function of interval. **(E)** eEPSCs evoked by 2 s, 80 Hz stimulation in the molecular layer; artefacts removed. **(F)** Amplitude of the first eEPSC in the train. **(G)** Normalised eEPSC amplitude (mean $\pm$ SEM) during 80 Hz trains; inset (G1): area under the curve per neuron. **(H)** Cumulative amplitude profile of the 2-s train with extrapolated fit estimating RRP_{syn}. **(I)** Bar graph of RRP_{syn} at excitatory synapses. **(J)** Bar graph of release probability (Pr) in STD and HFD mice.

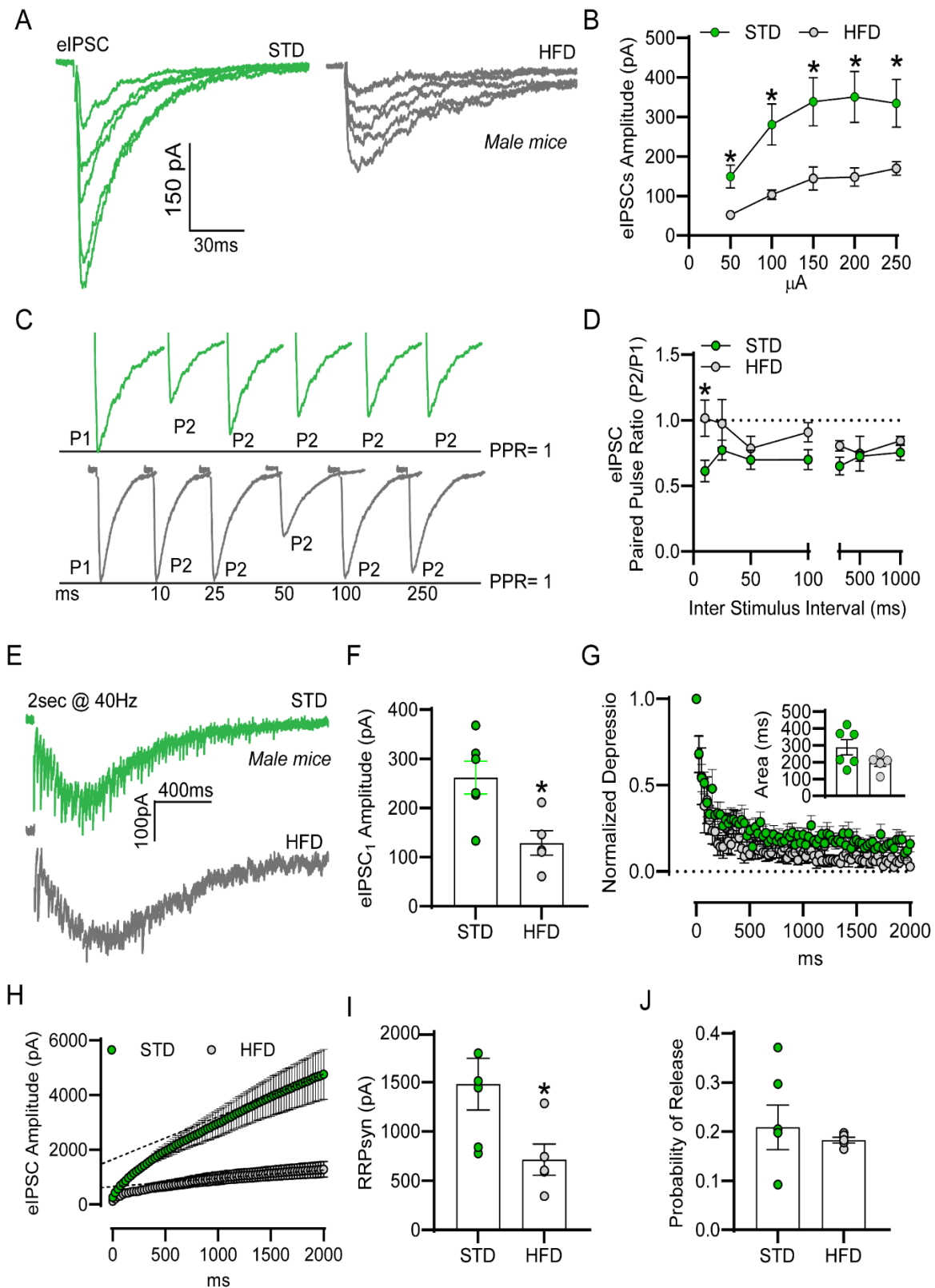


Figure 5. In male C57BL/6J mice, a high-fat diet decreases the amplitude of eEPSCs and modifies the paired-pulse ratio by reducing the size of the readily releasable pool and enhancing the probability of neurotransmitter release. (A) Representative eIPSC

traces from granule cells (GCs) of STD (green) and HFD (grey) mice evoked by inhibitory input (50–250 μ A, 100 μ s). **(B)** Input/output curves of eIPSC amplitude versus stimulation intensity. **(C)** Representative paired-pulse eIPSCs at interstimulus intervals (10–1000 ms). **(D)** Mean paired-pulse ratio (PPR $\pm$ SEM) versus interval. **(E)** eIPSCs evoked by 2 s, 40 Hz stimulation; artefacts removed. **(F)** Amplitude of the first eIPSC in the train. **(G)** Normalised eIPSC amplitude (mean $\pm$ SEM) during 40 Hz trains; inset (G1): area under the curve per neuron. **(H)** Cumulative amplitude profile of the 2-s train with extrapolated fit estimating RRP_{syn}. **(I)** Bar graph of RRP_{syn} at inhibitory synapses. **(J)** Bar graph of release probability (Pr) at inhibitory synapses in STD and HFD mice.

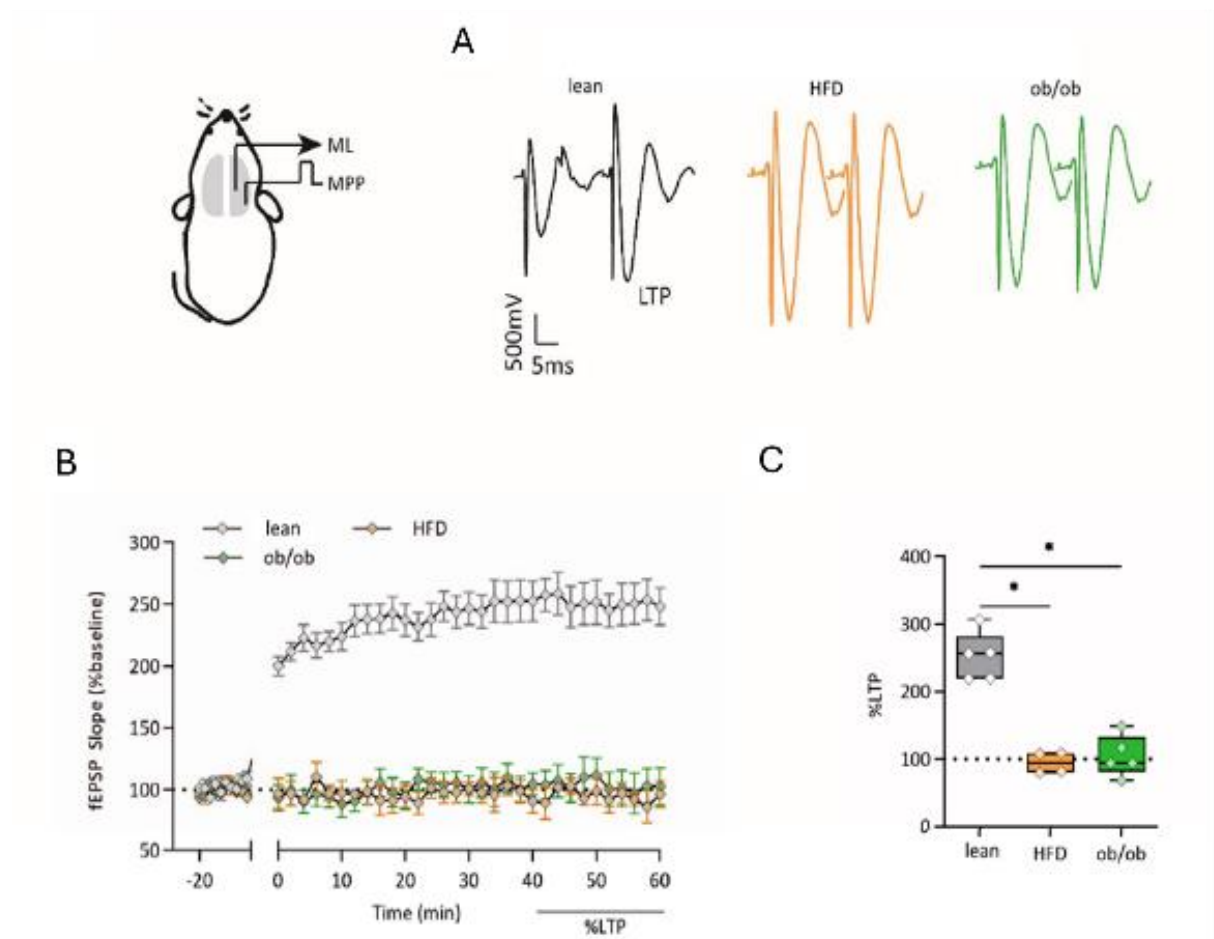


Figure 6. HFD alters hippocampal LTP in vivo. Schematic representation of the in vivo LTP recording, the stimulation electrode was placed in the MPP, the recording site was in the ML. **(A)** Representative traces recorded in lean, HFD, and ob/ob before and after the TBS. **(B)** Plot of the fEPSP slope recorded before and after the induction of the LTP (time

0) in the DG of $n = 5$ lean mice, $n = 4$ HFD mice, and $n = 5$ ob/ob mice; data are presented as mean values $\pm$ SEM, as the extent of LTP was calculated as a percentage of the baseline between the last 40 and 60 min of recording. **(C)** Box plot of the LTP in lean, HFD, and ob/ob mice.

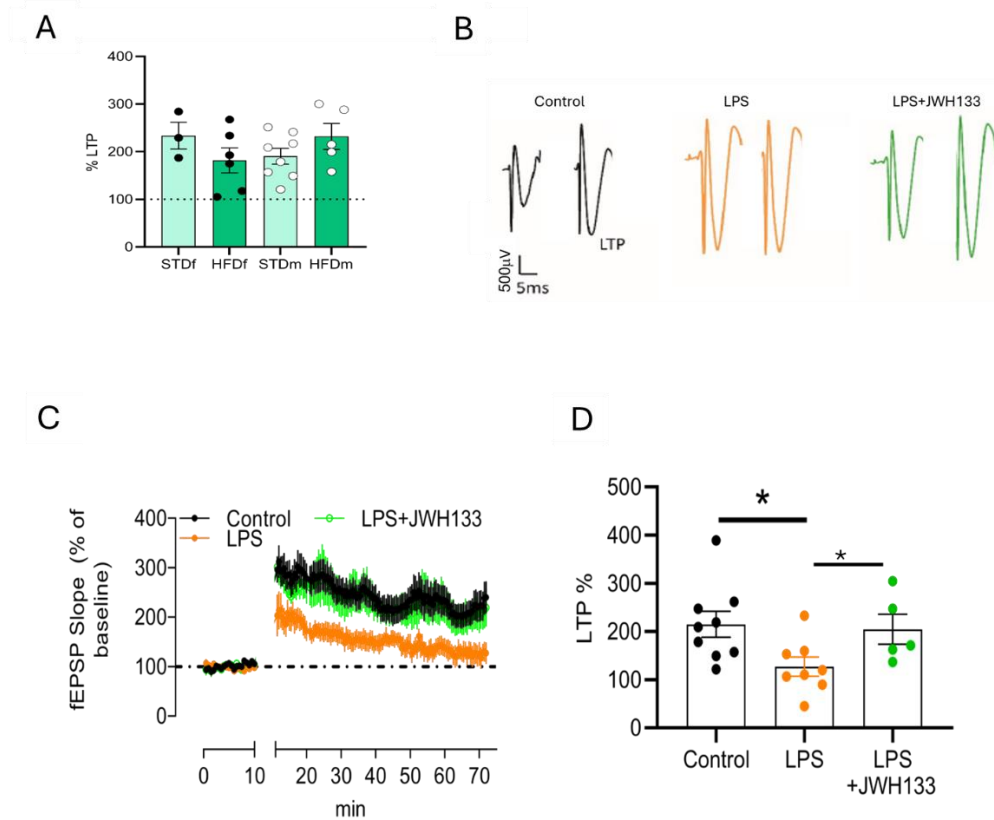


Figure 7. JWH133 rescues LTP inhibition due to LPS treatment in MPP – DG synapses. **(A)** Bar Graph of the LTP in female mice under standard diet (STDf), female mice under HFD diet (HFDf), in male mice under standard diet (STDm) and male mice under HFD diet (HFDm). **(B)** Representative traces recorded in control, LPS, and LPS+JWH133 before and after the high frequency train (HFS). **(C)** Plot of the fEPSP slope recorded before and after the induction of the LTP (time 0) in the DG. **(D)** Bar Graph of the LTP in control, LPS, and LPS+JWH133 slices

6.2.5 CB2 activation counteracts palmitic acid–induced lipotoxicity in microglia in vitro

To dissect the cellular mechanisms underlying the in vivo observations, BV2 microglial cells, an immortalized murine cell line, were exposed to palmitic acid (PA) as an in vitro model of lipotoxic stress, and the effects of CB2 modulation were evaluated using JWH-133 and the antagonist AM630. PA treatment induced marked lipid droplet accumulation, visualized by BODIPY Green staining, and increased production of reactive oxygen species (ROS), measured with CellROX Deep Red, accompanied by a reduction in microglial phagocytic capacity, assessed by uptake of pHrodo Green–conjugated *E. coli* bioparticles. These changes are consistent with a transition toward a metabolically stressed and functionally impaired microglial phenotype. Pre-incubation with JWH-133 prior to PA exposure significantly reduced lipid droplet accumulation and ROS levels, while preserving or partially restoring phagocytic activity, indicating that CB2 activation improves multiple aspects of PA-induced microglial dysfunction. In contrast, pre-incubation with AM630 did not improve microglial fitness; in combination with PA, the antagonist failed to reduce lipid accumulation or oxidative stress and did not rescue phagocytosis. Importantly, neither JWH-133 nor AM630 alone, in the absence of PA, produced significant changes compared with vehicle-treated controls in any of the measured parameters, underscoring that CB2 modulation exerts its protective effects specifically under lipotoxic challenge rather than altering basal microglial physiology. These in vitro results are consistent with the in vivo data, indicating that CB2 down-regulation or blockade weakens microglial resilience to metabolic stress, whereas CB2 activation enhances lipid handling capacity, redox balance, and phagocytic competence.

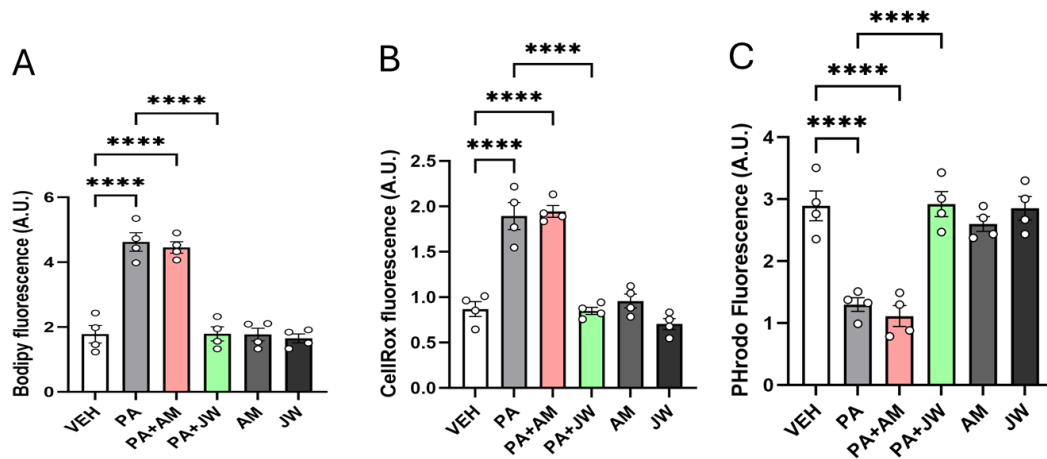


Figure 8. CB2 receptor activation reduces palmitic acid–induced lipid accumulation, oxidative stress, and improves phagocytosis in BV2 microglial cells

BV2 murine microglial cells the cells were plated in 24-well plates at a density of 4000 cells/cm². The cells were subsequently treated with: Vehicle, Palmitic Acid (PA, 1 μ M, 12 h), AM + PA (30 min pre-incubation with 10 μ M AM630 followed by 1 μ M PA for 12 h), JW133 + PA (30 min pre-incubation with 0.5 μ M JW133 followed by 1 μ M PA for 12 h), AM (30 min pre-incubation with 10 μ M AM630), and JW133 (30 min pre-incubation with 0.5 μ M JW133). The experimental conditions were monitored using the Incucyte® live-cell analysis system, which enables continuous, real-time analysis by maintaining optimal environmental conditions (37°C, 95% humidity, and 5% CO₂) and utilizing vital fluorescent dyes. The following parameters were assessed: **(A)** lipid droplet accumulation using Bodipy™ Green (1:1000), **(B)** reactive oxygen species (ROS) production using CellROX™ Deep Red (2.5 μ M), and **(C)** phagocytic capacity using pHrodo™ Green E. coli BioParticles™ conjugate.

7. Conclusions

The present set of unpublished data supports a comprehensive model in which HFD-induced obesity exerts a dual and converging pathogenic action on hippocampal structure and function. On one side, metabolic stress enhances Tau hyperphosphorylation at residue T231 and promoting a state of molecular vulnerability within hippocampal circuits. On the other side, the same obesogenic milieu progressively shifts hippocampal microglia toward a lipid droplet-rich, metabolically overloaded and functionally compromised phenotype, characterized by CB2 down-regulation, altered morphology, reduced synaptic coverage and a diminished capacity to support synaptic plasticity in the dentate gyrus. Electrophysiological analyses further reveal that HFD reduces excitatory synaptic strength by decreasing the readily releasable pool at perforant path synapses and weakens inhibitory transmission in a fashion compatible with a homeostatic attempt to preserve overall network excitability under chronic metabolic challenge, while *in vivo* recordings demonstrate that both diet-induced and genetic obesity impair LTP in the DG in a manner that is not fully reproduced in *ex vivo* slices, underscoring the indispensable contribution of circulating and paracrine inflammatory mediators to long-term synaptic dysfunction.

Within this intricately orchestrated pathophysiological framework, LPA1R and CB2 emerge not as isolated effectors but as pivotal, partially complementary determinants of hippocampal function and synaptic resilience. Pharmacological antagonism of LPA1R with AM095 restores both working memory capacity and CA3–CA1 LTP in obese mice, indicating that dampening the 2-AGP/LPA1R axis can reverse cognitive impairment and normalize activity-dependent synaptic strengthening in circuits critically involved in information encoding and retrieval. Conversely, selective activation of CB2 with JWH-133 rescues LTP in hippocampal slices exposed to LPS, a condition that mimics the inflammatory environment associated with obesity, thereby demonstrating that CB2 signaling can re-establish long-term synaptic plasticity in the dentate gyrus even in the presence of heightened immune tone. In parallel, *in vitro* experiments using BV2 microglial cells show that CB2 activation markedly reduces palmitic acid-induced lipid droplet accumulation, lowers oxidative stress and restores, at least in part, phagocytic competence, whereas CB2 antagonism fails to confer any protection, reinforcing the concept that CB2 is a crucial guardian of microglial metabolic fitness and immune functionality when cells are

exposed to sustained lipotoxic stimuli.

Taken together, these converging lines of evidence identify LPA1R and CB2 as key, interrelated regulators of hippocampal microglial and synaptic homeostasis in the context of HFD-induced obesity and suggest that the loss of CB2 expression in DG microglia contributes to the collapse of neuroprotective mechanisms that normally safeguard hippocampal circuits from metabolic and inflammatory insults. By demonstrating that LPA1R antagonism can reverse working memory deficits and CA3–CA1 LTP impairments, and that CB2 agonism can restore both microglial function and dentate gyrus LTP under inflammatory and lipotoxic conditions, this work points to both receptors as promising, and potentially synergistic, therapeutic targets for preserving synaptic integrity and preventing or attenuating cognitive decline associated with obesity and related neurodegenerative processes. In this light, the endocannabinoidome – through the dynamic interplay between CB2-dependent signaling and the generation and sensing of metabolic lipid mediators such as 2-AGP – emerges as a central integrative hub that coordinates the responses of neuronal networks and glial cells to chronic metabolic stress, offering a unifying framework to understand, and ultimately modulate, the complex crosstalk between energy imbalance, neuroinflammation and hippocampal dysfunction.

8. Overall Discussion and Concluding Remarks

This doctoral thesis proposes a unified view of the endocannabinoidome as a plastic and integrative system capable of translating dietary, metabolic, and environmental stimuli into coordinated adaptations of neuronal circuits that regulate energy homeostasis, synaptic plasticity, and cognitive functions. Although the different Tasks address apparently distinct domains, from dietary lipid quality to microbial signaling and photic stimuli, the findings converge toward a coherent model in which the endocannabinoidome operates as a shared molecular language through which the brain integrates peripheral and environmental inputs, thereby modulating neuronal excitability and behavior according to internal and external demands. In the murine model of high-fat diet–induced obesity, metabolic dysregulation is associated with a profound alteration of the microbiota–gut–brain axis and an imbalance in endocannabinoid tone within key regions such as the hypothalamus, mesolimbic system, and hippocampus. However, the data demonstrate that this dysfunction is not merely driven

by caloric excess, but rather by the quality of the lipids consumed. An olive oil–enriched diet, despite being isocaloric compared with a saturated fat–rich diet, prevents the typical alterations of the endocannabinoidome observed in HFD-fed mice. This protective effect is due to the administration of olive oil–derived lipoamino acids, OIGly and OIAla, which possess endocannabinoid-like structures and, through activation of PPAR α , modulate the hedonic consumption of palatable food. These findings indicate that the endocannabinoidome acts as a sensor of lipid quality, translating peripheral nutritional signals into modulation of mesolimbic circuits and preserving the functionality of neuronal networks that regulate arousal. In this context, it becomes evident that the components of the endocannabinoidome functionally cooperate, reinforcing the concept of an integrated lipid signaling network.

This perspective extends to signals of microbial origin. Acetate, a metabolite produced by the gut microbiota, acts as a molecular bridge between peripheral metabolic state and the activity of orexinergic neurons in the lateral hypothalamus. Under obese conditions, the reduction in acetate-dependent tone is associated with altered orexinergic regulation, impairing the control of food intake. Restoration of acetate levels re-establishes the functionality of these neurons, suggesting that the endocannabinoidome, being sensitive to the organism's overall metabolic state, participates in translating microbial signals into modulation of neuronal excitability. Within this framework, the microbiota–endocannabinoidome–brain axis represents a fundamental circuit through which the central nervous system monitors and integrates information regarding peripheral energy status.

To this metabolic dimension is added an environmental component. Exposure to blue light, characteristic of the modern environment, modulates hypothalamic 2-AG levels and influences the circadian activity of orexinergic neurons through an endocannabinoid feedback mechanism at the level of retino-hypothalamic glutamatergic afferents. This finding further expands the integrative paradigm outlined in the thesis, demonstrating that the endocannabinoidome not only responds to nutritional and microbial stimuli but also functions as an interface for photic signals. The transient modulation of 2-AG in response to blue light suggests a role for the endocannabinoidome in the fine-tuning between activation and stabilization of arousal circuits, with potential implications for circadian synchronization and metabolic alterations associated with chronic light pollution. Once again, stimuli of distinct nature converge on the same molecular nodes that regulate presynaptic neurotransmitter release and synaptic plasticity.

The conceptual continuity among these findings is synthesized within the framework of the ongoing study on synaptic and cognitive damage induced by a high-fat diet. In the early phases of metabolic stress, the endocannabinoidome appears to function as a compensatory system aimed at restoring homeostasis; however, under chronic exposure, this adaptive capacity becomes overwhelmed. Tau hyperphosphorylation in the hippocampus represents a critical transition from adaptation to vulnerability. Working memory deficits and alterations in synaptic plasticity demonstrate that persistent dysregulation of endocannabinoid lipid circuits can trigger a neurodegenerative cascade. In this context, the LPA1 receptor emerges as a critical mediator of obesity-associated synaptic damage, shifting the research focus from the regulation of food intake to the preservation of neuronal network integrity. The progression observed across the different Tasks highlights how molecular mechanisms that promote resilience under physiological conditions may, when chronically altered, contribute to the pathogenesis of cognitive deficits.

Taken together, these findings demonstrate that the endocannabinoidome constitutes a central hub at which dietary, metabolic, and environmental signals converge. The quality of dietary lipids, microbiota-derived metabolites, and photic stimuli coordinately modulate endocannabinoid pathways that regulate synaptic release, neuronal excitability, and both short- and long-term plasticity. This convergence suggests that obesity is not merely a peripheral metabolic disorder, but rather a neuro-metabolic condition characterized by a profound reorganization of synaptic modulatory systems. The endocannabinoidome therefore emerges as a molecular platform through which the brain integrates information concerning nutritional status, intestinal microbial composition, and the light environment, dynamically adapting circuits involved in energy balance, arousal, and memory.

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