UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

PhD Thesis

"Orexin-A-mediated control of VTA-NAc mesolimbic pathway contributes to obesity by endocannabinoid 2-AG-mediated disinhibition of dopaminergic neurons"

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Abbreviations

2-AG:	2-arachidonoylglycerol
AcbSh:	nucleus accumbens shell
AEA:	ethanolamide (*or* anandamide)
AgRP:	Agouti-related protein
AP:	adaptor protein
ARC:	arcuate nucleus
BBB:	blood brain barrier
BMI:	body mass index
CB_1R:	cannabinoid receptors type 1
CB_2R:	cannabinoid receptors type 2
CCK:	cholecystokinin
CPP:	conditioned place preference
CSF:	cerebrospinal fluid
DA:	dopamine
DAG:	diacylglycerol
DAGL:	diacylglycerol lipase
DIO:	diet-induced obesity
DMH:	dorsomedial hypothalamus
e.g.:	*exempli gratia*
eCBs:	endocannabinoids

Abbreviations

ECS:	endocannabinoid system
EPSCs:	excitatory postsynaptic currents
FAAH:	fatty acid amide hydrolase
GPCR:	G-protein coupled receptor
GPCR:	G protein-coupled receptor
GRK:	G protein-coupled receptor kinase
Hcrt:	hypocretin
HFD:	high fat diet
HPF:	highly palatable food
i.e.:	*id est*
Icv:	intracerebroventricular
IEB:	intestinal epithelial barrier
Ip:	intraperitoneal
IUPHAR:	International Union of Basic and Clinical Pharmacology
LepRs:	leptin receptors
LH:	lateral hypothalamus
MAGL:	monoacylglycerol lipase
mHTT:	mutant huntingtin
MSNs:	medium-size spiny neurons
NAc:	nucleus accumbens

Abbreviations

NAPE PLD:	NAPE selective phospholipase D
NAPEs:	N-acyl phosphatidylethanolamines
NCS:	neuronal calcium sensor
NMDARs:	N-methyl-D-aspartate receptors
NPY:	neuropeptide Y
NT:	neurotensin
OX_1R:	orexin 1 receptor
OX_2R:	orexin 2 receptor
OX-A:	orexin-A
OX-B:	orexin-B
PD:	Parkinson's disease
PeFLH:	perifornical area in the posterior lateral hypothalamus
PFC:	prefrontal cortex
PIP2:	phosphatidylinositol 4,5-bisphosphate
PLA_2:	phospholipase A_2
PLC:	phospholipase C
PPO:	prepro-orexin
PVN:	paraventricular nucleus
SN:	substantia nigra
TH:	tyrosine hydroxylase

Abbreviations

THC:	Δ^9-tetrahydrocannabinol
TRP:	transient receptor potential
VMH:	ventromedial hypothalamus
VTA:	ventral tegmental area
VTA:	ventral tegmental area
WAT:	white adipose tissue
WHO:	World Health Organization

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Abstract

Obesity is a widespread condition due to an imbalance between high food intake and low energy expenditure in combination with genetic, biological, environmental and behavioural factors. The rate of obesity has increased in last decades and it has been considered responsible for a deterioration in the quality of life and a decrease in life expectancy. A pivotal aspect of obesity is the consumption of food beyond homeostatic needs, known as "food addiction". This condition is driven by abnormal redundant surrounding edible stimuli and vulnerability to hedonic eating that lead to a lack of control over food intake and a progressive worsening reward homeostasis, shifting the effective doses of highly palatable food (HPF) to higher set points. Recent evidence show that the orexin system is implicated in reward and reinforcement processes, widely based on dopamine (DA). An endocannabinoid-mediated disinhibition of OX-A expressing neurons has been described in the lateral hypothalamus (LH) of leptin signalling-defective obese mice (*ob/ob* mice), with consequent elevation of orexin A trafficking and release to many LH target areas.

Here, we found enhancement of OX-A trafficking and release to VTA of obese *ob/ob* mice. We sought to investigate the effect of this increased OX-A release on dopamine trafficking in obese mice and the molecular mechanism through which the aberrant OX-A signalling could enhance DA synthesis in the ventral tegmental area (VTA) and its release to nucleus accumbens (NAc). Moreover, we want to investigate if the high dopaminergic tone is able to decrease the reward baseline sensitivity in obese mice by promoting the D2R desensitization in the NAc, the main target of VTA in the mesolimbic circuit. With this purpose, by exploiting morphological, pharmacological and biochemical approaches we found a significant increase of OX-A release to the VTA of obese mice concomitantly with elevation of endocannabinoid-mediated DA synthesis and release in the NAc, wherein triggers an overstimulation-induced desensitization of D2R by binding β-arrestin2.

These data show that OX-A is a powerful modulator of dopaminergic system and mesolimbic reward processes, which are strictly associated with the

hyperphagia that occurs in obese mice. These results are of special relevance since aberrant OX-A signalling during obesity could trigger the vicious circle underlying compulsive reward-associated feeding, because obese individuals overconsume food to counteract the reduced state of reward, thereby contributing to overweight and addiction.

Obesity is a worldwide widespread condition due to a dysregulated mechanism of feeding control in combination with genetic, biological, environmental and behavioural factors of individuals (*Wang et al., 2018*; *Al Massadi et al., 2019*). The prevalence of obesity nearly tripled over the last forty years, increasing up to 44 million of adults with body mass index (BMI) greater than or equal to 30 only in America *(Riva et al, 2006)* and reaching the 5% of U.S. population in the last decade for people with morbid obesity, a form of severe obesity (BMI over 40) *(Flegal et al, 2002)*. The World Health Organization (WHO) reports that in 2016 more than 650 million of adults were obese. Obesity is classified as the sixth most important risk factor that contributes to worldwide diseases (*Haslam and James, 2005*), and is due to complex multifactorial dynamics encompassing an imbalance between high food intake and low energy expenditure coupled to sedentary life style. This condition leads to an abnormal storage of fat and consequent excess of body weight, with an impairment of health-related quality of life and diminished average life expectancy. Obesity is associated to different chronic metabolic comorbidities, such as diabetes (mainly type 2), cardiovascular diseases (e.g. hypertension and heart disease), respiratory and gastrointestinal problems and several type of cancers (*Haslam and James, 2005*).

A key aspect of obesity is the consumption of food beyond homeostatic needs, other than eating from hunger (*Choi et al, 2010*). Overeating is driven by an abnormal amount of easily accessible highly palatable food (HPF) that increases energy intake and vulnerability to hedonic eating, mechanism known as “food addiction”. The term “food addiction” was introduced for the first time by Theron Randolph in 1956 referring to a psychopathological state in which continuous obtaining and eating palatable foods leads to a lack of control over food intake (*Randolph, 1956; Altman et al, 1996*). The persistent compulsive need for energy-rich food results from habits that become stronger with repetition of binge-like eating and increasingly harder for individuals to regulate. It implies that obese people lose the ability to moderate overeating and feel compelled to eat certain foods (especially

palatable, highly processed or caloric foods, with high-fat and sugar contents), due to the redundant surrounding edible stimuli, amongst which the accessibility and obtaining of palatable food (*Avena et al., 2008; Volkow et al., 2017*). Repeated exposure to HPF cause a compulsive food consumption along with decreasing food intake control in vulnerable individuals especially, with genetic or behavioural predisposing factors. In fact, three characteristics contribute to the diverse level of sensitivity and vulnerability to food: 1) differences in ability to take control over eating appealing food, i.e. the capacity to exert inhibitory self-regulation over the intention to eat HPF; 2) differences in susceptibility to rewarding properties of hedonic food; and 3) differences in predisposition to develop conditioned behavioural responses when people are exposed to HPF (*Volkow et al, 2008*). Food intake is highly regulated by the simultaneous function of two complementary drives: the homeostatic pathway and the more flexible hedonic one. The homeostatic system, essential for the survival, controls energy balance in response to caloric deficits and other metabolic needs following depletion of energy stores (*Liu and Kanoski, 2018*). The non-homeostatic pathway refers to feeding not governed by general energy deficit, that can exacerbates and take over the homeostatic pathway by increasing the craving for compulsive consumption of highly palatable food during periods of relative energy abundance (*Lutter and Nestler, 2009*). A wide variety of factors underlies the aberrant motivation to obtain HPF in obesity, including the palatability of food (usually highly processed and caloric), external conditioned cues (such as the sight and smell of food), social and behavioural circumstances (meals with other people or stress period), previous feeding-relevant experiences with hedonic food and availability of food (*Lau et al, 2017*). The hedonic impact of food is an essential aspect of addiction and sensory reward because both of its caloric content, its systematic consumption (due to its incessant and effortless access) and its rewarding value that overrides the homeostatic nutritional need (*Castro et al, 2016; Coccurello and Maccarrone, 2018*). Development of obesity and food addiction are strictly related to a progressive worsening

reward homeostasis, that leads to transition from casual physiological food intake to compulsive and uncontrolled urge to eat (*Johnson and Kenny, 2010; Volkow et al, 2017*). Overeating and consequent overstimulation of reward neuro circuitry trigger a counter-adaptive mechanism that decreases the baseline sensitivity of reward response. The hypofunction of reward inducted by diet contributes to obesity-associated food addiction by increasing the desire and motivation to consume HPF, in order to compensate this reduced state of reward (*Wang et al, 2002; Koob and Le Moal, 2005*).

Over the years, many scientists have claimed that obesity and eating disorders might share the same dynamic and properties of drug addiction. Consumption of highly palatable food and drug of abuse activate the mesolimbic neuro circuitry consisting of dopamine neurons located in the midbrain and brain areas of their projections, mainly nucleus accumbens (*Hyman et al, 2006; Trinko et al, 2007*). Both drugs and HPF are potent reinforcing stimuli that drastically alter the brain mechanisms underlying synaptic plasticity in mesolimbic circuit, by producing common functional effects (*Nestler, 2005; Volkow et al, 2011*). In particular, both in food and drug addiction, the enhancement of one type of reinforcer (energy-rich food or drug, respectively) provokes a conditioned learning, that is a conditioned association between the reinforcing stimulus and predicting cues, with the concomitant downregulation of other stimuli (*Carr, 2002; Volkow et al, 2017*). Moreover, the repeated stimulation by drugs or food leads to the reset of reward thresholds, shifting their effective doses to higher set points. Indeed, the recurrent administration of a reinforcer generates a compensation that is influenced by past experiences with the same stimulus and processed by memory circuits. The expected reward triggers two different behavioural responses: it overactivates motivational drive to eating or taking addictive drugs and, conversely, impairs the cognitive control circuit (*Volkow et al, 2008*). As a consequence, both hedonic eating and addictive drugs result in a loss of the ability to inhibit the seeking behaviour and to control the drive to consume the drug/food, despite the evidence of

overtly harmful consequences (*Hoebel et al, 2009; Dalley et al, 2011*). Food addiction, as well as drug addiction, is characterised by common behavioural stages: "bingeing", the escalation of food or drug intake; "withdrawal", the anxiety induced by food/drug deprivation; "craving" the increased responding for the reinforcer following its prolonged abstinence; and "sensitization", the enhanced locomotion in response to repeated doses of food or drug (*Koob and Le Moal, 2005; Vanderschuren and Everitt, 2005; Avena et al, 2008*). These characteristics imply that addicts on food or drug, apart from compulsion to take the substance, feel suffering and depressed from discontinuity of its use and persist with it despite the well-known negative consequences (*Riva et al, 2006*). It should be noted that the neurobiological regulation of food intake is even more complicated than the drug abuse one, because of its complex control by both homeostatic and non-homeostatic mechanism, i.e. central and peripheral signals and reward system, respectively (*Levine et al, 2003*). The epidemic situation of food addiction associated to obesity represents one of the major challenge in neuroscience and has triggered an extensive research and massive analysis of this phenomenon; studies over years have been investigating the molecules (such as hormones and neuromodulators) that attempt to maintain energy homeostasis and the neurobiological and peripheral mechanisms underlying the development of obesity (*Novelle and Diéguez, 2018*).

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Chapter 1

Orexinergic system

The orexinergic system is a key modulator of food intake and energy homeostasis that plays a pivotal role in several other physiological functions. The first description of orexines/hypochretins dates back to 1998 by two autonomous groups who discovered and identified independently this novel family of neuropeptides and their receptors in the central nervous system (CNS) (*de Lecea et al, 1998; Sakurai et al, 1998*). de Lecea and collegues, by using subtraction hybridization identified the mRNA sequence that encodes prepro-hypocretin, the putative precursor of two putative peptide transmitters, hypocretin 1 and 2. They termed these molecules "hypocretins", by combining two words: "hypo" for hypothalamus, given their nearly exclusive production in this brain area, and "cretin" for secretin, the gut hormone whereby shares a similar amino-acid sequence (*de Lecea et al, 1998*). Almost simultaneously, Sakurai and collaborators, searching for novel endogenous peptide ligands for multiple orphan G protein-coupled receptor (GPCR) HFGAN72 in rat brain, identified the same two neuropeptides, by using "reverse pharmacology" method. This research group named these peptides as "orexins" (orexin-A and orexin-B equivalent to hypocretin 1 and 2, respectively), after the Greek word "*orexis*", which means appetite, recalling the stimulatory action the central administration produces on food intake (*Sakurai et al, 1998*). However, it became soon clear that the pair of peptides isolated by both groups were identical and the two names are still in use. In particular, the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) suggests the use of word "hypocretin" for the genes encoding the molecules and "orexin" for peptides and receptors (*Gotter et al, 2012*). Thus, the genes encoding the common precursor of both peptides are *Hcrt* and those encoding the two receptors are *Hcrtr1* and *Hcrtr2*. On the other hand, the precursor is known as prepro-orexin (PPO), the two neuropeptides as orexin-A and orexin-B and the receptors as orexin-1 and orexin 2 receptor (OX_1R and OX_2R).

1.1 Orexin peptides

Structural analysis and molecular cloning studies revealed that both neuropeptides are produced from a common precursor polypeptide, prepro-orexin (PPO), composed by 130 amino acids (*de Lecea, 1998*; *Sakurai, 1999; Hungs et al, 2001*). The cDNA analysis revealed that the sequence of this precursor gene (*Hcrt*) is well conserved between rats, mice and humans; the human and mouse *Hcrt* sequences share 83% and 95% similarity with the rat one respectively (*Sakurai, 1999*). The human *Hcrt* is located on chromosome 17 and consists of two exons, the last of which comprises the whole coding region of the final mature peptides (*de Lecea et al, 1998; Sakurai et al, 1998*). The hypocretin precursor protein gives rise to two different mature neuropeptides, orexin-A (OX-A) and orexin-B (OX-B), by proteolytic processing (*Sakurai et al, 1998*) [*Fig. 1.1*].
After different structural analysis of purified neuropeptides, it is known that OX-A is a 33-amino acid peptide of 3562 Da, with N-terminal pyroglutamyl residue, C-terminal amidation and a region between the two ends including two intra-chain disulphide bonds that make the neuropeptide prone to misfolding induced by exposure to free radicals (*Nobunaga et al, 2014*). This structure is completely conserved among diverse mammalian species identified so far (human, mouse, rat, dog, pig, cow and sheep). Orexin-A is the most stable and lipophilic of the two neuropeptides. It has been demonstrated that it is able to cross the blood brain barrier (BBB) in both directions by simple diffusion, and it can be detected in plasma and cerebrospinal fluid (CSF) (*Kastin and Akerstrom, 1999*). Orexin-B is a 28 amino acid, linear peptide of 2937 Da. The C-terminal portion of OX-B has 46% homology of sequence with OX-A, whereas the N-terminal one is more variable. The amino-acid sequences of OX-A and OX-B are highly conserved phylogenetically in both mammalian and non-mammalian vertebrates, thus indicating their role as ancient neurotransmitters or neuromodulators (*Sakurai, 2007; Tsujino and Sakurai, 2009*).

1.2 Orexin receptors

The actions of orexin are mediated by two GPCRs belonging to the rhodopsin family, orexin-1 receptor (OX_1R) and orexin-2 receptor (OX_2R) (also known as Hcrtr1 and Hcrtr2), that share 64% of sequence identity (*Sakurai et al., 1998*). The OX_1R is 425 amino acids long synthesised from the chromosome 1 and OX_2R is 444 amino acids long and differently form from the chromosome 6. The two receptors show different binding affinities for the OX neuropeptides: OX_1R has an order-of-magnitude greater affinity for OX-A than for OX-B (it requires for a half-maximum response a concentration of 30 nM of OX-A but 2500 nM of OX-B), whereas OX_2R is relatively nonselective between the two peptides, since it binds both ligands with equal affinities (reviewed in *Kukkonen and Leonard, 2013*) [*Fig. 1.1*]. Likewise the peptides, also in the case of the receptors, both OX_1R and OX_2R genes are highly conserved among mammalian species (*Sakurai et al., 2007; Tsujino and Sakurai, 2009*). As GPCR, both receptors interact with G protein and they have been observed both pre- and post-synaptically (*van den Pol et al, 1998*). In particular, OX_1R couples to $G_{q/11}$ class of G-protein that triggers the activation of phospholipase C, with the consequent phosphatidylinositol cascade and influx of extracellular Ca^{2+}, presumably through a transient receptor potential (TRP), that finally leads to depolarization of neurons. OX_2R couples to $G_{q/11}$ or G_i classes of G-proteins, in spite of the physiological relevance of the pathway mediated by the latter, which has not been identified yet (*Zhu et al, 2003*).

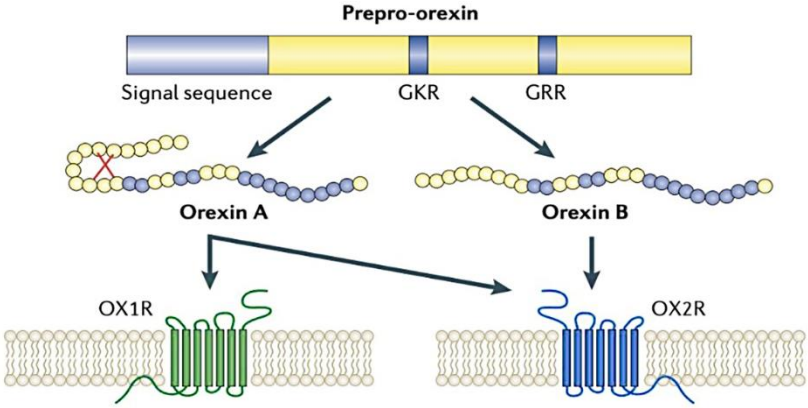

Fig. 1.1 | Schematic representation of the orexin system. Orexin-A (OX-A) and orexin-B (OX-B) are cleaved from their common precursor, prepro-orexin. OX-A contains two disulphide bridges, whereas OX-B is a linear peptide. The actions of orexins are mediated via two GPCR: orexin-1 (OX_1R) that is selective for orexin-A, and orexin-2 (OX2R) receptor, a nonselective receptor for both OX-A and OX-B.

1.3 Production and multiple projection areas

Orexins are synthesised by neurons strictly located in the hypothalamus, particularly in lateral hypothalamus (LH) and in the perifornical area of posterior lateral hypothalamus (PeFLH) (*de Lecea et al, 1998; Date et al, 1999*). Furthermore, orexin peptides and receptors are also spotted outside the CNS, such as in gastrointestinal tract and pancreas, where they affect insulin release, intestinal epithelial barrier (IEB) integrity, intestinal secretions and motility (*Kukkonen et al, 2002; Tunisi et al, 2019).*

LH is a large and heterogeneous area that acts as interface between somatomor, autonomic and endocrine homeostatic systems, by regulating cardiovascular, abdominal visceral and temperature functions (*Peyron et al, 1998*). Moreover, it represents a hub of central regulatory centres involved in the control of feeding, arousal, stress and reward by integrating neural and humoral information (*Bonnavion et al, 2016*). For this reason, LH is identified as a critical neuroanatomical area essential for the maintenance of energy homeostasis (*Stuber and Wise, 2016*). Recent studies using

anterograde and retrograde tracers suggest that lateral hypothalamic area (LHA) receives a vast array of afferent projections from brain region involved in its behavioural functions. Inputs to orexinergic neurons originate from various cortico-limbic structures, such as prefrontal, infralimbic and orbitofrontal cortex, amygdala, hippocampus, ventral tegmental area, nucleus accumbens (shell) and paraventricular thalamic nucleus (*Trivedi et al, 1998; Elias et al, 1998; Marcus et al, 2001*). Moreover, given its structural and functional complexity, there are extensive connections within the LH itself, in particular from anterior to posterior and medial portions of hypothalamus, namely from arcuate nucleus (*Ter Horst and Luiten, 1987; Berthoud and Münzberg, 2011*). Orexin neurons (in estimated number of ~ 3000 in rodents and approximately 70000 in human brains) (*Nambu et al, 1999*) send several projections widely distributed in the brain. This is consistent with the huge distribution of orexin receptors that have been found in many areas of brain by immunohistochemistry and in situ hybridization studies (*Peyron et al, 1998; Trivedi et al, 1998*). Orexinergic fibres are particularly dense within the hypothalamus and in multiple regions in forebrain (paraventricular and central medial thalamus, subthalamus), hindbrain (substantia nigra, ventral tegmental area, nucleus of the solitary tract and dorsal raphe). Other orexinergic efferent projections are also observed in amygdala, prefrontal cortex, basal ganglia, locus coeruleus and septal nuclei (*de Lecea et al, 1998; Date et al, 1999; Horvath et al, 1999*). The OX receptors are highly expressed in these regions, although the distribution patterns of OX_1R and OX_2R correspond in some regions (such as dorsal raphe and paraventricular thalamus) but are complementary and distinct in some others (e.g., locus coeruleus, hypothalamic ventromedial nucleus and central medial thalamus) (*Trivedi et al, 1998; Barson and Leibowitz, 2018*).

1.4 Functions

Orexins have been recognize as multi-tasking neuropeptides in a wide variety of diversified physiological functions. Thanks to terminal appositions from the arcuate nucleus of the hypothalamus, orexinergic neurons play a key role in regulation feeding behaviour (*Sakurai et al, 1999*). Indeed, Neuropeptide Y (NPY)- and Agouti-related protein (AgRP)-immunoreactive fibres, corresponding to leptin-responsive cells, are critical factors that link peripheral metabolic cues to central regulation of food intake, by innervating OX neurons. Several studies showed the essential contribution of orexins to food intake. Orexin knockout mice have a hypophagic phenotype in comparison with wild-type littermates (*Hara et al, 2001*); central injection of OX-A stimulates feeding consumption and, on the other hand, fasting upregulates PPO mRNA (*Sakurai et al, 1998*). Conversely, different research groups of Ida and Haynes demonstrated that central or intraperitoneal respectively administration of the selective OX_1R antagonist 1-(2-Methyylbenzoxanzol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride (SB-334867-A) significantly blunts food consumption and increases resting behaviour in rats (*Ida et al, 1999; Haynes, et al., 2000*). Moreover, upregulation of orexin signalling, sustained by factors like endocannabinoids and impaired leptin signalling, occurs in obese mice (*Imperatore et al, 2017*). The orexinergic system plays a pivotal role also in other important basic physiological functions, such as sleep/wake cycle and neuroendocrine balance as well as somatic motor control. It has been demonstrated the importance of orexin in wakefulness, because of loss of OX-containing neurons in LH producing narcolepsy (*Scammel, 2001; Nishino et al, 2000; Peyron et al, 2000; Thannickal et al, 2000*).

1.4.1 Role of orexin in food addiction and reward

The abundant limbic connections from and to limbic system suggest a fundamental role of orexin in food addiction and reward. Various reports conducted in mice under no caloric deficits have extended role of orexin system from the regulation of feeding homeostatic pathway to hedonic one (*Alcaraz-Iborra et al, 2014*). Recent studies showed that central injection of OX-A enhances the consumption of highly palatable food (HPF) (*Rodgers et al, 2001*), while systemic treatment with SB-334867 is able to reduce the HPF intake (*Ishii et al, 2005*). It has been suggested that the contribution of orexins to overeating and reward processing depends on the caloric status of the animals (*Furudono et al, 2006; Pich and Melotto, 2006*). Central administration of SB-334867 reduces responding for high-fat food or sucrose solution only in food-restricted rats (*Nair et al, 2008; Cason et al, 2013 (a); Kay et al, 2014*), but not in animals fed *ad-libitum* (*Cason et al, 2013 (b)*). Furthermore, the ability of OX_1R antagonist to inhibit HPF consumption only in animals under restricted access to food has been confirmed in a study of Piccoli and collegues (*Piccoli et al, 2012*). These results indicate that OX signalling influences consumption of food with high reinforcing value and might play a key role in compulsive eating behaviour (*Pich and Melotto, 2006, Piccoli et al, 2012*). Harris and collaborators have brilliantly demonstrated the pivotal impact of orexins on reward and addiction. They showed that activation of LH orexin neurons is strongly linked to preference for food or drug-associated cues, because exposure to the conditioned place preference (CPP) compartment (previously paired with food or drug) leads to over activation of orexinergic neurons (*Harris et al, 2005*). Conversely, mice lacking orexin gene fail to show a CPP for drug-paired environment (*Narita et al, 2006*). Moreover, the activation of orexinergic neurons in LH leads to a reinstatement of a drug-seeking behaviour; this effect is completely attenuated by pre-treatment with SB-334867 (*Harris et al, 2005*).

1.5 Factors that regulate orexinergic neurons

Many central and peripheral factors can influence and regulate the activity of orexin neurons (*Sakurai and Mieda, 2011*). Among neurotransmitters, epinephrine and norepinephrine hyperpolarize and inhibit orexin neurons via α_2-adrenoceptors and $5HT_{1A}$ receptors, respectively (*Li et al, 2002; Yamanaka et al, 2003(a); Yamanaka et al, 2006*), whereas several neuropeptides, including neurotensin, oxytocin, vasopressin, cholecystokinin and ghrelin depolarize and activate orexin neurons (*Tsujino et al, 2005*). The firing rate of OX neurons can be regulated also by metabolic signals. Hypoglycemia induces depolarization and excitation of orexin neurons; in contrast, hyperglycemia stops firing of orexin neurons by hyperpolarization (*Yamanaka et al, 2003 (b), Burdakov et al, 2006*). Moreover, hypoglycemia, induced by various methods like fasting, has revealed to increase mRNA for *c-Fos* and prepro-orexin in a subpopulation of orexinergic cells of the LH (*López et al, 2000; Briski and Sylvester, 2001; Kurose et al, 2002*). Among all these factors, leptin and endocannabinoid are considered to be the two main regulators of orexin expression in the hypothalamus. They exert two opposite effects on OX activity, presumably either via leptin and endocannabinoid receptors on orexinergic cells, directly, or by other feeding-controlling cells connected to orexinergic cells, indirectly (*Kukkonen et al, 2002*).

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Chapter 2

Endocannabinoid System

The endocannabinoid system (ECS) was discovered in the last decade of XX century when compounds present in psychotropic preparations of Cannabis sativa (xenobiotic cannabinoids) are found to bind receptors of this system. These findings raised the possibility of the existence of endogenous cannabinoids in mammalian tissues. The subsequent developments of studies have brought a huge advance in knowledge of the ECS components and molecular mechanisms underlying their pharmacological action both in physiological and pathological conditions. Although structurally different from xenobiotic cannabinoids, the endogenous ones act as cannabimimetic substances, i.e. have the same properties of Δ^9-tetrahydrocannabinol (THC), being able to functionally activate cannabinoid receptors characterised so far in animals (*Di Marzo and Fontana, 1995; Di Marzo, 1998*). This ECS consists of endogenous lipid ligands named endocannabinoids (eCBs), their G-protein coupled receptors type 1 (CB_1R) and type 2 (CB_2R), and multiple enzymes responsible for their synthesis, degradation, transport and accumulation (*Piomelli, 2003; Di Marzo, 2008*).

2.1 Endocannabinoids

Endocannabinoids are arachidonic acid-containing molecules (lipid compounds of eicosanoid family) derived from the degradation of membrane phospholipids through diverse pathways and produced on demand at the site of need (*Piomelli, 2003*). The first characterised eCB is N-arachidonoyl ethanolamide (AEA), the amide of arachidonic acid with ethanolamine, also named anandamide because of the Sanskrit name for bliss “ananda” (*Devane et al, 1992*). Since the isolation of anandamide, diverse other long-chain fatty acids have been isolated and studied. Amongst these, the most representative and better characterised is 2-arachidonoylglycerol (2-AG) (*Mechoulam et al, 1995; Sugiura et al, 1995*). In the CNS, eCBs are produced by specific enzymatic machinery in postsynaptic neurons following increased Ca^{2+} intracellular levels induced by depolarization (*Di Marzo et al, 1998*). AEA is synthesised from

membrane N-acyl phosphatidylethanolamines (NAPEs), via several pathways by enzymes like phospholipase A_2 (PLA_2), phospholipase C (PLC) and NAPE-selective phospholipase D (NAPE-PLD) (*Wang and Ueda, 2009*). On the other hand, 2-AG is synthesised by the activity of diacylglycerol lipase (DAGL) from arachidonic acid-containing diacylglycerol (DAG), cleaved from phosphatidylinositol 4,5-bisphosphate (PIP2) by PLC (*Murataeva et al., 2014*). Once produced "on demand", eCBs are rapidly released to act on cells situated close their place of synthesis in an autocrine or paracrine manner, and then rapidly inactivated due to the action of their degradation enzymes (*Di Marzo, 2009*). The inactivation of eCB signalling occurs by a fast removal from molecular targets (neuronal uptake) and consecutive intracellular hydrolysis mediated by specific enzymatic systems. AEA is degraded mainly by fatty acid amide hydrolase (FAAH); differently, the monoacylglycerol lipase (MAGL) principally hydrolyses 2-AG (*Di Marzo et al, 2004*). eCBs signalling can also terminated by a rapid diffusion across the plasma membrane facilitated by a saturable and selective transporter, the eCB membrane transporter (*Di Marzo et al, 1994; Hájos et al, 2004; Chicca et al, 2012*).

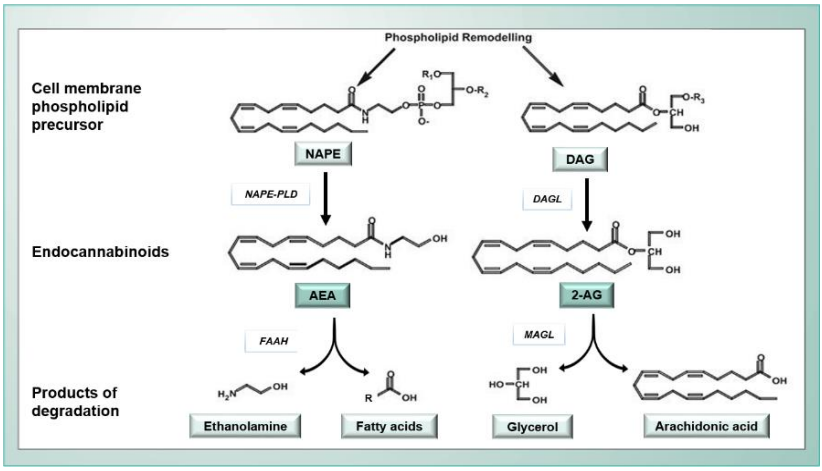

Fig. 2.1 | Biosynthesis and degradation pathway of endocannabinoids.
NAPE: N-acyl phosphatidylethanolamine; DAG: diacylglycerol; NAPE-PLD: NAPE selective phospholipase D; DAGL: DAG lipase; AEA: anandamide; 2-AG: 2-arachidonoilglycerol; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase.

2.2 Endocannabinoid receptors

Both AEA and 2-AG are able to bind two cannabinoid receptors subtypes, type 1 (CB_1) and type 2 (CB_2) receptor. The first receptor to be characterised was the CB_1R as reported in two studies of Devane and Matsuda groups. While screening the affinity of Δ^9-tetrahydrocannabinol (THC), the main psychoactive principle of cannabis, with an orphan GPCR, unexpectedly they identified and cloned CB_1R (*Devane et al., 1988; Matsuda et al., 1990*). Subsequently, to investigate also the non-psychoactive effects of cannabinoids, Munro and collaborators identified and cloned a second receptor for cannabinoids, CB_2R (*Munro et al., 1993*).
Both receptors belong to the family of GPCRs, constituted by a single polypeptide chain that crosses seven times the plasma membrane. In terms of structure, the receptors are characterised by the N-terminal with possible

sites of glycosylation on the extracellular part, followed by seven transmembrane helices connected by intracellular/extracellular loops and the C-terminal with potential phosphorylation sites on the intracellular part. CBRs are able to activate various signalling pathways through the GTP-binding $G_{i/o}$ proteins. Both AEA and 2-AG, after binding to CB_1 or CB_2 receptors, typically trigger the inhibition of cAMP formation, as seen in various *in vitro* studies (*Vogel et al, 1993; Lee et al, 1995; Pertwee, 1997*). Interestingly, several other intracellular actions of cannabinoids receptors have been suggested, especially of CB_1R. Glass and collaborators showed that CB_1R can activate adenylate cyclase via a G_s protein, but this stimulatory effect is usually masked by the more common G_i-mediated inhibitory one, dependent upon ligands concentration (*Fride et al, 1995; Glass et al, 1997*). Furthermore, eCBs were shown to activate a neuronal form of focal adhesion kinase (FAK+) in hippocampal slices of rats (*Rinaldi-Carmona et al, 1994*) and to inhibit the cell proliferation of human breast cancer through the suppression of prolactin receptor levels (*De Petrocellis et al, 1998*). The simplistic assumption that the two main cannabinoid receptors has specific different anatomical localisations, a "central" CB_1R and a "peripheral" CB_2R was sustained for almost a decade (*Pertwee, 1998; Di Marzo, 1998*). Nowadays, it is clear that both the receptors show a wide distribution and expression throughout the body of mammals, birds, reptiles and even fish (*McPartland et al, 2006*). In humans, CB_1R is widely expressed in the brain, where they are found both in neurons and astrocytes. This receptor are observed in areas associated with the regulation of energy homeostasis, like the hypothalamus, the brainstem and the mesocorticolimbic system, in the terminals of central and peripheral neurons and glial cells (*Egertová et al, 2003*). Like CB_1R, also CB_2R is expressed in the brain, mainly in non-neuronal cells (*Shohami et al, 2011*).

2.3 Retrograde neurotransmission of endocannabinoids

The ECS plays a key role in the modulation of synaptic transmission. Recent studies have highlighted the ability of eCBs to regulate short-term and long-term synaptic plasticity (*Castillo et al, 2012; Ohno-Shosaku and Kano 2014*). Endocannabinoids act as retrograde synaptic messengers that have an inhibitory action on afferent GABAergic or glutamatergic transmission, known as depolarisation-induced suppression of excitation (DSE) and depolarisation-induced suppression of inhibition (DSI). Fundamentally, both the events are triggered by postsynaptic depolarisation induced by the influx of Ca^{2+} and the consequent synthesis of 2-AG from lipid precursor. In virtue of its lipophilic property, 2-AG is able to cross plasma membrane and relocate to the presynaptic site, where it binds to its receptor. The activation of CB_1R induces a suppression of the neurotransmitter release, thereby provokes a transient inhibition of afferent synaptic currents. This mechanism of fine-tuning synaptic transmission is not long-lasting, hence is completely reversible in a range of 10 seconds (*Di Marzo, 2004*).

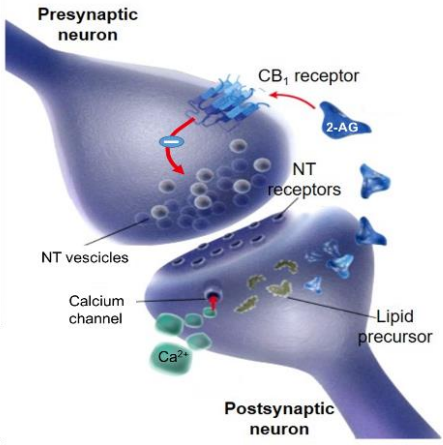

Fig. 2.2 Retrograde neurotransmission of endocannabinoids.

2.4 Cross-talk between orexin and endocannabinoids

A large number of emerging evidence support a functional and biochemical interaction between endocannabinoid and orexin systems. This cross-talk is possible because several anatomical studies show an anatomical overlap of both EC and OX receptors expression in the CNS. CB_1R and OX_1R, as well as OX_2R, co-expressions were described in brain areas involved in the regulation of appetite and energy homeostasis, reward sleep/awake cycle and nociception. Thus, they were found, with different densities, in all the hypothalamic nuclei, including LH, arcuate nucleus (ARC), dorsomedial hypothalamus (DMH) ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN) (*Marcus et al., 2001; Mackie, 2005*). The co-expression also occurs in prefrontal and infralimbic cortex, amygdala, hippocampus, ventral tegmental area, locus coerules and medial septal nucleus (*Cristino et al, 2017*). The overlapping distribution of two receptors was sustained by an *in vitro* study by Hilaret and collegues, that detected for the first time the increase of OX-A's potency (100-fold increase) to stimulate and activate ERK1/2 pathway via CB_1R, in a manner prevented by the CB_1R antagonist rimonabant (*Hilairet et al, 2003*). The authors explained this effect with the ability of OX_1R and CB_1R to form heteromeric complex, basing also on electron microscopy ultrastructural study that demonstrated that receptors are close enough to produce heteromers (*Hilairet et al, 2003; Ellis et al, 2006*). Regarding the biochemical aspect of interaction between the two types of receptors in the stimulation of appetite, it was demonstrated that central infusion of OX-A induces feeding in rats and surprisingly is reduced by blocking CB_1R with rimonabant (*Crespo et al., 2008*). OX-A has been found to act as modulator of endocannabinoid production since 2-AG biosynthesis occurs via Gq-PLC-DAGL pathway downstream to OX-A-mediated activation of OX_1R *(Imperatore et al., 2016; Berrendero et al., 2018*). As seen in *in vitro* model of OX_1R-expressing CHO cells and embryonic mouse hypothalamic neurons, OX-A, by binding OX_1R, triggers the activation of PLC and DAG production which is in turn converted in 2-AG by the biosynthetic enzyme DAGLα (*Turunen*

et al, 2012; Cristino et al, 2013). 2-AG rapidly released to the extracellular space may act in an autocrine or paracrine manner by binding CB_1R (*Turunen et al., 2012; Jäntti et al., 2013*).

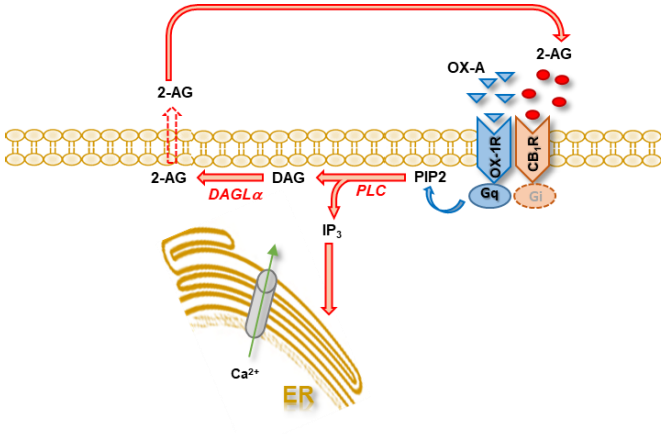

Fig. 2.3 OX-A signalling intracellular cascade regulates 2-AG biosynthesis and affects CB_1R signalling in autocrine and paracrine way.

2.5 Role of endocannabinoids in feeding behaviour

The effects of eCBs have been hardly studied in comparison to synthetic cannabinoids' (e.g. THC) most likely because of their rapid degradation in vivo (*Willoughby et al, 1997*). Over the last two decades, by testing eCB analogues and receptor antagonists, a growing body of evidence have demonstrated that ECS may be a contributing factor to physiological functions regulation (*Di Marzo, 1998*). Due to its thorough distribution in the whole body, ECS produce its effects in both central and peripheral tissues. Consequently, its alteration or disruption may contribute to several disorders in CNS and periphery.

Abel and colleagues were one of the first groups that studied a direct relation with cannabis in humans and its ability to increase appetite, or produce the phenomenon known as "munchies" (*Abel, 1975*). The ability of eCBs to

interact with CB_1R-expressing neurons in the LH was investigated in several studies that confirmed the role of ECS in the control of food intake. In 1991, it has been demonstrated that an intraperitoneally injection of Δ^9-THC stimulates feeding through an electrical stimulation of the LH (*Trojniar and Wise, 1991*). As well as synthetic or plant-derived cannabinoids, also AEA has a direct action on LH, since is able to induce *c-Fos* activation in hypothalamic neurons (*Fernández-Ruiz et al, 1997*). Moreover, Cota and collaborators have been demonstrated, using mice lacking the CB_1R, that the positive effects of eCBs on food intake is strictly related to the activity of CB_1R (*Cota et al., 2003*). Furthermore, hypothalamic 2-AG levels are correlated with the energy status of the body in a negative manner, since are increased during fasting and, in contrast, decreased during refeeding (*Kirkham et al., 2002*).

The role of ECS in feeding behaviour encompasses also the reward aspect of food intake. In fact, it has been reported that marijuana is able to enhance appetite for a tasty meal rather than a flavourless one. This observation leads to hypothesis that eCBS induce hyperphagia by increasing the food palatability (*Tart, 1970*). In particular, Berridge and collegues demonstrated that eCBs mediate the hedonic impact of food acting on striatal areas (*Berridge et al, 2010*). Accordingly, several studies reported the action of AEA in these regions. Indeed, injection of AEA in shell of nucleus accumbens increases the hedonic reaction in a taste reactivity test to sucrose (*Mahler et al, 2007*). Moreover, it has been demonstrated that AEA is able to inhibit both nigrostriatal dopamine synthesis (*Romero et al,1995*) and its electrically evoked releasefrom striatal slices in rats (*Cadogan et al, 1997*).

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Chapter 3

Leptin

Leptin is a non-glycosylated peptide hormone of 167-amino acids with a molecular weight of 16 kDa. It is encoded by the obese gene (*ob*, also known as *Lep*, for leptin) located on chromosome 7 and shows 84% of homology between the murine and the human form. Leptin was discovered by the geneticist Jeffrey Friedman in 1994 and was named leptin from the Greek word "leptos" that meaning "thin". Antecedent to the official discovery of leptin protein, in 1950, at Jackson Laboratory a lean mouse colony produced a strain of obese offspring, characterised by hyperfagia and high body weight (*ob/ob* mice) (*Ingalls et al, 1950*). The gene affected by the so-called ob mutation was identified several years later by positional cloning in Friedman's laboratory (*Zhang et al, 1994*) and it became soon clear that the recessive mutation in *ob/ob* mice was responsible for the obese phenotype.

3.1 Synthesis of leptin

Leptin is predominantly produced by adipocytes in white adipose tissue (WAT) but not exclusively; in fact, other tissues also produce the hormone even if in less quantity, including stomach (the lower part of fundic glands) mammary gland, ovaries, placenta, heart, skeletal muscle and brain (*Margetic et al, 2002*). Once synthetised, leptin is released in the blood in free form and bound to proteins (*Sinha et al, 1996*) and binds its receptors, LepRs, on target areas (*Marwarha and Ghribi, 2012*). The levels of circulating leptin are proportional to the amount of energy stored as fat in the body (adiposity), with plasma concentrations between a range of 1 to 100 ng/ml during fasting (*Considine et al, 1996*). As well as orexin, leptin is produced in a circadian manner, reaching the lowest levels at mid-afternoon and the highest at midnight. Moreover, its levels fluctuate in relation to several parameters (*Park and Ahima, 2015*): *i)* overfeeding and fasting, that induce an increase or decrease of leptin synthesis, respectively; *ii)* amounts of pro-inflammatory cytokines (e.g., TNF-α and IL-6) and *iii)* levels of other circulating hormone, as insulin, estrogen and thyroid hormone. Furthermore, leptin exhibits sexual dimorphism: women have

about three-fold higher plasma leptin even after controlling for body adiposity (*Saad et al, 1997*), probably due also to its higher production from subcutaneous fat than visceral one (*Montague et al, 1997*).

3.2 Receptors

Leptin receptors belong to the class I cytokine receptor superfamily and are encoded by diabetes (*db*) genes. The transcript under-goes alternate splicing to generate six multiple isoforms, characterised by high similarity in the extracellular domain but very little homology in their intracellular domain [*Fig. 3.1*]. Additionally, receptors are categorised into three different groups: short form, long form and secreted leptin receptor (*Fruhbeck, 2006*). Notably, the long form of the receptor (LepRb or ObRb) is the principle responsible for ligand binding and downstream activation of intracellular pathways. Leptin receptors are located mostly in the hypothalamus, the principal place of action on regulation of body weight, but also in other brain regions, such as the ventral tegmental area (*Schwartz et al, 1996; Hakansson et al, 1998; Hommel et al, 2006*). In the brain, in agreement with the literature, LepRs are clearly identified in neuronal and in non-neuronal cells, including astrocytes and microglia (*Hsuchou et al, 2009; Kim et al, 2014*).

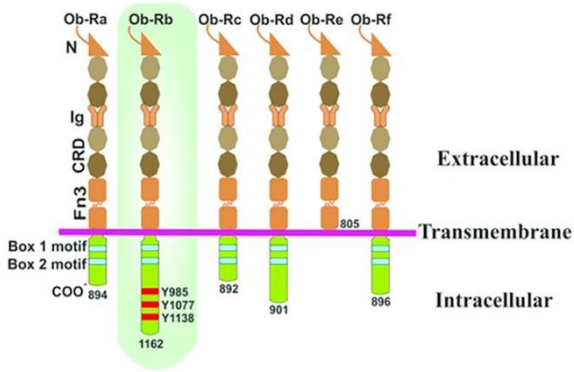

Fig. 3.1 | Six isoforms of the leptin receptor (from Ob-Ra to Ob-Rf). They are all identical in extracellular ligand binding domains, but different in C-terminus.

3.3 Functions

The hypothalamus has been identified as the major site of action of leptin in the brain. Once produced and released by adipocytes, it crosses the BBB through a specific and saturable transporter that goes underneath a mechanism of resistance in case of obesity (*Banks et al, 1999*).
Leptin exerts several physiological roles in the regulation of reproductive and immune function, bone metabolism, and neuronal function including cognition (*Dalamaga et at., 2013*). However, it is a well-established key molecule that contributes to regulation of food intake and metabolism, through an indirect inhibition action on orexinergic neurons (*Friedman, 2014*). In particular, the decreased levels of leptin induced by fasting lead to upregulation of prepro-orexin mRNA (*Sakurai et al, 1998*) and increase of orexinergic neuron activity (*Huang et al, 2007*). On the contrary, in obesity, the aberrant signalling of leptin, due to either leptin deficiency (in *ob/ob* mice, i.e. knock-out for leptin) or leptin resistance (in diet-induced obesity, DIO), causes a disinhibition of orexinergic neurons mediated by endocannabinoids, that leads to enhanced release of OX-A in target areas and consequent hyperphagia (*Cristino et al, 2013*).

3.3.1 Role of leptin in food addiction and reward

Leptin can exert a pivotal role also in food addiction and motivation to attain reward (*Figlewicz et al, 2004, 2006; Carr, 2007*). Leptin central administration reduces both dopamine release and incentive value of sucrose (that is enhanced by food restriction) (*Domingos et al, 2011*), thereby suppressing reward induced by food (*Fulton, 2000*). The injection of leptin in lateral hypothalamus induces a decrease in preference for high fat diet (HFD) in a conditioned place preference test (*Liu et al, 2017*). Conversely, the aberrant signalling of leptin can have a positive effect on food reward. It has been demonstrated that human subjects with both congenital deficiency in leptin and obese patients (with leptin resistance following

hyperleptinemia) display enhanced activation of brain regions involved in reward after seeing images of high-calorie food (*Farooqi et al, 2007; Jastreboff et al, 2014*). Reduced values of leptin induced by chronic mild stress increase the preference for a diet with a high content of fat (*Lu et al, 2006; Teegarden et al, 2008*). Several other studies have confirmed the important role of leptin in food reward, by using mice with viral-mediated reduction of LepRs within midbrain, that exhibit increased preference toward sucrose and efforts to obtain it (*Hommel et al, 2006*); or using mice knockdown for LepRs in LH, that show an enhanced intake of highly palatable food (*Davis et al, 2011*). The same effects have been observed also in drug addiction. In fact, Shen and collegues have demonstrated that blocking leptin signalling in midbrain, through either an intracerebroventricular (i.c.v.) injection of a LepR antagonist or long-term RNAi-mediated knock down of LepR, leads to enhancement of cocaine-conditioned reward (*Shen et al, 2016*).

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Chapter 4

Dopaminergic system

In 1957, the Swedish neuropharmacologist Carlsson uncovered the role of dopamine as a neurotransmitter, independent of its role as a precursor in the synthesis of catecholamines epinephrine and norepinephrine (*Carlsson, 1957*). Two years later, Ehringer and Hornykiewicz conduct several studies on the role and distribution of dopamine, showing that its concentration is markedly decreased in the striatum of human patients with Parkinson's disease (PD) (*Ehringer and Hornykiewicz, 1960; Hornykiewicz, 2002*). Over the years, the demonstration of the dopamine involvement not only in the motor and side effects of PD, but also in motivational disorders, including drug addiction, leads to a growing keen attention on dopamine and neural circuits driving motivated behaviour, and consequent numerous breakthroughs in neuroscience.

4.1 Dopamine

Dopamine (3-hydroxytyramine, DA) is a monoamine of the catecholamine family synthesised in the dopaminergic neurons mostly localised in ventral tegmental area (VTA). It is produced through the biosynthetic pathway of catecholamines, including also adrenaline and noradrenaline. The rate-limiting enzyme of DA synthesis is tyrosine hydroxylase (TH), that catalyses the hydroxylation of the amino-acid tyrosine to L-DOPA (l-3,4-dihydroxyphenylalanine), which, in turn, is rapidly converted in dopamine by aromatic amino acid decarboxylase [*Fig. 4.1A*]. TH has a multi-domain structure, constituted by a N-terminal regulatory domain (R), a catalytic domain (C) and a C-terminal with a coiled-coil domain (*Daubner et al, 2011*). The active site of C domain, constituted by about 330 amino-acids, is able to bind a ferrous iron atom (by a glutamate and two histidine residues), tetrahydrobiopterin and tyrosine (by two pair of three different residues). In fact TH, since belongs to the family of oxygenase, uses molecular oxygen (O_2) and tetrahydrobiopterin as cofactors for the hydroxylation of tyrosine aromatic ring (*Fitzpatrick, 1999*). The R domain, constituted by 160 amino-acids, is involved in the regulation of TH activity,

because the phosphorylation of its mobile portion makes the enzyme less susceptible to catecholamines feedback inhibition (*Daubner et al, 1992*). The phosphorylation at four different serine residues (at positions 8, 19, 31 and 40) by cAMP-dependent protein kinase (PKA) moves the R domain out of the active site of catalytic domain, making it accessible for tyrosine and thus leading to the activation of the enzyme (*Daubner et al, 2011*).
Once synthesised in cytoplasm, DA is transported into the synaptic vesicles through the action of vesicular monoamine transporter (VMAT). This protein regulate DA uptake in presence of proton pumps on the synaptic vesicle membrane that generate electrochemical gradient pumping two protons in the cytoplasm. Influx of Ca^{2+} in terminal bouton of the axon triggers the fusion of synaptic vesicle with the presynaptic membrane, leading to the release of DA in the synaptic cleft. At the end of transmission, DA is taken up by the presynaptic site and broken down by deamination mediated by monoamine oxidase (MAO) and methylation by catechol-O-methyl transferase (COMT), leading to the formation of inactive metabolites in two different sequences (*Eisenhofer et al, 2004*) [*Fig 4.1B*].

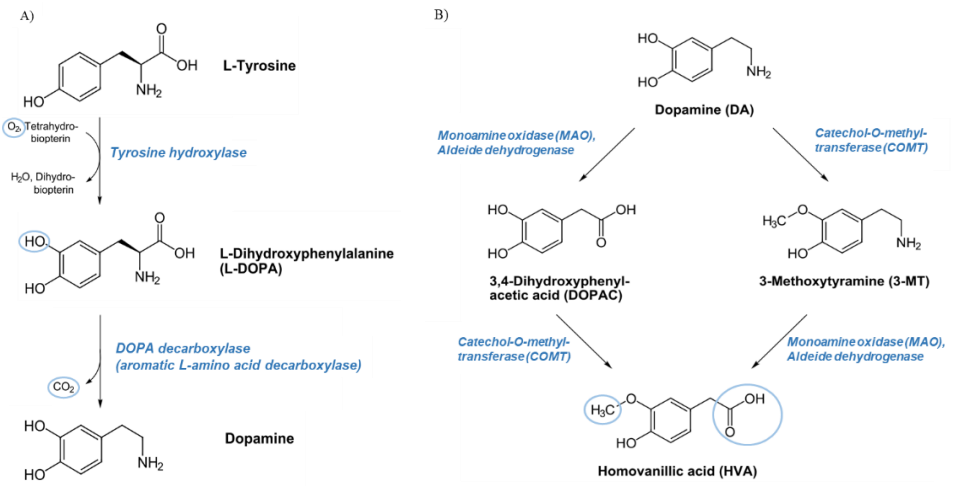

Fig. 4.1 | Biosynthetic (A) and biodegradative (B) pathway of dopamine.

4.2 Dopamine receptors

Once synthesised and released in synaptic cleft, dopamine interacts with its receptor expressed on postsynaptic sites. To date, in PubMed database there are more than 60000 results for "dopamine receptor", thus indicating the significant interest in studying and modulating dopaminergic transmission by the activity of dopamine receptors.

4.2.1 Genetic and structural properties of dopamine receptors

Dopamine receptors belong to the family of GPCRs that show a common structure of single polypeptide chain crossing the plasma membrane seven times and similar primary amino acid sequences. The first classification of DA receptors dated back in 1978 and was based on their ability to modulate the activity of adenylate cyclase (AC) in a positive or negative manner. In particular, the group of Spano firstly, and Kebabian and Calne soon after, demonstrated that DA receptors can be divided in two major groups, D1R and D2R subtypes (*Spano et al, 1978; Kebabian and Calne, 1979*). D1R subfamily, via coupling to $G_{s/olf}$ protein, activates AC, leading to the production of cAMP and consequent activation of protein kinase A (PKA). On the contrary, D2R subfamily, which is linked with $G_{i/o}$ proten, acts in terms of antagonism of AC-cAMP signalling. Nowadays, it is noteworthy that D2 receptors can activate also other intracellular signalling through βγ-complex of their coupled G-protein, such as modulation of various types of ion channels, release of Ca^{2+} in cytoplasm from intracellular deposits and the activation of MAP kinase system (*Bonci and Hopf, 2005*). Over the years, a growing interest has been focused on the biological and structural features of these receptors, leading to a more detailed classification that revealed the existence of multiple receptor subtypes in both D1 and D2 groups (*Andersen et al, 1990; Civelli et al, 1993*). Indeed, since the early '90s, by using genetic cloning techniques, has been possible to identify two receptors of D1R subfamily, D1 and D5 receptors (originally named as D1A

and D1B, respectively) (*Dearry et al, 1990; Tiberi et al, 1991; Sunahara et al, 1991*); and three receptors belonging to D2R group, D2, D3 and D4 receptors (*Bunzow et al, 1988; Sokoloff et al, 1990, 1992; Van Tol et al, 1991*). The main genetic differences between D1- and D2- classes concern the presence of introns in coding sequence of genes (*Nimik and Van Tol, 1992*). D1R gene, *DRD1*, and D5R gene, *DRD5*, located on 5 and 4 chromosome, respectively, do not express introns in their coding region. Consequently, there is only one isoform for both the receptors. On the other hand, genes for D2-class receptors, *DRD2*, *DRD3* and *DRD4*, that are located the first and the latter on chromosome 11 and the gene for D3R on chromosome 3, contain different number of introns in their coding sequence. In particular, *DRD2* expresses six introns, *DRD3* five introns and *DRD5* three introns (*Gingrich and Caron, 1993*). This genetic characteristic implies the generation of splice variants of all three receptors. The more studied and characterised isoforms of D2-class receptors are D2L (D2-long) and D2S (D2-short) variants of D2R, differing each other for 29 amino acid residues in third intracellular loop, for about 3 kDa (*Giros et al, 1989; Monsma et al, 1989*). Both D3 and D4 receptors have some splice variants, but some of them encode nonfunctional proteins (*Giros et al, 1991; Van Tol et al, 1992*). The presence or absence of protein isoforms provides the basis for different localisation of DA receptors. In fact, D1R and D5R are found exclusively on postsynaptic membranes of dopamine-responsive cells; conversely, D2-class receptors can be expressed both on presynaptic and postsynaptic sites. In particular, several studies have demonstrated that D2S isoform is mostly presynaptic localised and has a role as autoreceptor, whereas D2L isoform is the most predominant postsynaptic variant of D2Rs (*Usiello et al, 2000; De Mei et al, 2009*). The five DA receptors show diverse structural characteristics. They share different level of homology of their transmembrane domains, starting from 53% and 75% between D2 and, respectively D4 and D3, until the 80% between the two individual members of D1-class receptors (*Missale et al, 1998*). Both the classes of receptors express a similar number of amino acids at the N-terminal, whereas the C-

terminal of D2-class receptors is seven times smaller than the respective one of D1-class (*Gingrich and Caron, 1993; Missale et al, 1998*). Finally, D1 and D2 families of receptor have different pharmacological properties, since they exhibit a diverse sensitivity to dopamine agonists and antagonists. For example, selective antagonists for D4 and D3 receptors have an affinity 1000 and 100 folds, respectively, higher than the selectivity for the other receptors (*Sokoloff et al, 2006; Rankin et al, 2010; Rondou et al, 2010*).

Tab. 4.1 | Genetic and structural properties of dopamine receptors

Dopamine receptor subtype	**D1**	**D2**	**D3**	**D4**	**D5**
Gene symbol	*DRD1*	*DRD2*	*DRD3*	*DRD4*	*DRD5*
Chromosome gene map locus	5q35.1	11q23.1	3q13.3	11p15.5	4p16.1
Number of introns in the coding region	None	6	5	3	None
Presence of splice variants	None	Yes: D2S, D2L	Yes	Yes	None
Number of amino acids	446	D2S: 414 D2L: 443	400	387	477
Molecular weight (Da)	49.300	D2S: 47.347 D2L: 50.619	44. 225	41.487	52.951
G protein coupling	$G\alpha_s$, $G\alpha_{olf}$	$G\alpha_i$, $G\alpha_o$	$G\alpha_i$, $G\alpha_o$	$G\alpha_i$, $G\alpha_o$	$G\alpha_s$, $G\alpha_{olf}$

(Modified from Beaulieu and Gainetdinov, 2011)

4.2.2 Dopamine receptors expression

Dopamine receptors are widely distributed in both the CNS and periphery, with different level of density (*Beaulieu and Gainetdinov, 2011*). As regards the D1-class of receptors, D1R is expressed in brain mostly in mesocorticolimbic and nigrostrital areas, including PFC (prefrontal cortex), NAc (nucleus accumbens), SN (substantia nigra), striatum, amygdala and

olfactory bulb; and, in lower amounts, in hippocampus, cerebellum, hypothalamus and thalamus. D5Rs are localised with low density in various cortical areas, i.e. prefrontal, premotor, cingulated and entorhinal cortex, as well as hypothalamus, hippocampus and SN. Regarding the class 2 of DA receptors, D2R, similar to D1R, is expressed at very high levels in mesocorticolimbic and nigrostrital areas, such as NAc, cortex and striatum. It has also been detected in SN, VTA, olfactory tubercle, hypothalamus, hippocampus and amygdala. D3R has a lower distribution in CNS in comparison to D2R (*Missale et al, 1998; Sokoloff et al, 2006*). It has been observed mostly in NAc, olfactory tubercle, and, in minor density, in VTA, SN, striatum and cortical areas. The lowest DA receptor expressed in the brain is D4R, which has been localised in cortex, hippocampus, hypothalamus, cortex and also in SN and dorsal striatum (*Missale et al, 1998; Rondou et al, 2010*).
In peripheral areas, both D1- and D2-subtypes of receptors have been detected at different level of density in cardiocirculatory system (i.e., heart and blood vessels), sympathetic ganglia, gastrointestinal tract and in various other areas, such as retina, kidney and adrenal glands (*Missale et al, 1998; Aperia, 2000; Witkovsky, 2004; Li et al, 2006; Iversen and Iversen, 2007*).

4.2.3 Role of dopamine receptors

One of the principal and most studied effects of dopamine is its role in locomotor activity. All five receptors participate to this function, although each of them contributes in a different way. The mainly involved receptors are D1, D2 and D3R, whereas D4 and D5 receptors seem to make only a minimal contribute (*Missale et al, 1998; Sibley, 1999*). D1R plays a well-defined positive stimulatory role of locomotor activity. Conversely, D2Rs and D3Rs show a dual action depending on receptor localisation. In fact, when expressed presynaptically on somata, dendrites or axons of DA neurons, they act as autoreceptors, that, through a negative feedback mechanism, reduce the release of dopamine from axon terminal. On the

contrary, the activation of receptors localised on postsynaptic sites of non-dopaminergic neurons leads to stimulation of locomotion. Moreover, D2S presynaptic receptors exhibit a higher sensitivity to DA than D2L postsynaptic receptors; thereby DA and DA agonists show a biphasic effect, because low doses trigger the activation of autoreceptor, leading to decrease of locomotor activity, whereas high doses of DA agonists induce the activation of postsynaptic receptors, with the consequent hyperlocomotion (*Puglisi-Allegra and Cabib, 1997*). Many other effects of dopamine signalling depend on the activation of D1, D2 and D3 receptors. Because of DA pivotal role in reward, the dopamine receptors mainly expressed in the brain areas involved in reinforcement and reward mechanisms appear strictly related to this function. Several studies have shown that is possible to regulate the behavioural responses to both natural and drug reward stimuli through the pharmacological and genetic modulation of DA receptors (*Hyman et al, 2006; Di Chiara and Bassareo, 2007; Koob and Volkow, 2010*). The receptor that has been demonstrated to be mostly involved in schizophrenia and bipolar disorders is D2R, since it is the target of all clinically effective antipsychotic drugs (*Snyder et al, 1970; Roth et al, 2004*). Both D1R and D2R are likely to play a fundamental role in cognitive functions such as learning and working memory, thanks to their wide expression in the prefrontal cortex (*Goldman-Rakic et al, 2004; Xu et al, 2009*). All five subtypes of receptors seem to play similar role in many other DA functions, such as sleep, attention, reproductive behaviours and decision making (*Missale et al, 1998; Vallone et al, 2000; Iversen and Iversen, 2007*).

Furthermore, DA activity in periphery is sustained principally by D1Rs and D2Rs. DA is critically involved in hormonal regulation; in particular, through D2Rs, it contributes to control the secretion of prolactin from pituitary gland and aldosterone from adrenal gland, whereas, thanks to D1Rs expression on renal cells, it regulates renin secretion from kidney (*Missale et al, 1998; Aperia, 2000; Iversen and Iversen, 2007*).

4.2.4 Desensitization of D2 receptor

In addition to growing knowledge of structural and pharmacological properties of dopamine receptors, significant progress has begun to explain the complexity and plasticity of intracellular pathway downstream their activation.

It is well known that dopamine receptors, since being GPCRs, can undergo dynamic regulation upon longer-lasting activation mediated by prolonged exposure to agonists. The main mechanism of this regulation is the desensitization of DA receptors. Several studies have shown that drug of abuse, as well as excess of highly palatable food (HPF), can alter the expression of dopamine receptors in mesolimbic regions of the brain (*Avena et al, 2008*). Both of them are able to imbalance the ratio between D1R and D2R, by decreasing the availability of D2Rs and its binding capacity in various brain areas, in particular in striatum. In fact, it has been demonstrated that striatal D2Rs are down-regulated in rats with diet-induced obesity and that animals knock-down of striatal D2R, when exposed to extended access to HPF, are more vulnerable to compulsive-like eating (*Johnson and Kenny, 2010*). The desensitization-dependent reduction of D2R is a neuroadaptative response to overconsumption of food. It has been demonstrated an inverse correlation between body weight of human subjects and striatal D2R density. Indeed, obese individuals show a decreased expression of D2Rs in striatum (*Wang et al, 2001; Stice et al, 2008*); on the contrary, it has been reported that patients that suffer from anorexia nervosa express an enhanced level of striatal D2Rs (*Frank et al, 2005*). Same changes have also shown in drug addiction. In fact, Park and collegues have demonstrated that chronic cocaine intoxication decreases the density of D2R, as seen by an upregulated cAMP signalling pathway, which suggest a predominance D1R over D2R signalling (*Park et al, 2013*). These results are consistent with preclinical (*Nader et al, 2006; Thanos et al, 2007*) and clinical (*Volkow et al, 2009*) studies, that show a significant reduction of striatal D2R availability in cocaine abusers. The down-regulation of D2Rs in both food and drug addiction contributes to the hypofunction of reward

induced by extended access to drug or diet. This deficit in reward processing represents a counter-adaptive mechanism of reward neuro circuitry that decreases threshold of baseline sensitivity to oppose its prolonged stimulation in obesity or drug abuse. This means that an aberrant vicious circle underlies compulsive food and/or drug seeking behaviours, because subjects overconsume food or drug to counteract this reduced state of reward, thereby contributing to obesity and addiction (*Wang et al, 2001; 2002; Koob and Le Moal, 2005*).

The down-regulation of D2R is dependent from β-Arrestin binding and involves other intracellular proteins that contribute to its desensitization. After long-lasting activation of D2R, G protein-coupled receptor kinase (GRK) phosphorylates the receptor at specific residues on intracellular and C-terminal domains. Specifically, among all members of GRK family, the mostly involved in D2R phosphorylation is GRK2 (*Kim et al, 2001*), which in turn can be negatively regulated by protein neuronal calcium sensor-1 (NCS-1) (*Kabbani et al, 2002*). The phosphorylated D2R becomes target for binding of β-Arrestin protein. Like GRKs, β-Arrestin family counts various isoforms, among which β-Arrestin2 has reported to be the key protein directly involved in agonist-dependent internalization of this receptor (*Gainetdinov et al, 2004*). The association between β-Arrestin2 and D2R has a dual role in receptor desensitization: on one hand, it prevents further receptor activation, in spite of its constant stimulation mediated by DA; moreover, it induces the recruitment of clathrin through adaptin-2, a clathrin adaptor protein (AP-2) (*Laporte et al, 2002*). Thereby, this process triggers internalization of D2R through endocytosis into clathrin-coated vesicles, pinched off the cell membrane via GTP-ase activity of dynamin2 (*Kabbani et al, 2004*). As a result, the D2R is translocated from the extracellular versant of plasma membrane to cytosolic compartment and it can undergo subsequent recycling on cell surface or degradation via endosomal-lysosomal system (*Ferguson et al, 1996; Ferguson, 2001; Claing et al, 2002*).

4.3 Ventral tegmental area and mesolimbic circuit

4.3.1 Input and output of VTA

The VTA is a semi-circular structure lying along the midline in the midbrain that contains somata of neurons producing mainly dopamine (*Oades and Halliday, 1987*) and also cholecystokinin (CCK) (*Studler et al, 1981*) and neurotensin (NT) (*Kalivas and Miller, 1984*). The activity of dopaminergic neurons in VTA is regulated by intrinsic and extrinsic inputs (*Di Chiara, 1995; Watabe-Uchida et al, 2012*). Intrinsic afferences include signals from dopamine neurons themselves, through the paracrine release by their dendrites in VTA of both dopamine, necessary for self-inhibitory mechanism that regulates dopamine production, and also CCK and NT, responsible for modulation of dopamine neurons activity (*Llinás et al, 1984; Kalivas, 1993; Sibley, 1999*). It has been demonstrated that VTA receives tonically inhibitory inputs from *pars reticulata* of SN (*Johnson and North, 1992*). Dopaminergic neurons are also regulated by extrinsic inputs that arise from other brain regions (*Beier et al, 2015*). Among these, it receives dense projections from the lateral hypothalamus, amygdala and shell and core regions of NAc (*Grace et al, 2007; Faget et al, 2016*). Moreover, VTA receives inhibitory inputs derived from striatal medium-size spiny neurons (MSNs) and excitatory ones from prefrontal cortex, through the pathways activated by GABA and glutamate receptors expressed on dopamine neurons, respectively (*Kalivas, 1993; Ferrada et al, 2017*). On the other hand, dopaminergic neurons of VTA send a huge amount of projections to several areas of the brain. Densely innervated areas are striatum, both dorsal (nucleus caudatus and putamen) and ventral striatum (mainly nucleus accumbens), prefrontal cortex, substantia nigra, central amygdaloid nucleus and bed nucleus of stria terminalis (*Björklund and Dunnett, 2007*). Thanks to all these connections, dopaminergic system is implicated in several functions, including food and drug reward, psychomotor functions and motivational behaviour.

4.3.2 Dopaminergic pathways

Four main dopaminergic pathways have been identified in the brain [*Fig. 4.2*]: *i)* the mesolimbic pathway and *ii)* the mesocortical pathway (often collectively named "mesocorticolimbic pathway"); *iii)* the nigrostriatal pathway; and *iv)* the tubero-infundibular pathway (*Anden et al, 1964; Dahlstroem and Fuxe, 1964*). The mesocolimbic pathway arises from A_{10} group of dopaminergic neurons in the ventral tegmental area and sends dopaminergic fibres to the nucleus accumbens. It is the primary brain circuit that governs addiction, reward-mediated drive, reinforcement and motivational behaviour. The NAc is considered a master regulator of motivational drive, able to translate it in motor behaviour (*Wise, 2004; Salamone et al, 2005; Soares-Cunha et al, 2016*). The mesocortical pathway is constituted by dopaminergic inputs originating in the same A_{10} neuronal group of VTA that innervate the prefrontal cortex. It is involved in cognitive ad emotional control following the cortical integration of information about past reward responses (*Bidwell et al, 2011; Arnsten, 2015*). The nigrostriatal pathway arises from A_9 cell group of dopaminergic neurons in the substantia nigra and sends fibres to the dorsal striatum; it is involved in motor functions of dopamine, as part of a basal ganglia motor system (*Haber, 2003; Struzyna et al, 2018*). Finally, the tuberoinfundibular pathway, originating in A_8 neuron group of infundibular nucleus and projecting to the pituitary gland, is critical for the modulation of prolactine secretion (*Grattan, 2015*).

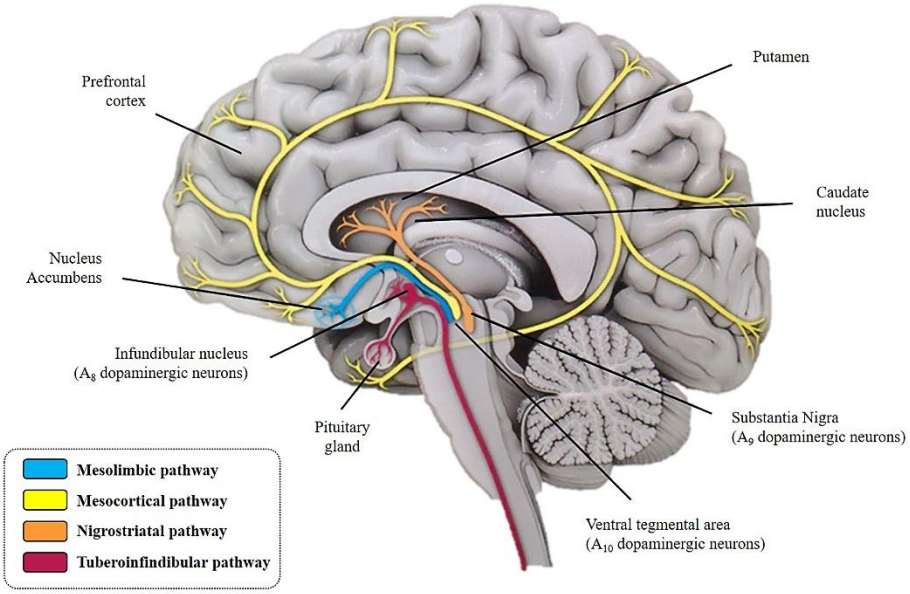

Fig. 4.2 | Four main dopaminergic pathways. For each circuit is indicated the source of dopaminergic neurons and the projecting area.

4.3.3 Mesolimbic circuit: role of dopamine in reward system

In particular, the midbrain projections from DA neurons in VTA to the NAc represent the key neural substrate upon which highly palatable food (HPF) and drug of abuse exert their actions. Therefore, the mesolimbic pathway is often referred to "reward system" of the brain (*Berland et al, 2016*). The alterations of this hedonic reward circuit in association with those of homeostatic hypothalamic system underline the mechanism of food addiction and obesity. Maladaptations in these two systems are suggested to be both the cause and the consequence of obesity (*Rothemund et al, 2007; Stice et al, 2008; Volkow et al, 2013*). Indeed, compulsive eating might result from adaptative responses of both circuits, which in turn derive from chronically exposure to HPF in vulnerable individuals (*DiLeone et al, 2012*

Volkow et al, 2013). In this context, a particular relevance has the contribute of D2 receptors expression in the projecting areas of mesolimbic circuit. It is well known that the availability of D2R in striatum modulate eating behaviour in normal weight subjects. The reduction of its levels has been positively related to both the body weight of obese individuals and the likelihood of eating when subjects are stressed or exposed to negative emotions (*Volkow et al, 2003*). To evaluate how decreased striatal D2R availability is implicated in facilitation of overeating, it has been investigated the activity of prefrontal cortical regions involved in inhibitory control and emotional processing, through the analysis of brain glucose metabolism (*Volkow et al, 2008*). The expression of D2 receptors in obese patients correlates with the metabolic activities of dorsolateral prefrontal cortex and cingulate gyrus, that are severely decreased in morbid obesity. This relationship can explain the loss of control on feeding behaviour and the poor emotional self-regulation during binge-compulsive eating. Conversely, it has been reported an enhanced activation of medial prefrontal cortex upon exposure to food-related stimuli in obese subject in combination with enhanced release of DA in striatum, that leads to desire for food, cravings and compulsive feeding (*Gautier et al, 2000;Volkow et al, 2002; Wang et al, 2004*).

4.4 Physiological and pathological roles of dopamine

Due to the huge extension of dopaminergic network, dopamine plays pivotal roles in various functions in human body. It can exert a dual role in both physiological and pathological conditions.

Dopamine is critically involved in several central functions in CNS, including modulation of reward and addictive behaviour, voluntary movements, working memory and attention. DA is a key factor that contributes to the inhibition of prolactine production from the pituitary gland. It is involved in moods such as depression, cognition, mania, learning and in the regulation of sleep and food intake. DA participates also in the

control of peripheral functions, such as regulation of kidney, pancreas, and gastrointestinal system; it has also a crucial role in the functioning of cardiovascular, immune and sympathetic systems. Moreover, it is involved in hormonal regulation and participates to the modulation of olfaction and retinal processes (*Snyder et al, 1970; Missale et al, 1998; Sibley, 1999; Carlsson, 2001; Iversen and Iversen, 2007*).

Because of its involvement in a huge variety of physiological functions, alterations or dysfunctions of dopamine system are strictly related to several human disorders. As demonstrated by the study of Ehringer and Hornykiewicz, the first evidence in the implication of dopamine disorder in health disease was the decrease of dopamine content in the caudate and putamen of subjects suffering from Parkinson's disease (PD), due to the loss of dopaminergic neurons in SN (*Ehringer and Hornykiewicz, 1960*). Indeed, few years later, Cotzias and collaborators developed a treatment for PD patients, that is already used today, based on high-dose oral DOPA, the precursor of dopamine that, unlike DA, is able to cross BBB (*Cotzias et al, 1967; Hardebo and Owman, 1980*). DA is thought to be involved also in another neurodegenerative disorder, Huntington's disease. In this disorder, the early accumulation of toxic mutant huntingtin (mHTT) in the dopaminergic striatal neurons suggests an important role of DA in the development of the pathology (*Jakel and Maragos, 2000; Cyr et al, 2006*). Since DA participates in cognitive and behavioural modulation, its dysregulation is involved in attention deficit hyperactivity disorder (ADHD) and Tourette's syndrome (*Mink, 2006; Swanson et al, 2007; Gizer et al, 2009*) and, although with less straightforward evidence, also in bipolar disorders and depression. It has suggested a crucial role of DA in schizophrenia, since most of antipsychotic drugs block the type 2 of dopamine receptors, thereby decreasing the postsynaptic response of DA (*Snyder et al, 1970; Seeman et al, 1976; Carlsson et al, 2001*). Because of its role in reward-seeking behaviour and motivation, it is not surprising that the abnormal release of dopamine during addiction has a pivotal role in this pathological condition (*Hyman et al, 2006; Di Chiara and Bassareo, 2007;*

Koob and Volkow, 2010). Drugs of abuse increase extracellular levels of DA in different ways depending on the pharmacological class of drugs (*Di Chiara, 1995)*. For example, amphetamines increase DA release from synaptic vesicles (*Cadoni et al, 1995*); narcotic analgesics and ethanol stimulate the firing rate of DA neurons through a disinhibitory mechanism (*Kiyatkin, 1988; Brodie et al, 1990*); and cocaine blocks the reuptake of DA from synaptic cleft (*Carboni et al, 1989*). Furthermore, in cocaine addiction it has been reported an up-regulation of D1R expression and capacity of binding (*Unterwald et al, 1994, 2001*) and, conversely, a lower density of D2R in NAc (*Moore et al, 1998*). Similar changes in dopamine receptor availability and functionality have been demonstrated also in food addiction. In particular, in rats with intermittent access to sugar or restricted access to sucrose, it has been reported an enhanced D1R and reduced D2R binding in the NAc (*Colantuoni et al, 2001; Bello et al, 2002*).

4.5 Interaction between orexin and dopamine in addiction

Orexins play a prominent role in conditioned responses to stimuli associated with food and drug rewards by regulating synaptic plasticity of DA neurons and dopaminergic neurotransmission in the VTA (*Harris et al, 2005*).

4.5.1 Interactions between orexinergic and dopaminergic systems

Anatomical and functional interactions occur among orexinergic and dopaminergic systems since:
i) dendrites and somata of dopaminergic neurons receive OX-positive afferences. Neurons of LH labeled with the anterograde tracer biotinylated dextran amine (BDA) have seen to project to VTA, in close proximity to cell bodies of dopaminergic neurons. The same result has been confirmed also by the observation of retrogradely-labeled fibres in VTA arising from lateral and perifornical area of the hypothalamus (*Fadel and Deutch, 2002*);

ii) orexin increases the firing rate of different groups of neurons in VTA. The enhanced firing rate of A_{10} dopaminergic neurons is coupled with depolarization not blocked by tetrodotoxin, a voltage-gated sodium channel blocker, thus indicating a direct effect on DA neurons (*Korotkova et al., 2003*). This group of dopaminergic neurons is involved in the mesolimbic circuit, since a recent study has reported an OX-A-mediated increased firing of neurons in VTA projecting to the shell region of nucleus accumbens (AcbSh) (*Baimel et al, 2017*);
iii) blocking of D2R reduces hyperlocomotion and stereotypy induced by intracerebroventricular orexin. OX-A and drugs like amphetamine share the ability to induce hyperlocomotion, grooming and stereotypy in rats, typically considered behaviours of enhanced dopaminergic tone (*Ida et al, 1999*). These effects are inhibited by the i.p. administration of haloperidol, a D2R antagonist, thereby suggesting the involvement of dopaminergic system in behavioural responses induced by OX-A (*Nakamura et al., 2000*). This is the reason why compounds that enhance extracellular dopaminergic levels in target regions, thanks to their capacity to promote one of the principal effects of OX-A, arousal, are used in treatment of narcolepsy (*Wisor et al, 2001*).

4.5.2 OX-A is involved in dopaminergic mesolimbic reward circuit

A huge amount of evidence supports the role of orexin in food/drug addiction and its involvement in mesolimbic reward system. Mice with orexin deficiency (knock-out for *Hcrt*) show a lower consume of sucrose when available ad libitum compared to littermate wild-type mice, regardless of their locomotor activity (*Matsuo et al, 2010*). Clinical observations have reported that patients suffering from narcolepsy, due to almost complete loss of orexinergic neurons, despite long-term medical treatment with amphetamines and other wakefulness-stimulating substances, rarely develop addiction for stimulant abuse (*Nishino et al, 2000; Peyron et al, 2000*). As demonstrated by Harris and collaborators, orexinergic neurons are

strongly activated by conditioned preferences for different rewards associated with food or drug, such as cocaine or morphine. Indeed, conditioned rats, that show a preference for CPP compartment previously paired with cocaine or food, exhibit an increased *c-Fos* activation (an immediate early gene used as a marker of neuronal stimulation) in LH orexinergic neurons; on contrary, animals showing no preference for drug-paired environment have *c-Fos* levels similar to controls (*Harris et al, 2005*). The activation of these neurons is strictly associated with pathological hyperarousal typical of compulsive and addictive behaviours (*Boutrel et al, 2005; de Lecea, 2012*). These and many other findings supported the "motivational activation" of orexinergic neurons hypothesis relating to drug addiction, because of OX-A critical role in driving the associated preferences and regulation of motivated responses for drugs (*Aston-Jones et al, 2009; Borgland et al, 2009; Mahler et al, 2014*).

Multiple extra-hypothalamic limbic brain areas, including the nucleus accumbens and ventral tegmental area, are suggested to be involved in orexin stimulatory effect on intake of HPF or drug. The central injection of OX-A directly in ventral tegmental area induces burst firing of dopaminergic neurons, through the movement of N-methyl-D-aspartate receptors (NMDARs) from intracellular or extrasynaptic sites to the neuron synapses, thus leading to potentiation of excitatory postsynaptic currents (EPSCs) (*Borgland et al, 2006*). The following increased release of dopamine in target areas induced by OX-A is prevented by administration of NMDA antagonist, as demonstrated by Wang and collegues (*Wang et al, 2009*). Intra-VTA injection of OX-A increases the intake of diet with high contents of fat and sucrose solution in ad-libitum fed rats (*Terrill et al, 2016*) and reinstates an extinguished drug-seeking behaviour after prolonged cocaine or morphine withdrawal (*Harris et al, 2005*). OX-A causes a 300% enhancement in hedonic reactions to sucrose taste, and is able to increase the intake of palatable chocolate M&Ms® when injected in rostral half of Nac medial region (*Castro et al, 2016*). Moreover, fourth intracerebroventicular (icv) OX-A injection increases intake of sucrose

solution (*Kay et al, 2014*) and when administered in paraventricular nucleus of the thalamus, orexin leads to an enhanced release of dopamine in the NAc, thus promoting reward-related feeding (*Choi et al, 2012*).

4.5.3 OX-A signalling in reward is mediated by OX_1R

Although both OX_1R and OX_2R are expressed on mesolimbic regions, the neurobiological effects of OX-A in drug reward seeking (such as cocaine or morphine) is due primarily by its binding to OX_1R, whereas OX_2R is more likely involved in sleep/wake cycle regulation and is suggested to participate in ethanol self-administration (*Harris et al, 2005; Borgland et al, 2006; Barson et al, 2015; Baimel et al, 2015*). Furthermore, endogenous OX-A appears to promote consumption of highly palatable food mainly through the activation of OX_1R. A role for the OX_1R in mediating intake of palatable food is supported by the findings that systemic injection of its antagonist, SB-334867, reduces intake of a high-fat diet under both ad libitum and limited-access feeding paradigms. In particular, as demonstrated by the two groups of Zheng and Terrill, blockade of orexin signalling in VTA reduces opioid-driven feeding behaviour (*Zheng et al, 2007*) and drinking of sucrose solution (*Terrill et al, 2016*). Injection of SB-334867 in VTA has also a crucial role in drug addiction, since it induces a decrease in reinstatement of drug-seeking elicited by cues (*Harris et al, 2005*) and reduces the overflow of dopamine induced by cocaine in NAc (*Wang et al, 2009*). Blockade of OX_1R activation in other central regions, through administration of SB-334867 in fourth ventricule or using rats knock-down for *Hcrtr1* in the paraventricular thalamus, confirmed its role in modulation of hedonic feeding and preference in the consumption of a high-fat diet (*Choi et al, 2012; Kay et al, 2014*). Furthermore, it has been reported that i.p. injection of the OX_1R antagonist reduces acute intake of HPF and the binge-like consumption of sucrose, by reducing *c-Fos* activation in the VTA (*White et al, 2005; Alcaraz-Iborra et al, 2014; Valdivia et al, 2014*).

4.5.4 Orexinergic system is activated by and promotes compulsive feeding

In addition to inducing palatable food intake, endogenous orexin itself is found to be strongly responsive to the consumption of highly palatable food. Orexin neurons are activated by physiological homeostatic stimuli including hypoglicemia and caloric restriction. Moreover, they are activated also in response to environmental cues that induce feeding, even during periods of relative energy abundance, thus indicating the ability of orexin system to respond and be activated in anticipation of rewards induced by HPF (*Choi et al, 2010*). Both orexin gene expression and peptide levels are affected in a time-dependent manner. In fact, brief stimulation of orexin neurons by glucose inhibits their firing by triggering hyperpolarization; in contrast, long-lasting stimulation induces orexin neurons depolarization and consequent increase of their spontaneous firing (*Burdakov et al, 2004, 2005*). Acute HFD consumption activates orexin neurons that project to the VTA, suggesting a crucial role of orexin signalling and mesolimbic circuit in the full manifestation of overconsumption of HPF (*Valdivia et al, 2014*). The activation of this LH-VTA pathway increases motivational behaviour that drives the craving for appetitive reinforcers. A recent study has shown that optically activation of LH-projecting fibres to dopaminergic neurons in VTA promotes compulsive reward seeking even in absence of physiological energy needs, but does not affect the homeostatic food intake. In fact, the inhibition of this pathway leads to a reduction of compulsive seeking of sucrose, but not food consumption in hungry mice (*Nieh et al, 2015*). Supporting this hypothesis, Piccoli and collaborators have showed that blockade of OX-A signalling inhibits compulsive eating of HPF in rats exposed to chronic stress induced by cyclic food restriction, characterised by exposition of animals to HPF, but avoiding their access to it. The antagonist of OX_1R is unable to suppress intake of food with high content of sugar in control animals unexposed to food restriction (*Piccoli et al, 2012*).

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Chapter 5

Aims

We sought to elucidate the effect of the aberrant OX-A signalling on dopamine trafficking in the ventral tegmental area of obese mice.

The functional interaction between orexinergic and endocannabinoid system has a dual crucial role in obesity and reward processes, since the massive 2-AG release in LH sustains the overproduction of OX-A which occurs during obesity (*Cristino et al, 2013*) and, on the other hand, is involved in the disinhibition mechanism in dopaminergic neurons of VTA.

In particular, we investigated if:

1) the aberrant OX-A signalling from LH to VTA could enhance DA synthesis and its release to NAcc in obese mice;

2) a 2-AG-dependent inhibition of CB_1R inhibitory inputs to TH-positive neurons could be triggered by enhanced OX-A signalling in the VTA of obese mice.

Moreover, we investigated the molecular mechanism through which aberrant dopaminergic signalling, sustained by high orexinergic tone in obesity, reduced the reward sensitivity making the mice prone to develop the food addiction.

With these aims, by exploiting morphological, pharmacological and biochemical approaches, we sought to unravel the role of OX system in the modulation of dopamine neurotransmission that underlies the rewarding circuitry in obese mice, promoting reward-associated food seeking behaviours (food addiction) and hyperphagia.

Cristino L, Busetto G, Imperatore R, Ferrandino I, Palomba L, Silvestri C, Petrosino S, Orlando P, Bentivoglio M, Mackie K, Di Marzo V, 2013. Obesity-driven synaptic remodeling affects endocannabinoid control of orexinergic neurons. Proc Natl Acad Sci U S A. 110(24):E2229-38.

Chapter 6

Materials and methods

6.1 Animal and drugs

Experiments were performed under institutional approval and according to the guidelines of the institutional ethical code and the Italian (D.L. 116/92) and European (Official Journal of European Community L358/1 12/18/1986) regulations for the care and use of laboratory animals.
Adult (16-wk-old) male C57BL/6j mice were purchased from Charles River Laboratories (Sulzfeld, Germany); male mice with spontaneous nonsense mutation of the ob gene for leptin (*ob/ob*, JAX mouse strain) B6.Cg-Lepob/J and WT ob gene expressing homozygous siblings of different ages were obtained from breeding ob gene heterozygotes, and genotyped with PCR. Since orexin levels exhibit a diurnal fluctuation (concentrations increase during the dark period or active phase (i.e. ZT13- 24) and decrease during the light period or rest phase (*Yoshida et al, 2001*), the animals were maintained under a 12h light:12h dark cycle, light on at 8:00 PM, i.e. ZT0, for at least 4 weeks before killing at ZT20-22. B6.Cg-Tg(TH-GFP)21-31 (C57BL/6JJcl) mice were used for the electron microscopy analysis. All the mice were housed in controlled temperature and humidity conditions, and fed ad libitum.
Wt and *ob/ob* mice were injected intraperitoneally (i.p) with several treatments [Tab. 6.1].

Tab. 6.1 Treatments for the animal models

Treatment	Concentration	Time	Product	Company
Vehicle - DPBS	1X	2h	14190094	Thermo Fisher
Leptin	5 mg/kg	2h	L4146	Sigma Aldrich
SB334867	30 mg/kg (wt mice) 60 mg/kg (*ob/ob* mice)	1h (before OX-A injection) 3h (*per se)*	1960	Tocris
Orexin-A	40 µg/kg	2h	1455	Tocris

6.2 Immunohistochemistry-DAB

Animals were then euthanised under isoflurane anaesthesia, and perfused transcardially with 0.1M phosphate buffer saline (PBS) followed by 4% paraformaldehyde/0.1M phosphate buffer, pH7.4 (PB). Brains were removed, fixed and cryoprotected with 30% sucrose (#S8501, Sigma Aldrich) in PB and were cut by a Leica CM3050S cryostat into 10µm-thick through the coronal plane. The sections were collected in three alternate series and manteined frozen until being processed.
The sections were rinsed 3 times, 5 min each, with tris-buffered saline solution (TBS) (0.1M, pH 7.3) and to block the endogenous peroxidase activity the slides were immersed in a solution of 0.75% H_2O_2 for 5 min at room temperature (RT) because significantly reduces the nonspecific background. To block the nonspecific binding sites and obtain an optimal tissue permeabilisation, the tissue sections were incubated with the TBS/0.4% Triton (TBS-T) blocking buffer, containing the primary antiserum (Normal Serum), for 30 min at RT. The sections were incubated overnight in a humid box at RT with primary antibody goat anti-Orexin-A (Santa Cruz) or mouse anti-TH (Millipore) diluted 1:200 in TBS-T [*Tab. 6.2*]**.** The following day, the sections were rinsed 3 times, 5 min each, with TBS and then incubated for 2h at RT with specific secondary rabbit anti-goat or goat anti-mouse immunoglobulin (H+L) conjugated with biotin (Vector Laboratories, Burlingame, CA) diluted 1:100 in TBS-T [*Tab. 6.3*]. For the peroxidase reaction, the sections were rinsed again 3 times, 5 min each, with TBS and incubated with avidin-biotin complex (ABC) for 1 h. Followed by an incubation with the chromogen substrate 3,3'-diaminobenzidine-4 (DAB) (Sigma Fast, Sigma-Aldrich, Louis, MO U.S.A.) for 1 or 2 minutes when the intensity of the signal were appropriate for imaging. To finish the reaction the sections were rinsed in TBS to stop the DAB reaction and the section dehydrated by subsequent slides immersion in alcohol 50% (2 min), 75% (2 min), 95% (2 min), 100% I (2 min), 100% II (2 min). The slices clarified by immersion 2 times in xylene 10 min each and then mounted with DPX (dibutyl phthalate xylene).

The densities of OXA and TH-immunoreactive signals in the ventral tegmental area (VTA) were evaluated by quantifying the immunosignal density in each region of interest (ROI) area ($3x10^3$ μm^2). The optical density (OD) of the background was acquired from areas adjacent to the measured area (i.e., a portion of tissue devoid of stained cells or fibres), to assign the zero as the value of the background. After the background density was subtracted, the OD was obtained using the formula: $OD = \log_{10} (255/I)$, where the "I" is the pixel intensity value given by the program.

Tab. 6.2 List of primary antibodies for immunohistochemistry-DAB

Name	Clonality	Product #	Company	Diluition used
OX-A	Goat polyclonal	sc-8070	Santa Cruz	1:200
Tyrosine hydroxylase (TH)	Mouse monoclonal	MAB318	Millipore	1:200

Tab. 6.3 List of secondary antibodies for immunohistochemistry-DAB

Name	Product #	Company	Diluition used
Biotinylated rabbit anti-goat immunoglobulin (H+L)	BA-5000	Vector Laboratories	1:100
Biotinylated goat anti-mouse immunoglobulin (H+L)	BA-9200	Vector Laboratories	1:100

6.3 Cell count

Quantitative analysis of the relative abundance of TH-positive neurons was performed per μm^2 of one of three alternate series selected from the VTA and was representative of the entire area from bregma -2.92 mm to -3.88 mm. (n = 32 sections/mouse; n= 3 mice/genotype).

6.4 Immunofluorescence

Animals were then euthanised under isoflurane anaesthesia, and perfused transcardially with 4% (wt/vol) paraformaldehyde /0.1M phosphate buffer (PB) pH 7.4. Brains were removed, fixed and then transferred into a 30% sucrose (#S8501, Sigma Aldrich) in PB and were cut with a Leica CM3050S cryostat into 10μm-thick serial sections in the coronal plane, collected in alternate series and frozen until being processed.

The tissue sections were rinsed 3 times with 0.1M PB (pH 7.4) and incubated with 1% normal donkey serum dissolved in the permeabilization buffer PB-Triton X-100 0.3% (PB-T) for 30 min at RT to permeabilize the cell membrane and block the nonspecific binding sites. Then, the sections were incubated overnight in a humid box at RT with the following primary antibody diluted in PB-T [*Tab. 6.4*]: goat anti-OX-A (Santa Cruz); mouse anti-TH (Millipore); guinea pig anti-diacylglycerol lipase-α (anti-DAGLα) (kindly provided by Prof. Ken Mackie); goat anti-OX_1R antibody (Santa Cruz); guinea pig antivesicular glutamate transporter (anti-VGluT1) (Synaptic Systems); guinea pig anti-vesicular GABA transporter (anti-VGAT) (Synaptic Systems); rabbit anti-CB_1R antibody (anti C terminus 461-472, Abcam); rabbit anti-dopamine 2 receptor (Biorbyt); goat anti-beta arrestin2 (Santa Cruz).

The following day, the slides were rinsed 3 times, 5 min each, with PB and then incubated for 2h at RT with specific Alexa-488, -546 or -350 secondary donkey anti-IgGs (Invitrogen LifeTechnology) [*Tab. 6.5*]. Then, the sections were rinsed 3x for 5 min each, with PB, counterstained with nuclear dye DAPI (4',6-diamidino-2-phenylindole), rinsed again 3x for 5 min each with PB and, then, mounted with Aquatex, the aqueous mounting medium that stabilizes the tissue sample and stains for long-term usage. The sections were analysed by confocal microscopy (Nikon Eclipse Ti2). The images were acquired with a digital camera DS-Qi2 (Nikon) and processed by Image analysis software NIS-Elements C (Nikon, Florence, Italy), n=6–10 z stacks were collected every 0.5μm throughout the area of interest of each acquired field to be processed by the imaging deconvolution software. For

each section, the optical density zero value was assigned to the background (i.e., a tissue portion devoid of stained cell bodies or fibres).

Tab. 6.4 List of primary antibodies for immunofluorescence

Name	Clonality	Product #	Company	Diluition used
OX-A	Goat polyclonal	sc-8070	Santa Cruz	1:100
TH	Mouse monoclonal	MAB318	Millipore	1:100
VGlut1	Guinea pig polyclonal	135 304	SYSY	1:200
OX_1R	Goat polyclonal	sc-8072	Santa Cruz	1:100
CB_1R	Rabbit polyclonal	ab23703	Abcam	1:100
D2R	Rabbit polyclonal	AB5084P	Merck	1:100
VGAT	Guinea pig polyclonal	131 004	SYSY	1:200
β-Arrestin 2	Goat polyclonal	sc-6387	Santa Cruz	1:100
DAPI	DNA staining	D9542	Sigma Aldrich	1 µg/ml

Tab. 6.5 List of secondary antibodies for immunofluorescence

Name	Product #	Company	Diluition used
Alexa Fluor 594 donkey anti-goat IgG (H+L)	A11058	Life Technologies	1:50
Alexa Fluor 488 donkey anti-mouse IgG (H+L)	A21202	Life Technologies	1:50
Alexa Fluor 488 goat anti-guinea pig IgG (H+L)	A11073	Life Technologies	1:100
Alexa Fluor 350 donkey anti-mouse IgG (H+L)	A10035	Life Technologies	1:50
Alexa Fluor 594 donkey anti-rabbit IgG (H+L)	A21207	Life Technologies	1:50
Alexa Fluor 488 donkey anti-goat IgG (H+L)	A11055	Life Technologies	1:50

6.5 Correlative light and electron microscopy (CLEM): OX_1R and CB_1R immunoreactivity in preembedding electron microscopy

Under isoflurane anaesthesia, mice were perfused transcardially with 3% paraformaldehyde/0.5–1% glutaraldehyde (vol/vol) in PB. Double preembedding immunogold labelling was performed on the VTA sections (50-µm-thick) of TH-eGFP mice fed ad libitum. The VTA sections containing TH-eGFP neurons were selected by observation at fluorescent microscope being easily recognisable under 488nm excitation vawelenght. The selected sections were incubated free-floating overnight at 4°C with the primary antibodies (rabbit anti-CB_1R antibody, anti C terminus 461-472, Abcam, and goat anti-OX_1R, SantaCruz), all diluted 1:100 in donkey serum blocking solution with 0.02% saponin. Subsequently, the sections were incubated in a mixture of 6nm (for CB_1R) and 10nm (for OX_1R) gold-conjugated secondary antibodies (Aurion), diluted 1:30 in donkey serum blocking solution with 0.02% saponin. Sections were then treated with 0.5% OsO4 in PB for 30 min at 4 °C, dehydrated in an ascending series of ethanol

(50%, 70%, 96%, 100%) and propylene oxide and embedded in TAAB 812 resin (TAAB). During dehydration, sections were treated with 1% uranyl acetate in 50% ethanol (vol/vol) for 15 min at 4 °C. Ultrathin (50nm thickness) sections were cut by vibratome (Leica), collected on Formovar-coated, single- or multiple-slot (50-mesh) grids and stained with 0.65% lead citrate. Electron micrographs were taken with the TEM microscope (FEI Tecnai G2 Spirit TWIN). The TEM observation was limited to series sectioned up to 0.6–0.8 μm depth from the external surface of preembedded immunolabeled tissue. Additional sections were processed in parallel as controls of reaction by omitting both or one of the primary antibodies from the mixture. No labeling was detected in the control material.

6.6 Lipid extraction and 2-AG measurement

After treatments, the lean and obese mice were euthanised by cervical dislocation, the brains removed and the brain areas rapidly dissected. Tissue samples were pooled and homogenised in 5 vol chloroform/methanol/Tris HCl 50mM (2:1:1 by volume) containing 50 pmol of d5-2-arachidonoylglycerol (d5-2-AG) as internal standards. Homogenates were centrifuged at 13,000 × g for 16 min (4°C), the aqueous phase plus debris were collected and four times extracted with 1vol 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 analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (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). Values are expressed as pmol/mL of lipid extracted.

6.7 Dopamine measurement in VTA and NAc

After treatments, the lean and obese mice were euthanised by cervical dislocation, the brains removed and the study brain regions, the ventro-tegmental area and nucleus accumbens, rapidly dissected from each brain on ice. The Dopamine levels were measured with the mouse dopamine enzyme-linked immuno specific assay (ELISA) kit (Cusabio Biotech, Wuhan, China), following the manufacturer's instructions. Briefly, both the brain areas were rinsed with 1X PBS, homogenized in 1 ml of 1X PBS and stored overnight at -20°C. After two freeze-thaw cycles, performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g, 2 - 8°C. The supernatant was removed and assayed immediately.
The absorbance of each well was measured at a wavelength of 450 nm using a 96-well microplate spectrophotometer (Multiskan GO; Thermo Scientific, Waltham, MA). This assay can detect mouse dopamine in the range of 5–1000 pg/ml.

6.8 Co-immunoprecipitation assay and Western blotting

After treatments, the lean and obese mice were euthanised by cervical dislocation, the brains removed and the nucleus accumbens rapidly dissected on ice and homogenized in the appropriate ice cold lysis buffer. Protein concentrations in the supernatants were determined in duplicate using the Lowry protein assay (#55000111, Bio-Rad). The co-Immunoprecipitation was performed with the Dynabeads Protein G-Kit (Invitrogen Life Technology) according to the manufacturer's instruction. Briefly, the Rabbit anti-Dopamine D2 Receptor polyclonal antibody (Millipore, catalog #AB5084P) was incubated with dynabeads precoupled with protein G that act as suspendable solid support that can be fixed by the use of a magnet, 10' with rotation at RT. The beads with attached antibodies (Dynabeads-Ab complex) were washed with specific washing buffer. D2R was immunoprecipitated via the incubation of the D2R-containing sample with

Dynabeads-Ab complex for 10' with rotation at RT. After 3x washes with washing buffer, immunoprecipitated D2R was extracted from Dynabeads-antibody complex via a resuspension with elution buffer for 2' at RT.
Samples were then prepared with 4XLaemmli sample buffer (#1610747, Bio-Rad) completed with 0,1% 2-Mercaptoethanol (#31350010, Thermo Fisher) and then boiled at 95°C for 10 minutes to load them into the wells of the gels. The samples were run on 12% gels made from the resolving gel, 40 ml of 30% Acrylamide (#1610158, Bio-Rad), 25ml of 1,5M Tris-HCl (pH 8.8), 1ml of 10% Sodium dodecyl sulfate (SDS) (#L5750, Sigma Aldrich), 500 µl of 10% ammonium persulfate (APS) (#1610700, Bio-Rad), 50 µl of N,N,N′,N′-Tetramethylethylenediamine (TEMED) (#1610800, Bio-Rad) and 33,5 ml of distilled water. All the components were mixed and poured between two glass plates of 0,75mm, topped with 100% methanol to avoid contact of the gel with air which prevents polymerisation. Upon polymerisation of the resolving gel, a 4% stacking gel was prepared by mixing 3,3 ml of 30% Acrylamide, 6,3ml of 0,5M Tris-HCl (pH 6.8), 250 µl of 10% SDS, 125 µl of 10% APS, 25 µl of TEMED and 15 mL of distilled water. The stacking gel solution was added on the resolving gel between the glass plates after removal of the methanol and a comb was inserted for loading the 10 or 15 well formation. Enough incubation time, approximately 30 minutes was allowed for the gel to harden and use. The gel was placed in electrophoresis cassettes and completely covered with 1x running
buffer (25 mM Tris, 190 mM glycine (#G8898, Sigma Aldrich) and 0.1%SDS). The first lane of the gel was loaded with 5 µl of Precision Plus Protein All Blue Standards as a ladder (#161-0373, Bio-Rad), and 20 µl of sample with the same amount of protein were loaded in the remaining lanes. Samples were run at 80V for 20 min and subsequently at 120V until the front dye would diffuse out of the gel. Gels were removed from the glass plates and washed in 1X transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol). A 0.2 µm PVDF membrane (#1620177, Bio-Rad) was activated for 10 seconds in methanol and then washed in 1x transfer buffer. Gel and membrane were assembled in a wet transfer cassette covered in 1x transfer

buffer and the gel proteins were blotted onto PVDF membrane at 100V for 60 min at 4°C. The membranes were blocked with 5% skim milk powder (#70166, Sigma Aldrich) for 1h shacking at room temperature, and incubated overnight at 4°C with rabbit anti-Dopamine D2 Receptor antibody (Millipore, catalog #AB5084P) or rabbit anti-β-Arrestin2 antibody (Cell Signaling Technology, catalog #3857) dissolved in 5% milk in TBS (20mM Tris, 150 mM NaCl). Membranes were washed 3x with TBS-T (TBS with 0.1% Tween 20, #1706531, Bio-Rad) and then incubated for 1 hour at room temperature with goat anti-rabbit IgG (H+L)-HRP conjugate antibody (Biorad, #1706515). The membranes were again washed 3x with TBS-T and their reactive bands were detected by chemiluminescence after a 5 min incubation without light with ECL (#170-5061, Bio-Rad) and visualised using Chemidoc MP Imaging System (#17001402, Bio-Rad).
The images were analysed and quantified using ImageJ software (imagej.nih.gov/ij/). The intensity of the bands of immunoblotted β-Arrestin2 protein was normalised with the D2R protein as loading control. An example of the quantification protocol can be found online (https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/using-imagej-quantify-blots).

6.9 Statistical analyses

Data are expressed as mean ± SEM and were analysed with GraphPad Prism 6 software, version 6.05 (GraphPad, Inc.). Statistical differences among groups were determined by either Student's t-test or two-way ANOVA followed by post hoc Tukey tests for comparison among means. A level of confidence of $P<0.05$ was employed for statistical significance.

Yoshida Y, Fujiki N, Nakajima T, Ripley B, Matsumura H, Yoneda H, Mignot E, Nishino S, 2001. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. Eur J Neurosci. 14(7):1075-1081

Chapter 7

Results

7.1 Increase of OX-A content in fibres and TH expression in the VTA of *ob/ob* mice.

To investigate the effect of OX-A on dopamine trafficking in obese mice, we carried out an immunohistochemical study to analyse OX-A and TH expression in the ventral tegmental area (VTA) of *ob/ob* (n = 6 mice per group) and wt (n = 6 mice per group) mice.

7.1.1 Enhanced OX-A levels in the VTA of ob/ob mice

In *ob/ob* mice, we found an increased expression of OX-A in the fibres projecting from LH to VTA [*Fig. 7.1A*]. As shown in *Fig. 7.1A3* and *Fig. 7.1A4*, orexin fibres of obese mice exhibited a higher positivity and varicosity than wt mice, which showed a lower expression of OX-A [*Fig. 7.1A1 and A2]*, in both the magnifications. Optical densitometry analysis confirmed these data, showing a higher density of OX-A signal in *ob/ob* than littermate wild-type mice [*Fig. 7.1B*].

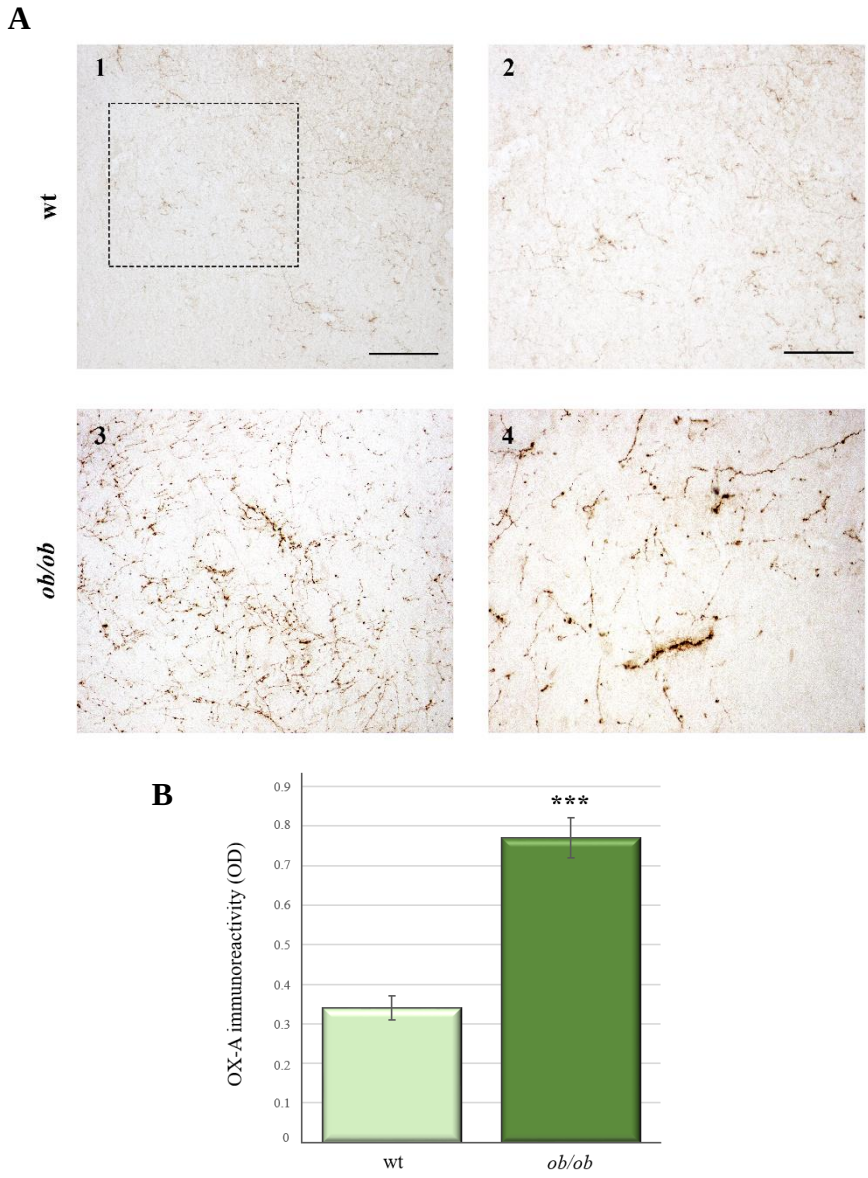

*Fig. 7.1 | **(A)** Representative peroxidase-based OX-A immunoreactivity staining in the VTA of wt and obese ob/ob mice showing a dense plexus of orexinergic fibres intensely immunoreactive in the latter mice. Higher magnification of fields depicted in right side. [Scale bar: 150 µm (A1 and A3), 50 µm (A2 and A4)]. **(B)** Bar graph of OX-A peroxidase-based optical density. (OD). Data were from n = 6 mice per group fed ad libitum and were means ± SEM; ***P<0.001 vs wt mice.*

7.1.2 TH levels are increased in the VTA of ob/ob mice

Dopamine neurons in the VTA are crucial in the regulation of reward and reinforcement behavioural processes. In this area, we examined the differences in the tyrosine hydroxylase (TH) expression, the rate-limiting enzyme of dopamine synthesis, between obese and wt mice.
We found enhancement of TH expression in the dopaminergic neurons of *ob/ob* mice in comparison to wt mice [see *Fig. 7.2A1 and A2 for wt; Fig.7.2A3 and A4 for ob/ob*]. The quantitative analysis of immunoreactivity (ir) in VTA confirmed the enhancement of TH expression in obese mice compared to lean mice [*Fig. 7.2B*]. These data suggested that an augmentation of dopamine levels occurred in the VTA of *ob/ob* mice.
Cells counts within the entire volume of VTA demonstrated that the enhancement of dopamine concentration was not accompanied by a quantitative increase of neurons in the area in both genotypes. The number of TH-ir neurons (± SEM) was 117,56 ± 6,9 for wt mice and 133,89 ± 12,1 for *ob/ob* mice, with no significant difference [*Fig. 7.2C*].

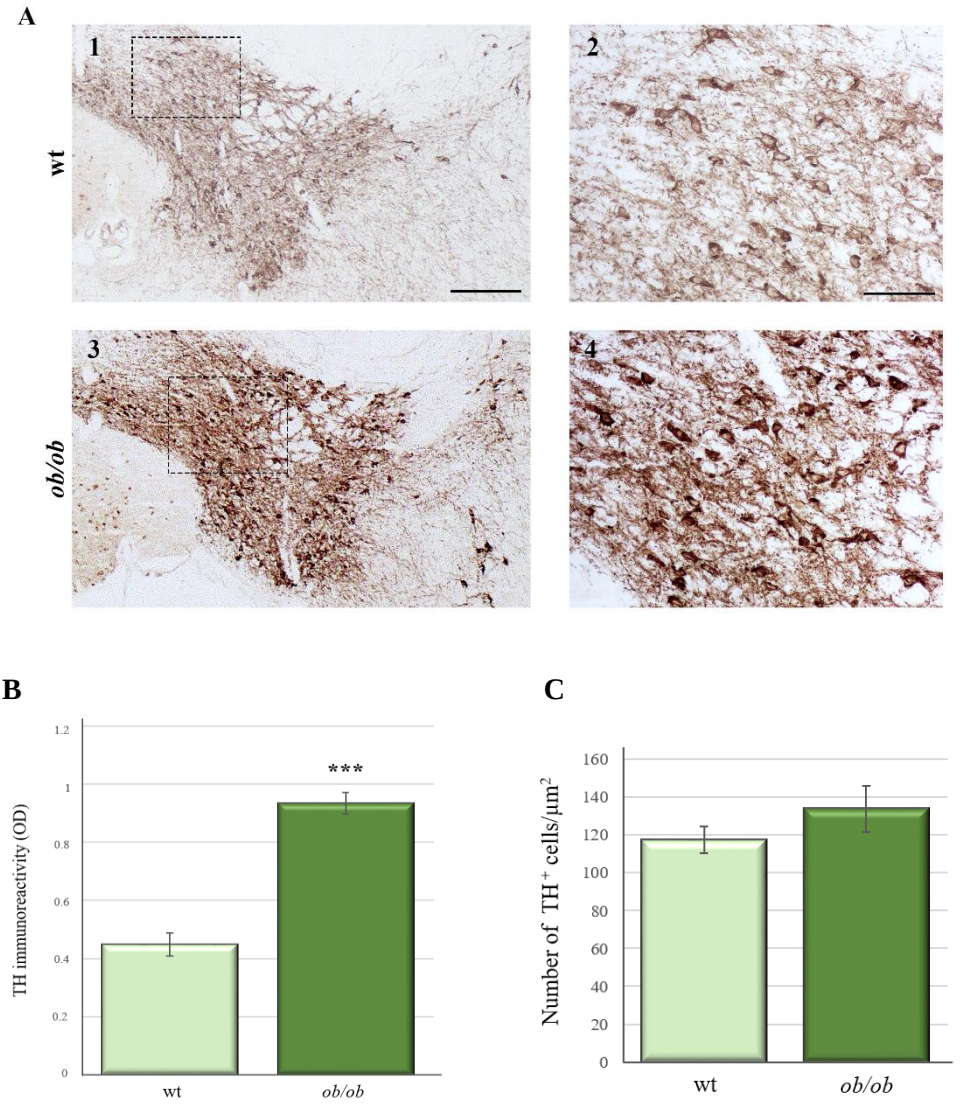

*Fig. 7.2 | (**A**) Representative peroxidase-based TH staining in the VTA of wt and ob/ob mice. Higher magnification of fields depicted in right side. [Scale bar: 300 µm (A1 and A3), 100 µm (A2 and A4)]. Please note enhancement of TH peroxidase immunolabeling in ob/ob mice (**B**) Bar graph of TH peroxidase-based optical density (OD). (**C**) Bar graph showing the mean number of TH–expressing neuron. Data were from n = 6 mice per group and are means ± SEM; ***P<0.001 vs wt mice.*

7.1.3 Elevation of OX-A release in VTA occurred concurrently with an increased expression of TH in the dopaminergic neurons of obese mice

In order to analyse simultaneously OX-A and TH expression in the VTA and to investigate the relationship between OX-A levels and dopamine production, we performed an immunofluorescence study on wt and *ob/ob* mice. In the normal weight mice, a low expression of OX-A in the orexinergic fibres arising from LH was found in concomitance with reduction of TH expression in the dopaminergic neurons [*Fig. 7.3A and B*]. In *ob/ob* mice, we detected a higher immunodensity of OX-A in fibres projecting from LH to VTA, accompanied by an increased TH expression in the area [*Fig. 7.3C*]. As shown in *Fig.7.3C* and in high magnification in *Fig.7.3D*, presynaptic terminals with an elevated OX-A content were apposed to somata of dopaminergic neurons, which exhibited an enhanced immunolabeled signal of TH. These results showed that in the VTA of obese mice the elevation of OX-A release occurred concurrently with an increased expression of TH in the dopaminergic neurons.

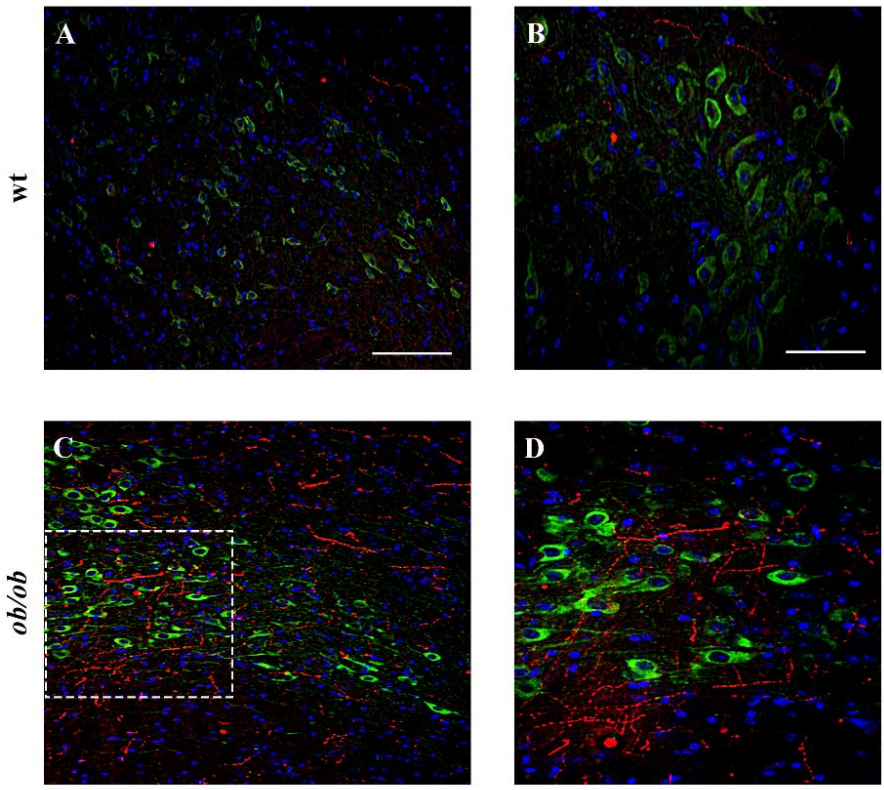

*Fig. 7.3 | Confocal microscopy images showing OX-A immunolabeled axon terminals apposed to TH-expressing neurons of adult wt and ob/ob mice. Red signals indicated OX-A immunofluorescence, green signals is referred to TH immunofluorescence, blue signal to DAPI. Please note enhancement of OX-A and TH immunolabeling in the VTA of ob/ob mice (**C** and **D**) compared to wt mice (**A** and **B**). Higher magnification of fields depicted in boxed areas. [Scale bar: 100 μm (A and C), 50 μm (B and D)]. Data were from n = 6 mice of each group.*

7.2 The anatomical and molecular substrate of VTA which underlies a functional cross-talk between orexin and endocannabinoid system: structural and ultrastructural evidence

7.2.1 Confocal study

In order to study the molecular substrates of the OX-A/eCBs/dopamine interaction at TH neurons in the VTA, we performed multiple OX-A/DAGL-α/TH, OX-A/VGluT1/TH and CB_1R/VGAT/TH immunofluorescence.

Multiple TH-DAGLα-OX_1R immunofluorescence of VTA [*Fig. 7.4A*] revealed the immunoexpression of DAGLα, the main biosynthesizing enzyme of 2-AG, close to OX_1R immunoreactivity on the plasma membranes of TH-positive neurons. These data provided anatomical evidence for the molecular substrate which underlies the functional cross-talk between 2-AG and OX-A in the VTA.

Orexinergic fibres originating from LH and projecting widespread to many different areas of the brain, are mainly excitatory (*Rosin et al, 2003*). In accordance, VGluT1, the main vesicular transporter of glutamate at presynaptic excitatory inputs, was found to colocalise with OX-A immunolabeling in fibres apposed to TH positive neurons, as revealed by TH-OX-A-VGluT1 [*Fig. 7.4B*]. Along with excitatory orexinergic inputs, dopaminergic neurons are regulated also by inhibitory inputs coming from striatal areas (*Kalivas, 1993*). To further study the anatomical pathway underlying the control of OX-A/dopamine circuit in the VTA, we performed TH-VGAT-CB_1R immunofluorescence. We found inhibitory CB_1R-immunolabeled GABAergic synaptic endings apposed to soma of TH-positive neurons in VTA. Indeed, these axon terminals exhibited VGAT, the protein responsible for the storage of GABA inside the synaptic vesicles, and expressed the CB_1 receptor on their cell membrane [*Fig. 7.4C*].

These data suggest the hypothesis that, because of the neuronal arrangement of VTA, a cross-talk between orexin, endocannabinoid and dopamine occurs in obesity. In fact, is well-known that upregulation of orexin signalling,

sustained by factors like endocannabinoids and impaired leptin signalling, occurs in obese mice (*Imperatore et al, 2017*). OX-A, released in excess from LH axon terminals in the synaptic cleft, interacts with the OX_1R receptor expressed on the dopaminergic neurons in VTA. Hence, it activates the orexin intracellular pathway that, through PLC activation and DAG production, leads to 2-AG biosynthesis, mediated by DAGLα. Given its lipophilic nature, 2-AG acts through retrograde neurotransmission to modulate the synapse in the VTA. In fact, by interacting with the CB_1 receptor expressed on the inhibitory presynaptic terminal, it prevents the release of GABA, thus disinhibiting the dopaminergic neurons, which would be more stimulated to produce and release dopamine [*Fig. 7.4D*].

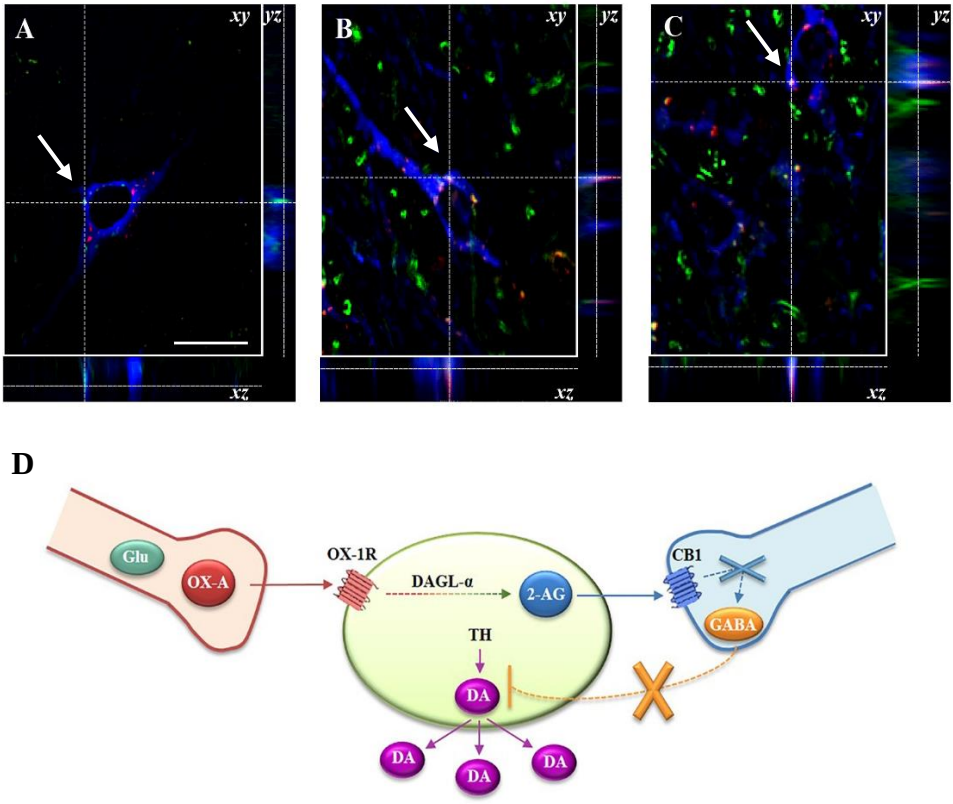

*Fig. 7.4 | Confocal microscopy images (maximum intensity projections) of three multiple immunostained sections of the VTA. (**A**) Representative image of a dopaminergic neuron showing the expression of OX_1R (red), DAGL-α (green) and TH (blue), overlapping as indicated by arrow. (**B**) Representative image of a dopaminergic neuron showing OX-A/VGluT1/TH co-expression as white signals as a merge of OX-A (red), VGlut1 (green) and TH (blue) overlapping immunofluorescences. An OX-A/VGluT1 synaptic contact with TH neuron is indicated by arrow. (**C**) Representative image of a TH neuron (blue) in the VTA receiving CB_1R immunolabeled puncta (red) colocalising with VGAT immunoreactive inputs (green). Merged signals are indicated by arrows and are represented for each single optical section (xy). The intersection between the white lines represents overlapping contact from the orthogonal projections at higher magnification, as reported in the lateral insets showing yz and xz combination of the optical sections [scale bar: 30 μm]. Data were from n = 6 mice of each group. (**D**) Scheme of the anatomical substrate depicting the functional cross-talk between OX-A and 2-AG in the dopamine neurons of VTA.*

7.2.2 Correlative light and electron microscopy (CLEM) study

To further demonstrate the anatomical substrate wherein orexin, endocannabinoids and dopamine interplay, we performed a CLEM study of OX_1R and CB_1R immunolabeling in the VTA of TH-eGFP mice in collaboration with the advanced Microscopy and Image Core, Electron Microscopy division headed by Dr. Polishchuck at the Tigem Institute of the Research Area NA/3 of Pozzuoli.
CLEM analysis revealed TH-eGFP somata of a neuron, identified according to math e methods (*chapter 6*), receiving CB_1R positive puncta (6 nm immunogold particles) forming symmetrical (putative inhibitory) contact. On the same neurons, co-expression of OX_1R labeling (10 nm immunogold particles) was found at post-synaptic site of asymmetrical (putative excitatory) inputs [*Fig. 7.5*].

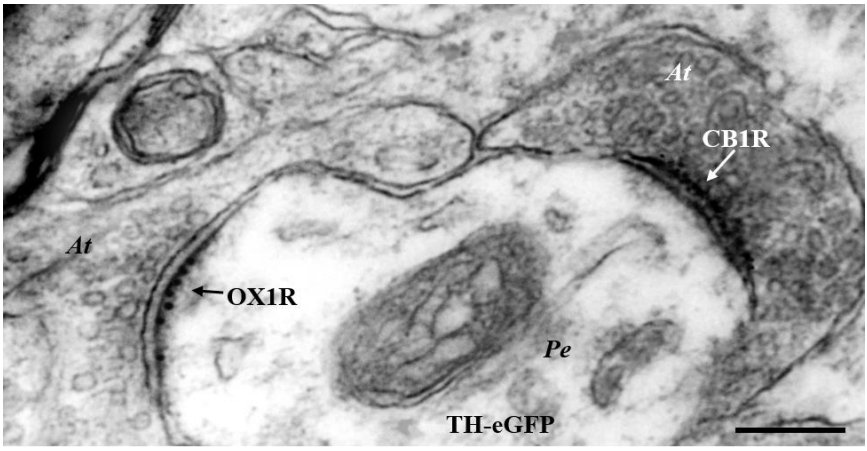

Fig. 7.5 | Representative electron micrograph showing double OX_1R/CB_1R immunogold reactivity by silver enhancing in TH-eGFP mice. On the left, asymmetrical, putative excitatory, axosomatic synapse between an axon terminal (At) opposite to the perikaryon (Pe) of a neuron that exhibited marked OX_1R immunogold labeling at the edges of the postsynaptic density. On the right, symmetrical, putative inhibitory, axosomatic synapse between an axon terminal (At), which exhibited marked CB_1R immunogold labeling in the presynaptic membrane opposite to perykaryon of a TH-eGFP neuron. CB_1R labeling was evident at the edges of presynaptic membrane specializations. [Scale bar: 150 nm].

7.3 2-AG levels are increased in the VTA of obese mice

These experiments were carried out in collaboration with Dr. Piscitelli, of the Endocannabinoid Research Group at ICB, CNR, Pozzuoli, who performed the analysis by LC-APCI-Mass spectrometry. Enhancement of 2-AG levels was found in the VTA of *ob/ob* mice in comparison to littermates wild-type mice, as well as in the VTA of wt mice injected with OX-A in comparison to the wt-vehicle injected mice and in a manner prevented by SB-334867 injection. In the VTA of SB-334867-treated *ob/ob* mice we found decreased levels of 2-AG, similar to the vehicle-injected wt mice. Notably, these data demonstrated that the increase of the endocannabinoid tone in VTA is due specifically to OX-A via OX_1R, since it can be modulated by SB-33487, a specific antagonist of OX-A pathway [*Fig. 7.6*].

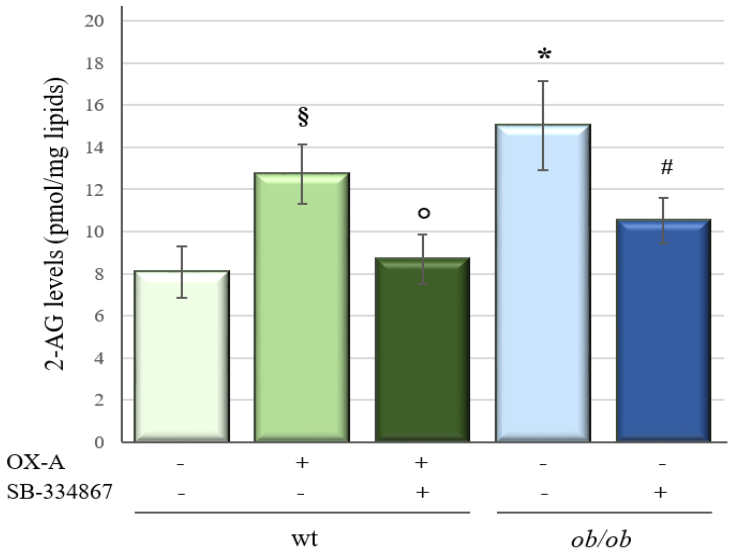

*Fig. 7.6 | Levels of 2-AG in VTA of wt mice, treated with vehicle, OX-A or SB-334867 + OX-A, and ob/ob mice. OX-A injection (40 µg/kg, i.p., 2h) increased production of 2-AG in the VTA of wt mice similar to ob/ob mice, via OX_1R, since pre-treatment with SB-334867 (30 mg/kg, i.p., 1h before OX-A injection) restored the basal level of the endocannabinoid. Data were from n = 6 mice per group and were means ± SEM; $^{§}P<0.05$ wt+OX-A vs wt mice; °P<0.05 wt+SB-334867+OX-A vs wt+OX-A; *P<0.05 ob/ob vs wt mice; #P<0.05 ob/ob+SB-334867 vs ob/ob mice.*

7.4 Dopamine levels are enhanced in VTA and NAc of obese mice and are partially modulated with specific treatments

Once we fully studied the anatomical substrate and the molecular pathway that allow the interaction between OX-A and 2-AG in VTA, we moved forward to the quantitative analysis of dopamine, in order to investigate the contribute of OX-A in the production and release of dopamine during obesity.

7.4.1 The augmentation of DA production in VTA of ob/ob mice is dependent on OX-A signalling

ELISA assay was performed to determine DA production in the VTA, which is the brain region containing TH-immunoreactive or dopamine-synthesising neurons. We carried out a comparison between the levels of DA in both wt and obese mice injected with vehicle or different molecules [*Fig. 7.7*].

First of all, we found that basal level of DA in *ob/ob* mice were significantly higher than wt mice, because of the strictly direct correlation among dopaminergic tone and body weight. Treatment with OX-A in wt mice increased DA concentration in a manner sensitive to SB-334867, since the pre-treatment with the antagonist of OX_1R inhibited the effect of OX-A. Moreover, the DA levels were found to be increased in the VTA of wt mice injected with leptin that avoided the release of OX-A from LH and consequently, as well as SB-334867, had an inhibitory effect on orexin signalling [*Fig. 7.7A*]. Furthermore, we investigated DA production also in *ob/ob* mice with various treatments. Unlike wt mice, injection of OX-A in obese mice did not alter levels of DA in dopaminergic neurons, because of the elevated basal concentration of OX-A in vehicle-treated *ob/ob* mice. This augmentation was reverted after treatment with the antagonist of OX_1R, the SB-334867, alone or before OX-A, thus demonstrating the direct involvement of orexin in dopamine production. Finally, we analysed DA

concentration in obese mice treated with leptin. Since *ob/ob* mice model is characterised by leptin deficiency, the injection of this peptide hormone was able to reduce the levels of DA, through an indirect mechanism that involved the inhibition of orexin production in LH [*Fig. 7.7B*].

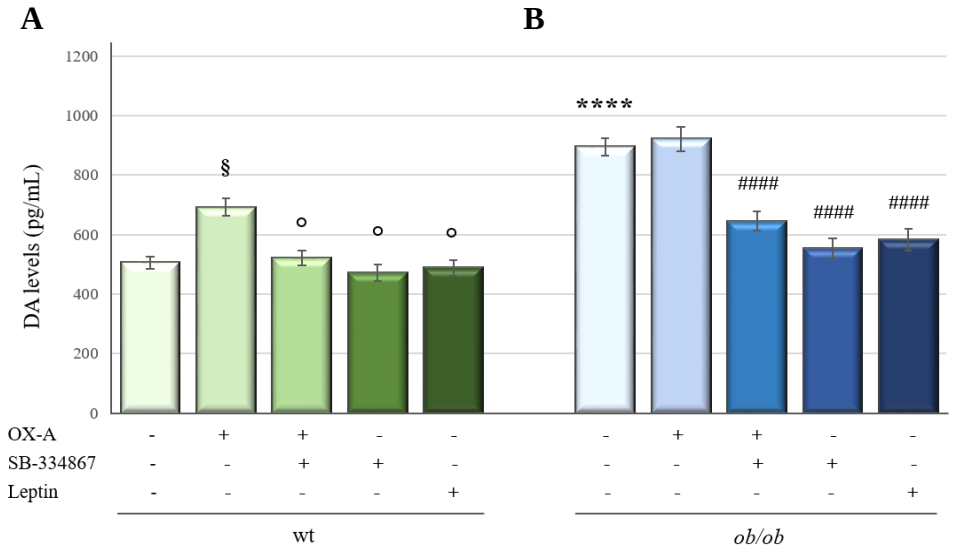

*Fig. 7.7 | Bar graph reporting the results of ELISA assay for dopamine determination in the VTA of wt, lean (**A**) and ob/ob (**B**) mice. DA concentrations in ob/ob mice were significantly increased compared to wt, in a manner sensitive to leptin (5 mg/kg, i.p., 2h) and prevented by the selective antagonist of OX_1R, SB-334867(60 mg/kg, i.p., 3h per se or 1h before OX-A injection). Treatment with OX-A (40 µg/kg, i.p., 2h) increased DA levels in wt but not in ob/ob mice. Data were from n = 6 mice per group and were means ± SEM; $^{§}P<0.05$ wt+OX-A vs wt mice; °P<0.05 wt treated vs wt+OX-A; ****P<0.0001 ob/ob vs wt mice; $^{####}P<0.0001$ ob/ob treated vs ob/ob mice.*

7.4.2 DA release is increased in NAc of ob/ob mice and is modulated by several treatments

One of the principal target area of VTA is NAc. In fact, the dopaminergic neurons send wide projections to the nucleus accumbens, forming the so-called mesolimbic reward pathway, that represents the key neural substrate upon which excessive feeding, mainly highly palatable food intake, exerts its action. On these bases, we next investigated the levels of dopamine in the NAc of lean and obese mice.
As might be reasonably expected from the analysis of dopamine production in VTA, also in NAc the levels of DA in wt mice were constitutively lower than the *ob/ob* mice treated with vehicle. The i.p. injection of OX-A was able to increase the concentration of DA in wt mice. The administration of SB-334867, alone or in combination with OX-A, by blocking OX_1R activity, prevented an excessive release of DA from VTA to NAc. Furthermore, DA concentrations in lean mice treated with leptin were found to be comparable to vehicle-injected wt mice [*Fig. 7.8A*]. In *ob/ob* mice, because of the intensified production of DA that occurred both in VTA of vehicle and in OX-A treated animals, the levels of this monoamine in the NAc were significantly raised. To confirm that the increase of DA levels was due specifically to high concentration of OX-A, we injected animals with SB-334867 *per se* or as pre-treatment of OX-A, or leptin. Both the treatments, thanks to their ability to block OX-A signalling, induced a severe reduction of DA levels, reaching values comparable to basal level of wt mice [*Fig. 7.8B*]. All these data demonstrated the implication of OX-A through an EC-mediated mechanism in the regulation of dopamine production and the modulation of this system through different treatments that specifically block OX-A signalling.

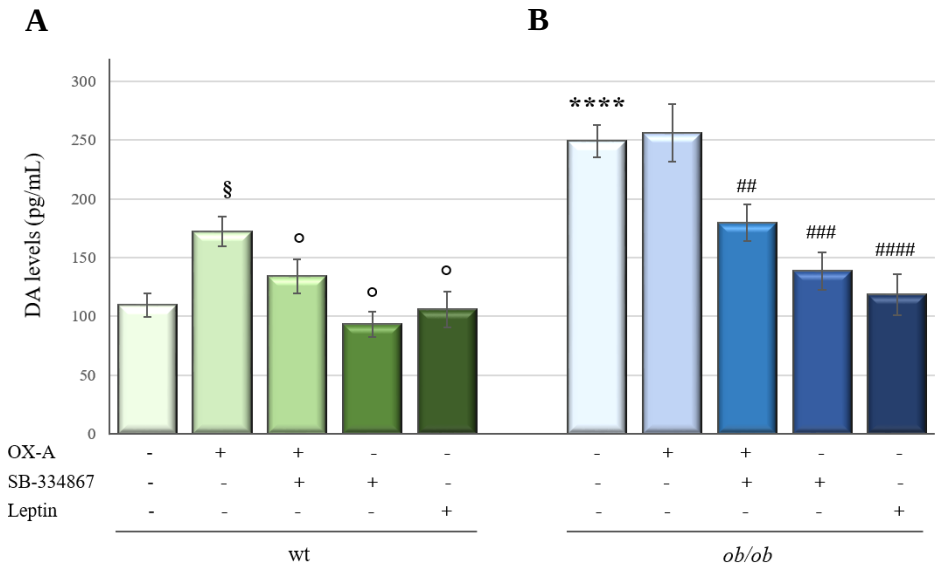

*Fig. 7.8 | Bar graph reporting the results of dopamine quantitative analysis in the NAc of wt, lean (**A**) and ob/ob (**B**) mice. DA levels in ob/ob were constitutively higher than wt mice. This increase was reverted by administration of leptin (5 mg/kg, i.p., 2h) or SB-334867 (60 mg/kg, i.p., 3h per se or 1h before OX-A injection). Treatment with OX-A (40 µg/kg, i.p., 2h) increased DA levels in wt but not in ob/ob mice. Data were from n = 6 mice per group and were means ± SEM; §P<0.05 wt+OX-A vs wt mice; °P<0.05 wt treated vs wt+OX-A; ****P<0.0001 ob/ob vs wt mice; ##P<0.005 ob/ob+SB-334867+OX-A vs ob/ob mice; ###P<0.001 ob/ob+SB-334867 vs ob/ob mice; ####P<0.0001 ob/ob+leptin vs ob/ob mice.*

7.5 The augmentation of DA signalling leads to β-arrestin mediated desensitization of D2R in obese mice

Several studies reported that both excess of highly palatable food (HPF) and drug of abuse can alter the expression of dopamine 2 receptors in mesolimbic regions of the brain, as a neuroadaptative response to overconsume of food or drug that contributes to the hypofunction of reward (*Wang et al, 2001; 2002; Koob and Le Moal, 2005; Avena et al, 2008; Johnson and Kenny, 2010*).
In order to investigate whether the high levels of dopamine in *ob/ob* mice can affect D2R expression in NAc and the molecular pathway through which this receptor could be down-regulated, we performed a double immunostaining of D2R and β-arrestin2 in the NAc of wt and obese mice. In the neuronal soma of wt mice, we observed separated immunolabeled signals of receptor and β-arrestin2. Conversely, *ob/ob* mice showed a strong colocalisation between D2R and β-arrestin2, as seen by their merged immunefluorescent signals [*Fig. 7.9A*]. These results indicated that D2R can undergo β-arrestin 2-mediated desensitization in NAc upon longer-lasting activation induced by prolonged exposure to dopamine [*Fig 7.9B*]. These data suggested that the extended access to food during obesity, by causing the enhancement of dopaminergic tone and overstimulation of D2R, triggers a desensitization of this receptor in the mesolimbic regions and ensuing impairment in the rewarding processes.

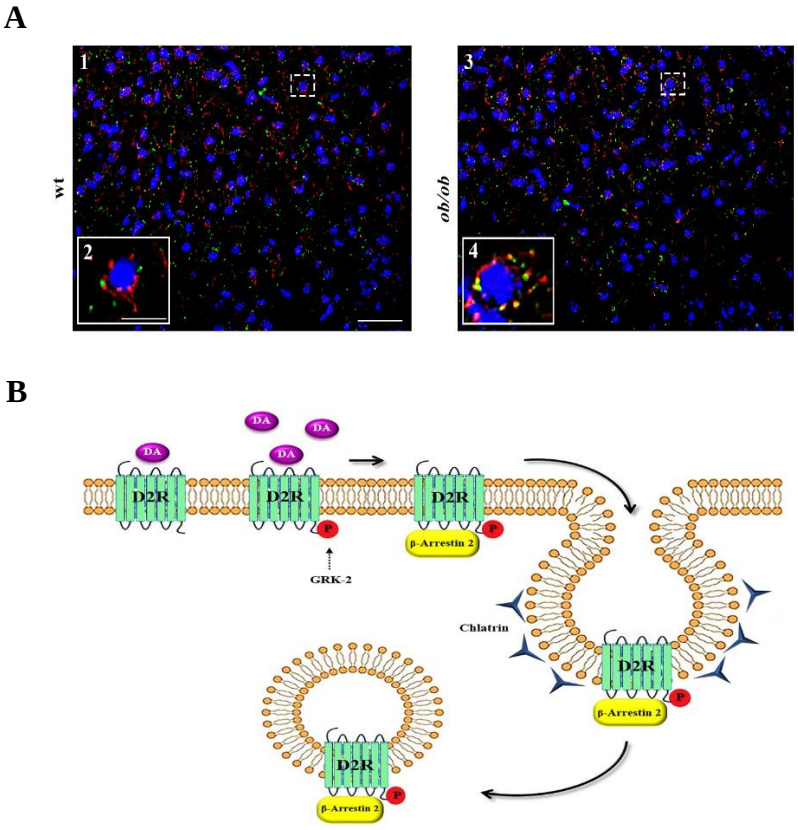

*Fig. 7.9 | **(A)** Confocal microscopy images showing D2R (red) and β-arrestin2 (green) immunoreactivities in the somata of neurons in the NAc of wt **(A1)** and ob/ob mice **(A3)**. Note the high D2/β-arrestin2 colocalisation (yellow) in ob/ob mice in comparison to wt mainly at the plasmatic membrane as revealed by the high magnification of a representative cell in the dotted boxed area in A1 and A3 and depicted in the respective inset as A2 and A4. Data are from n = 6 mice per group. [Scale bar: scale bar: 60 µm (A1 and A3), 30 µm (A2 and A4)]. **(B)** Scheme of the D2R internalization mechanism in the NAc. Following continuous activation of the D2R receptor by DA binding, and its phosphorylation by G protein receptor kinase-2 (GRK-2), signalling via β-arrestin2 may occur at the plasma membrane, in clathrin-coated pits or in endosomes after internalization of the receptor.*

7.6 The aberrant OX-A signalling in obesity sustains D2R desensitization through the formation of β-arrestin2-D2R complex

Since we found a marked desensitization of D2R in NAc of obese mice, we wanted to test the direct implication of OX-A in this mechanism. At this purpose, we treated wt and *ob/ob* mice with OX-A and its specific signalling inhibitors. In agreement with the immunohistochemical results, we provided direct evidence of aberrant OX-A signalling in D2R internalization, via co-immunoprecipitation assay and subsequent immunoblotting [*Fig. 7.10A and B*]. We found a significant increase in β-arrestin2/D2R complex with a corresponding increase in the β-arrestin2/D2R ratio in the immunoprecipitate-complex of *ob/ob vs* wt mice. The treatment of lean mice with OX-A increased the coupling of β-arrestin2 to D2R, in a manner similar to *ob/ob* mice, but not as high as obese littermates, that had constitutively a much higher orexin levels. On contrary, SB-334867 administrated alone or before OX-A, inhibited the formation of β-arrestin2/D2R complex, thus demonstrating the involvement of OX-A and its OX_1 receptor in D2R desensitization. In the same way, wt mice injected with leptin showed a low β-arrestin2/D2R ratio in the immunoprecipitate-complex, compared to basal condition of wt mice treated with vehicle [*Fig. 7.10B, left panel*]. In obese mice all pharmacological treatments were able to reduce the formation of β-arrestin2/D2R complex in comparison to vehicle- treated *ob/ob* mice. In particular, blockade of OX-A pathway with SB-334867, both alone or in combination with OX-A, prevented the binding of β-arrestin2 to D2R. Lastly, treatment with leptin, by inhibiting the excessive release of DA from VTA and the consequent overstimulation of D2R in NAc, was able to reduce the β-arrestin2/D2R ratio in the immunoprecipitate-complex of *ob/ob* mice [*Fig. 7.10B, right panel*].

These data suggested the pivotal role of OX-A in the hypofunction of reward induced by overeating mediated by dishinibition of dopaminergic neurons and subsequent hyperdopaminergia causing D2R desensitization in the mesolimbic area.

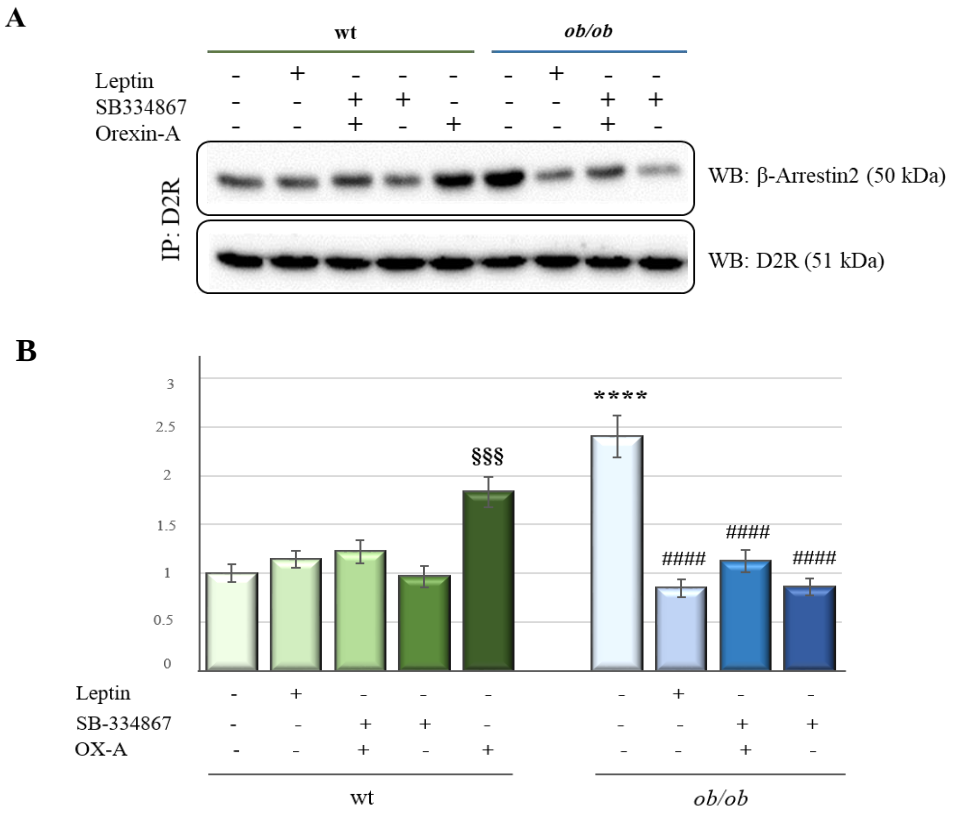

*Fig. 7.10 | OX-A-induced increase of dopamine levels caused binding of D2R to β-arrestin2 in the Nucleus Accumbens. (**A**) Representative immunoblots from D2R/β-arrestin2 co-immunoprecipitation assays in wt and ob/ob mice. OX-A treatment (40 µg/kg, i.p., 2h) in wt mice strongly increased the formation of D2R/β-arrestin2 complex in comparison to vehicle-treated mice. In ob/ob mice the binding of D2R to β-arrestin2 was sensitive to treatment with leptin (5 mg/kg, i.p., 2h) and SB-334867 (60 mg/kg, i.p., 3h per se or 1h before OX-A injection). (**B**) Densitometric analysis of the D2R/barrestin2 complex. Data were mean ± SEM; §§§P<0.0001 wt+OX-A vs wt mice; ****P<0.0001 ob/ob vs wt mice; ####P<0.0001 ob/ob treated vs ob/ob mice.*

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Chapter 8

Discussion

Orexins play a prominent role in conditioned responses to stimuli associated with food and drug rewards by regulating functional activity of DA neurons in the VTA (*Harris et al, 2005; Tung et al, 2016*). Dopaminergic neurons are innervated by orexinergic fibers (*Fadel and Deutch, 2002*) and those projecting to the shell of nucleus accumbens undergo an OX-mediated increase of firing rate (*Baimel et al, 2017*). Although both OX_1R and OX_2R are expressed at mesolimbic regions, mainly OX-A promotes consumption of highly palatable food through OX_1R activation (*Harris et al, 2005; Borgland et al, 2006; Barson et al, 2015; Baimel et al, 2015*). Accordingly, intra-VTA injection of OX-A increases intake of diet with high contents of fat and of sucrose solution in ad-libitum fed rats (*Terrill et al, 2016*) and hedonic reactions to sucrose taste when injected in the rostro-medial NAcc (*Castro et al, 2016*). Moreover, i.c.v. OX-A injection in the PVN of thalamus increases intake of sucrose solution (*Kay et al, 2014*) and release of DA in the NAc, thus promoting reward-related feeding (*Choi et al, 2012*). Acute HFD consumption (*Valdivia et al, 2014*) or optogenetic stimulation of LH-projecting fibres to dopaminergic neurons in VTA (*Nieh et al, 2015*) promote a compulsive reward seeking that is inhibited by OX_1R antagonist in rats exposed to chronic stress induced by cyclic food restriction (*Piccoli et al, 2012*). Accordingly, systemic SB-334867 injection reduces drinking of sucrose solution (*Terrill et al, 2016*), acute intake of highly palatable food and binge-like consumption of sucrose, together with reduced c-Fos activation in the VTA (*White et al, 2005; Alcaraz-Iborra et al, 2014; Valdivia et al, 2014*). Noteworthy, Hcrtr1 knock-down rats undergo a reduction of hedonic feeding and preference in the consumption of a high-fat diet (*Choi et al, 2012; Kay et al, 2014*).

Endocannabinoids, especially 2-arachidonoylglycerol (2-AG), have been implicated in food seeking behaviours and obesity in a manner prevented by type 1 cannabinoid receptor (CB_1R) antagonist. 2-AG is synthesiszed on demand to produce retrograde inhibition of neurotransmitter release at presynaptic CB_1 receptors. We and others demonstrated that 2-AG can be

generated when OX_1Rs, that are GqPCRs, are activated via OXs (*Ho et al, 2011; Cristino et al, 2013*).

In this PhD thesis we have demonstrated that OX-A is abundantly released from the LH to the VTA of obese *ob/ob* mice in comparison to lean wt mice. According to others, OX_1Rs have been found widely distributed in the vast majority of VTA dopaminergic neurons wherein the diacylglycerol lipase alpha (DAGL-α, the 2-AG synthesising enzyme) is located postsynaptically, apposed to the CB_1R-expressing GABAergic terminals (*Riegel et al, 2004; Matyas et al, 2008; D'Addario et al, 2014*). Therefore, we have found that aberrant OX-A release in the VTA of *ob/ob* mice, by promoting overactivation of postsynaptic OX*1*Rs in dopaminergic neurons, possibly by stimulating GqPCR-mediated phospholipase C (PLC)-DAGL enzymatic cascade, enhances 2-AG levels. This, in turn, inhibits GABA release by binding CB_1 receptors at the inhibitory inputs to DA neurons, a mechanism that is antagonized by OX_1R [*see schematic figure 7.4D*].

Furthermore, we have demonstrated that this OX-A/2-AG cascade-mediated disinhibition of VTA dopaminergic neurons contributes to enhancement of dopamine levels in the VTA and NAcc of obese *ob/ob* mice in a manner partially lowered by SB-334867 treatment or completely prevented in wt mice before OX-A injection. Enhanced levels of dopamine synthesis have been further confirmed by increase of TH-immunoractive expression in the VTA of *ob/ob* mice in comparison to wt. This effect was accompanied to a β-arrestin2-mediated desensitization of D2R dopamine receptor at NAc, in a manner prevented by antagonist of OX_1R in obese mice or before OX-A injection in wt lean mice.

In summary, in this study, by exploiting morphological, biochemical and pharmacological approaches, we demonstrated that in obese mice the aberrant OX-A release from LH to VTA triggers a food-intake / food-reward addictive behaviour caused by desensitization of the D2 receptors in the Nucleus Accumbens and prevented by treatment with OX_1R receptor antagonist.

Taken together, these results suggest that orexin A indirectly decreases GABA release by engagement of presynaptic CB_1R by 2-AG, which is synthesised from DAG by postsynaptic DAGL after PLC activation, upon acting on postsynaptic OX_1Rs of VTA dopaminergic neurons. In addition to inhibiting GABA release (disinhibition) through 2-AG, OX-A indirectly reduces the activity of VTA-NAc neuronal pathway by D2R desensitization in the NAcc shell.

In conclusion, these results suggest that endogenous OX-As released under severe obesity like that of *ob/ob* mice trigger OX_1R-PLC-DAGLα-2-AG-CB_1R-mediated disinhibition of VTA dopaminergic neurons by contributing to β-arrestin2 mediated desensitization of D2R at the VTA target neurons in the NAcc. This phenomenon could underlie food addictive behaviours.

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