The Endocannabinoid System in Leptin-Driven Changes of Orexinergic Signaling Under Physiological and Pathological Conditions

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Abstract In this chapter, the neuroanatomical overlap in the distribution of orexin and endocannabinoid systems, as well as the functional interaction between leptin-driven synaptic rewiring of orexinergic neurons and the orexin receptor-mediated activation of 2-arachidonoylglycerol synthesis, will be presented in the context of their role in the regulation of appetite, reward, sleep/wake, and analgesia. This chapter attempts to piece together what is known about this important cross talk and points out its potential therapeutic implications.

Abbreviations

2-AG	2-arachidonoylglycerol
AA	arachidonic acid
AC	adenylyl cyclase
AEA	anandamide
AgRP	agouti-related peptide
ARC	arcuate nucleus
BBB	blood-brain barrier
cAMP	cyclic adenosine monophosphate
CART	cocaine- and amphetamine-regulated transcript
CB1	cannabinoid receptor 1
CNS	central nervous system
CSF	cerebrospinal fluid
CTB	cholera toxin B subunit
DA	dopaminergic
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DMH	dorsomedial hypothalamic nucleus
DR	dorsal raphe nucleus

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DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
ECs	endocannabinoids
ERK	extracellular signal-regulated kinases
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
GPCRs	G-protein-coupled receptors
Hcrt	hypocretin
HFD	high-fat diet
IP3	inositol trisphosphate
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LH	lateral hypothalamus
LHA	lateral hypothalamic area
LPA	lysophosphatidic acid
MAGL	monoacylglycerol lipase
MAPKKK	mitogen-activated protein kinase kinase kinase
MCH	melanin-concentrating hormone
MSH	melanocyte-stimulating hormone
NAcc	nucleus accumbens
NMDA	*N*-methyl-D-aspartate receptor
NPY	neuropeptide Y
NSCCs	nonselective cation channels
OX-1R	orexin-1 receptor
OX-2R	orexin-2 receptor
OX-A	orexin-A
OX-B	orexin-B
PA	phosphatidic acid
PAG	periaqueductal gray
PAP	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PFCx	prefrontal cortex
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA1	phospholipase A1
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
POMC	pro-opiomelanocortin
PPO	prepro-orexin
PPT	pedunculopontine nucleus
PVN	paraventricular nucleus

REMS	rapid eye movement sleep
RHT	retinohypothalamic tract
ROCCs	receptor-operated calcium channels
SOCCs	store-operated calcium channels
SON	supraoptic nucleus
THC	tetrahydrocannabinol
TMN	tuberomammillary nucleus
TRP	transient receptor potential channels
TRPV	vanilloid transient receptor potential channels
VMH	ventromedial hypothalamus
VTA	ventral tegmental area

1 Endocannabinoids and Cannabinoid Receptor Type 1: An Overview

The endocannabinoids (ECs) are arachidonic acid-containing messengers generated by esterase and phosphoesterase action, produced on demand at the site of need. The most important ECs are *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al. 1992; Sugiura et al. 1995). In the CNS, ECs are often synthesized by postsynaptic neurons in response to a depolarization-induced increase in intracellular Ca^{2+} levels before being rapidly released to act in an autocrine or paracrine manner (Di Marzo 2009). Through the paracrine mode of action, ECs bind CB1 at presynaptic sides and inhibit both inhibitory and excitatory neurotransmission, thereby inducing a retrograde suppression of inhibition (DSI) or suppression of excitation (DSE), respectively, of the cellular target (Kano et al. 2009). After EC synthesis and release, EC signaling is terminated by neuronal reuptake and intracellular hydrolysis of AEA and 2-AG by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. A plethora of studies report that the ECs modulate other neurotransmitters like endogenous opioids (Robledo et al. 2008; Parolaro et al. 2010), dopamine and adenosine (Carriba et al. 2007; Ferré et al. 2009; Fernández-Ruiz et al. 2010). Interestingly, strongly emerging evidence points to a cross talk between ECs and orexins in the overall regulation of appetite, nociception, and sleep/wake cycle, thus opening new horizons to potential therapeutic applications of currently existing drugs targeting dysfunctions of these functions. The cannabinoid receptors of type 1 (CB1) are the major cannabinoid receptors expressed in the brain, where they are found both in neurons and astrocytes. CB1 belongs to the superfamily of G-protein-coupled receptors (GPCRs), which inhibit adenylyl cyclase (AC) activity with consequent decrease of cAMP and reduction, among others, of inhibitory c-Raf phosphorylation by PKA activity (Melck et al. 1999; Davis et al. 2003). Through Gi, CB1 regulates ion channels via activation of K^+ channels and inhibition of N-,

P-/Q-, and L-type voltage-gated Ca^{2+} channels (Deadwyler et al. 1995; Hampson et al. 1995). However, CB1 activation is also able to regulate AC via Gs, as well as Ca^{2+} fluxes and phospholipases with subsequent modulation of mitogen-activated protein kinases (p42/p44, p38, and c-Jun N-terminal kinase) regulating nuclear transcription factors (Howlett et al. 2002).

2 Orexin/Hypocretin: Discovery and Characterization

The discovery of orexin/hypocretin neuropeptides and their receptors dates back to 1998 and is attributed to two independent groups. By using subtraction hybridization, de Lecea with colleagues identified the mRNA sequence encoding prepro-hypocretin, the putative precursor of two putative peptide transmitters produced in the mammalian hypothalamus. Simultaneously, while searching for endogenous peptide ligands for multiple orphan G-protein-coupled receptor HFGAN72, Sakurai and Yanagisawa identified the same neuropeptides (Sakurai et al. 1998). De Lecea et al., named these peptides "hypocretins" by combining the suffix "hypo" of hypothalamus, the main source of these peptides in the brain, and "cretin" of "secretin," the gut hormone with high amino acid homology with hypocretins (de Lecea et al. 1998). Sakurai et al., referred to these peptides as orexins from the Greek word "orexis" meaning appetite, in view of their stimulatory action on food intake following central administration (Sakurai et al. 1998). It soon became clear that the peptides isolated by these two groups were identical and both sets of names are still in use. However, the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) recommends the use of "orexin" for peptides and receptors and "hypocretin" for the genes encoding them (Gotter et al. 2012). Thus, the peptides are known as prepro-orexin (PPO), orexin-A and orexin-B, and the receptors as OX_1 and OX_2 receptors (OX-1R and OX-2R, respectively), while the rodent genes encoding PPO and the receptors are *Hcrt*, *Hcrtr1*, and *Hcrtr2*, respectively. Mammalian (human, pig, dog, rat, mouse) PPO is composed of 130–131 aa (de Lecea et al. 1998; Sakurai et al. 1998). OX-A is a 33-amino acid peptide of 3562 Da with two intrachain disulfide bonds (Sakurai et al. 1998), which make the peptide particularly prone to misfolding following exposure to free radicals like nitric oxide (Nobunaga et al. 2014). OX-B is a 28-amino acid of 2937 Da with 46% sequence homology with OX-A. The amino acid sequences for OX-A and OX-B are highly conserved in mammals: OX-A is identical in pigs, dogs, rats, mice, and humans, whereas in OX-B there are few changes in one or two amino acids among these species (Kukkonen 2013). Since OX-A and OX-B are very ancient neuromodulators, a strong phylogenetic conservation of their amino acid sequences occurs among nonmammalian vertebrates (Sakurai 2007; Tsujino and Sakurai 2009). Both OX-A and OX-B are cleaved from the precursor PPO (de Lecea et al. 1998; Sakurai et al. 1998), whose expression is restricted to a small number of neuronal cell bodies in the LHA (Peyron et al. 1998), which send widely distributed projections to the rest of the brain (Peyron et al. 1998; Date et al. 1999; Nambu et al. 1999). Indeed, each brain region contains at

least one of the two orexin receptors, except for the cerebellum (Trivedi et al. 1998). Orexinergic neurons send widespread projections to other neurons of the brain, including dopaminergic neurons of the reward areas (VTA, NAcc), noradrenergic neurons controlling arousal at LC, serotonergic neurons of the DR, cholinergic neurons of the striatum, and multiple populations of neuropeptide-expressing neurons in the cerebral cortex, mesopontine tegmental nuclei, periaqueductal gray, tuberomammillary nucleus, septal nucleus, and paraventricular thalamus (Peyron et al. 1998; Horvath et al. 1999). Orexinergic neurons also project to regions regulating energy balance in the brain (ARC, VMH, DMH, PVN, LHA) (Lee et al. 2013) and periphery (pancreas, gastrointestinal system, liver, brown and white adipose tissue) (Wu et al. 2004; Grabauskas and Moises 2003; Oldfield et al. 2002; Adler et al. 2012). Notably, the orexins colocalize with many neuropeptides, such as prolactin (Risold et al. 1999), pentraxin (Reti et al. 2002), dynorphin (Chou et al. 2001), and galanin (Håkansson et al. 1999). Many orexin neurons are also glutamatergic as shown by their expression of the vesicular glutamate transporters (Rosin et al. 2003; Torrealba et al. 2003). Of the two peptides, OX-A is the most lipophilic, has been detected in the CSF and plasma, and is able to cross the BBB in both directions (Kastin and Akerstrom 1999).

3 Orexin Receptors: Common Signaling with CB1

Orexins act via OX-1R and OX-2R, which are GPCRs belonging to the rhodopsin family (Sakurai et al. 1998). OX-2R couples to the G_q, G_s, or $G_{i/o}$ subclasses of G proteins and has equal affinity for OX-A and OX-B (reviewed in Kukkonen and Leonard 2014). OX-1R preferentially couples to G_q (Sakurai et al. 1998) and is activated ten times more potently by OX-A than by OX-B. In many regions of the brain, OX-1R and/or OX-2R are distributed complementary to OX-positive projections, especially in the prefrontal and infralimbic cortex, CA of hippocampus, amygdala, BST, paraventricular thalamic nucleus, anterior hypothalamus, DR, VTA, LC, LDT/PPT nucleus (Trivedi et al. 1998; Lu et al. 2000; Marcus et al. 2001), ARC, TMN, DMH, PVN, LH, and medial septal nucleus (Lu et al. 2000; Marcus et al. 2001). OX-1R and OX-2R are also present in peripheral tissues (kidney, adrenal, thyroid, testis, ovaries, lung, pituitary, and jejunum) (Jöhren et al. 2001). Ca^{2+} signaling is among the first functional response triggered by OX-A-mediated stimulation of OX-1R (Sakurai et al. 1998). Indeed, orexin receptors regulate receptor-operated calcium channels (ROCCs), which include nonselective cation channels (NSCCs, such as transient receptor potential (TRP) channels) and Na^+/Ca^{2+} exchanger channels (Louhivuori et al. 2010). In neurons and other excitable cells, unlike CB1, OX-1R also activates N-type voltage-gated Ca^{2+} channels (Uramura et al. 2001). OX-A-mediated elevation of intracellular Ca^{2+} concentration is dependent from many different pathways, including ROCCs, SOCCs, and IP3-mediated Ca^{2+} release (Fig. 1). Unlike CB1, which inhibits AC activity, OX-1R regulates AC activity in both a positive and negative manner (Tang et al. 2008). Both CB1 and OX-1R activate ERK1/2 (Milasta et al. 2005; Ammoun

et al. 2006a) and PI3K (Ammoun et al. 2006a; Skrzypski et al. 2011). Upon activation by OX-1R, PKC activates NSCCs (Xia et al. 2009), PLD (Jäntti et al. 2012), and L- and N-type Ca^{2+} channels and inhibits the inwardly rectifying K^+ channels (Nakajima and Nakajima 2010). Notably, OX-1R modulates the CB1-mediated synaptic plasticity (Borgland et al. 2006; Selbach et al. 2010; Yang et al. 2013) by being able to activate PKC which, in turn, inhibits CB1 activity (Garcia et al. 1998; Uramura et al. 2001). Furthermore, OX-1R activates PLC, PLA2, and PLD (Lund et al. 2000; Johansson et al. 2007, 2008; Turunen et al. 2010, 2012; Jäntti et al. 2012). PLC activation leads to the production of DAG, which can be directly deacylated by DAGL generating a monoacylglycerol (possible 2-AG) or phosphorylated to phosphatidic acid (PA). Both DAG and PA are important lipid mediators with many targets (Fig. 1). PLA2 hydrolyzes glycerophospholipids at the *sn*-2 position by releasing a free fatty acid, mainly arachidonic acid (AA), a precursor of prostaglandins and leukotrienes. AA inhibits the activity of K^+ and Na^+ channels and of L- and N-type Ca^{2+} channels, while it can activate TRP channels such as the vanilloid TRPs, TRPV1 and TRPV4 (Kukkonen 2011) (Fig. 1) (reviewed in Meves 2008). Orexin receptors activate PLD, which produces PA and free choline starting from phosphatidylcholine (PC) as a substrate. PA activates many kinases such as phosphatidylinositol-4-phosphate 5-kinase, which generates PIP2, thereby stimulating mitogen-activated protein kinase kinase kinase (MAPKKK) Raf1 and protein kinase Cζ. Furthermore, PA can be converted to DAG by phosphatidic acid phosphohydrolase (PAP) or in lyso-PA (LPA) by PLA1 or PLA2 (Fig. 1). Orexin

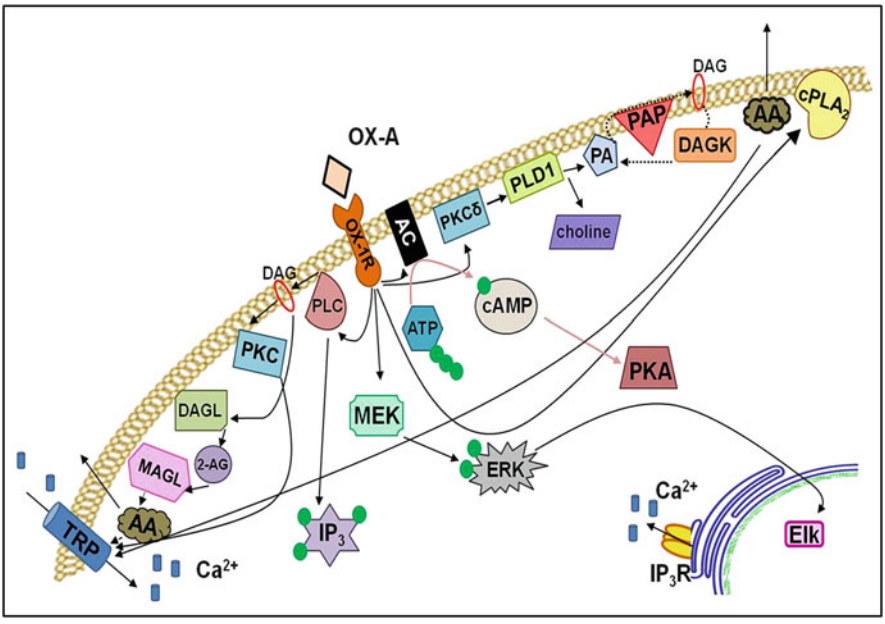

Fig. 1 Simplified overview of orexin receptor signaling

receptor-induced production of DAG and PA may affect CB1 activity through the formation of 2-AG, but the physiological relevance of this remains to be shown.

4 Anatomical Overlap Between Cannabinoid and Orexin Receptor Distribution in the Brain

To the best of our knowledge, no extensive study has been yet performed to describe the anatomical map of cannabinoid and orexin co-distribution in the brain. Evidence from separate studies concerning CB1 or OX-1R or OX-2R suggests an overlap of expression in critical areas involved in the overall regulation of appetite and metabolism, reward, nociception, and sleep/wake cycle. Thus, OX-1R and OX-2R, as well as CB1, are expressed with different densities in all the hypothalamic nuclei, including ARC, DMH, LHA, PVN, SON, and VMH. Prefrontal and infralimbic cortex, CA of hippocampus, amygdala, BST, paraventricular thalamic nucleus, DR, VTA, LC, LDT/PPT nucleus TMN, and medial septal nucleus also express both cannabinoid and orexin receptors (Fig. 2).

5 Molecular Interactions Between Cannabinoid and Orexin Receptors

In 2003, Hilairet et al. provided for the first time, in CHO cells stably co-transfected with CB1 and OX-1R, evidence for a functional cross-talk between these receptors. Indeed, co-expression of both receptors enhanced the ability of OX-A to stimulate

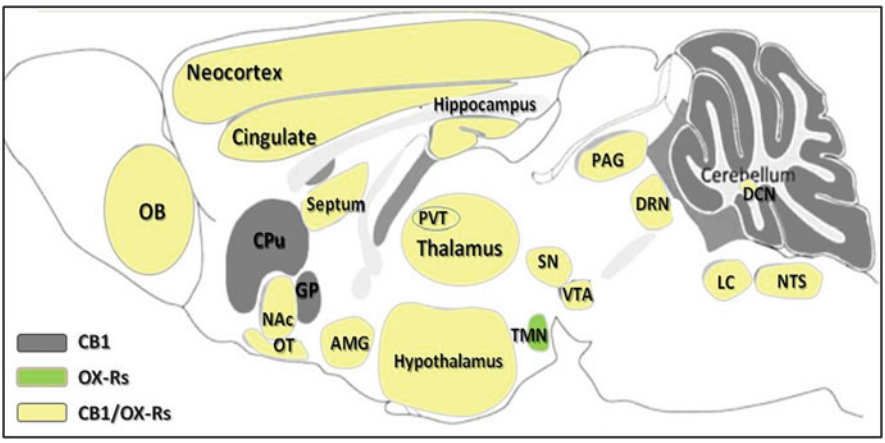

Fig. 2 Anatomical map showing different densities of cannabinoid and orexin receptor co-distribution in the brain

the ERK1/2 pathway ~100-fold more as compared to what is observed when only one of the receptors is expressed in cells and in a manner prevented by SR141716A (a CB1 antagonist/inverse agonist, also known as rimonabant), as well as by pertussis toxin. The authors explained this effect with a direct functional involvement of CB1 in the formation of heteromeric complexes with OX-1R also on the basis of electron microscopy study showing these receptors closely enough to form hetero-oligomers in CHO cells (Hilairet et al. 2003). This possibility was further investigated by Ellis et al. (2006) by using HEK293 cells co-expressing both receptors and displaying spontaneous OX-1R and CB1 internalization following cell exposure to OX-A. In this study, rimonabant reduced the potency of OX-A to activate the MAP kinases ERK1/2 in cells expressing both receptors. Likewise, the OX-1R antagonist SB674042 reduced the potency of the CB1 agonist WIN55,212-2 to phosphorylate ERK1/2. More importantly, orchestrated OX-1R and CB1 trafficking occurred in HEK293 cells following inducible expression of these receptors since (i) treatment with rimonabant resulted in the re-localization of CB1 to the cell surface and (ii) treatment with SB674042 (a selective OX-1R antagonist) induced redistribution of both receptors to the cell surface. Further evidence concerning physical interactions between OX-1R and CB1 was demonstrated by labeling covalently the extracellular domains of CB1 and OX-1R with SNAP-tag® and CLIP-tag™, respectively (Ward et al. 2011). By this approach CB1/OX-1R heteromerization was clearly identified by single-cell fluorescence resonance energy transfer (FRET) assay, which established that CB1 and OX-1R were close enough to produce true heteromers (dimers or oligomers). Besides heteromerization, a further mechanism has been proposed to explain CB1-dependent potentiation of OX-A signaling to ERK1/2. This is based on the enhancement of 2-AG synthesis by OX-A-mediated activation of the PLC-DAGL pathway via OX-1R (Turunen et al. 2012). Since both OX-1R and CB1 activate ERK1/2 (Bouaboula et al. 1995; Ammoun et al. 2006b), the heterologous or constitutive expression of OX-1R and CB1 in cells very strongly potentiates OX-A signaling to ERK1/2 (Turunen et al. 2012; Jäntti et al. 2013; Kukkonen and Leonard 2014). Therefore, the orexinergic potentiation of ERK1/2 phosphorylation in OX-1R-/CB1-CHO-transfected cells could be due to CB1 receptor activation by 2-AG and hence to the amplification of OX-1R intracellular signaling by endocannabinoid activation of CB1, instead of receptor di-/oligomerization. Emerging studies in rodents provide pharmacological evidence that OX-A enhances 2-AG levels in different regions of the brain including LH (Cristino et al. 2013a, b), PAG (Cristino et al. 2016), and ARC (Morello et al. 2016), possibly by Gq(OX-1R)-PLC-DAGLα-2-AG pathway which is crucial in the modulation of autocrine and paracrine functions and regulation of synaptic transmission (Fig. 3).

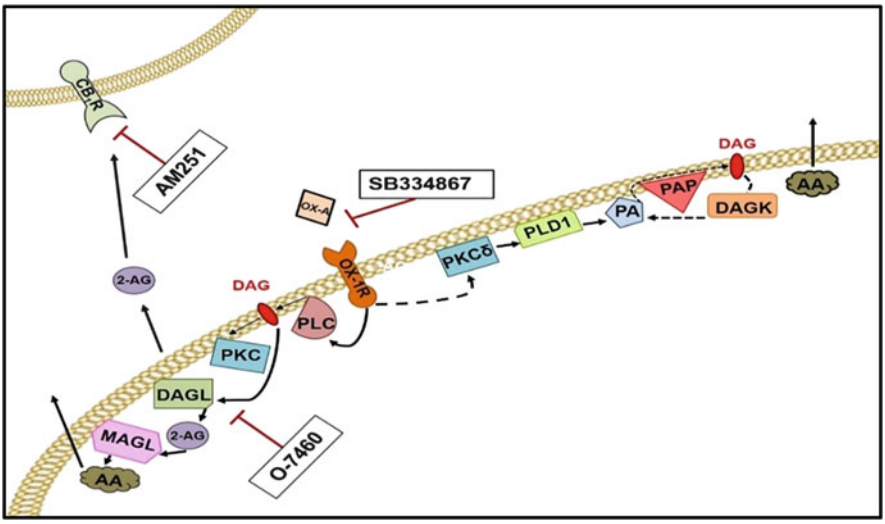

Fig. 3 OX-A signaling cascade regulates 2-AG synthesis and affects CB1 signaling in autocrine and paracrine way

6 Functional Interaction Between Cannabinoid and Orexin Receptors Under Leptin Signal Deficiency: Emerging Studies

Because of their local fast and fine regulation of production, endocannabinoid levels change according to rapid changes in metabolic requirements of the body. Thanks to the pivotal study of Di Marzo and colleagues (Di Marzo et al. 2001), an inverse relationship between leptin and the endocannabinoids (2-AG or AEA) has been established in the hypothalamus of leptin signaling-deficient obese (*ob*/*ob* and *db*/*db*) mice. A previous study found that the CB1 antagonist/inverse agonist rimonabant was able to reduce the food consumption in fasted mice (Colombo et al. 1998). Subsequently, both AEA and 2-AG levels were found to increase or decrease in fasted and sated mice, respectively (Kirkham et al. 2002), whereas CB1-deficient mice were shown to exhibit a leaner phenotype and resistance to diet-induced obesity, also because of lower food intake (Cota et al. 2003). Leptin is a protein of 167 amino acids mainly produced by adipocytes (Fox 2006) and released into the bloodstream. Leptin crosses the BBB by a saturable system (Banks et al. 1996) and signals to the brain to stop eating by inhibiting NPY/AgRP neurons and activating POMC/CART neurons in the arcuate nucleus of the hypothalamus (Schwartz et al. 2000; Sahu 2003) (Fig. 4). Notably, the amount of the circulating leptin is proportional to adiposity and body weight, both in mice and humans (Considine et al. 1996), and leptin administration in rodents causes a profound decrease in food intake and weight loss (Friedman and Halaas 1998). Rodents with genetic defective leptin signaling are obese, as in the case of *db*/*db* mice (knockout

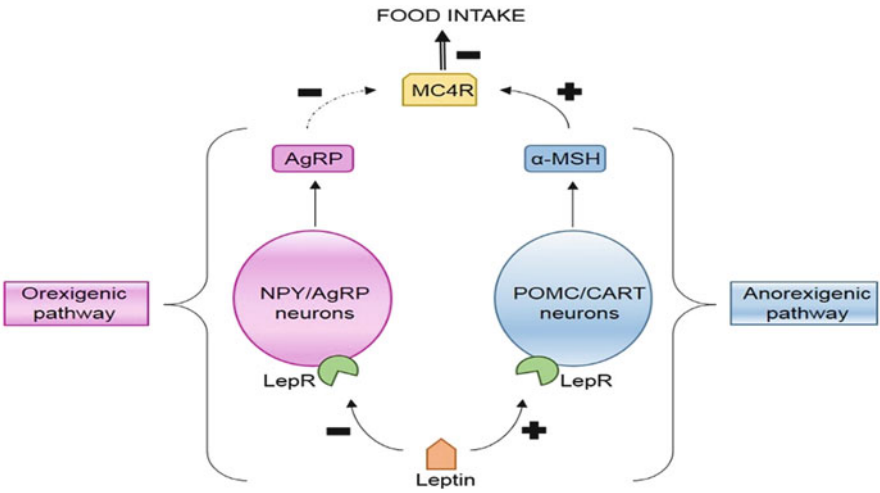

Fig. 4 Schematic representation of leptin signaling at hypothalamic neurons of ARC

for the Ob-Rb gene coding the leptin receptors) or *ob*/*ob* mice (knockout for the *Ob* gene coding for leptin) (Hill et al. 2010).

6.1 Appetite and Energy Homeostasis

Unlike anatomical studies, growing data are accumulating to unravel the functional cross talk between orexinergic and cannabinoid systems in the regulation of appetite and energy balance. In this regard, the first study concerning the regulation of appetite was by Crespo et al. (2008). By using i.c.v. injection of orexin in pre-fed rats, they reported a dose-dependent increase in the short-term feeding behaviors. The authors also tested the effect of the CB1 inverse agonist SR141716 (rimonabant) when given alone and observed a decrease in feeding behaviors. Finally, the combined effect of orexin and SR141716 was tested. Intraperitoneal injection of SR141716, 10 minutes prior to i.c.v. orexin injection, blocked the orexin-induced feeding already at doses that did not reduce feeding when used alone (Crespo et al. 2008). Similar to a CB1 agonist, also OX-A or OX-B administration stimulates food intake in mice, whereas the OX-1R antagonist, SB334867, reduces feeding (Sakurai et al. 1998; Haynes et al. 2000; Shiraishi et al. 2000). As with endocannabinoid levels, an inverse relationship occurs between circulating leptin and orexin levels. Indeed, fasting results in the upregulation of PPO mRNA (Sakurai et al. 1998), also in obese mice (Yamanaka et al. 2003). However, the regulation of food intake by endocannabinoids at the hypothalamic level results more complex than what was initially believed, because of the occurrence of bimodal orexigenic *vs.* anorexigenic effects of CB1 activation, depending on its

expression at glutamatergic or GABAergic inputs. In this scenario, the study of Huang et al. (2007) is clearly understandable, as the authors suggest that fasting-related reduction of leptin levels controls arousal by increasing of orexinergic neuron activity. By using patch-clamp recordings, Huang et al. demonstrated that WIN 55,212-2, a cannabinoid agonist, was able to depolarize MCH neurons, whereas it reduced the spontaneous firing of OX neurons in a manner prevented by AM251 and tetrodotoxin. Using conditions inducing DSI or DSE applied to MCH or OX neurons, the authors revealed that both MCH and OX neurons release endocannabinoids and are innervated by CB1-expressing inhibitory and excitatory inputs, respectively. Notably, the regulation of food intake controlled by endocannabinoids appears further complicated by the observation that hypothalamic circuits are affected by synaptic rewiring regulated by several circulating hormones (leptin, ghrelin, glucocorticoids, etc.). Cristino et al. (2013a, b) investigated the obesity-associated changes in the hypothalamic circuits of orexinergic neurons in the LH. This study employed leptin signaling-deficient mice such as *ob*/*ob* mice and mice with leptin insensitivity in the ARC caused by high-fat diet (HFD)-induced obesity. It was found that, in these obese mice, in comparison with respective lean control mice, (i) CB1-expressing presynaptic inputs to orexin-A neurons change from predominantly excitatory to inhibitory inputs; (ii) OX-A neurons are able to synthetize 2-AG more than in lean control mice because of elevation of DAGLα expression; and (iii) DSI occurs at OX-A neurons with consequent disinhibition of OX-A neurons and elevation of OX-A trafficking and release to many LH target areas like ARC, PVN, PAG, NAcc, and VTA. The authors also preliminarily investigated the mechanism of the synaptic remodeling at OX-A neurons and found that lack of leptin signaling in the ARC of obese mice, where from most of the fibers innervating orexinergic neurons originate, was the most likely cause. Leptin treatment reversed the synaptic remodeling only in *ob*/*ob* and not in HFD mice, indicating that this phenomenon is a consequence of leptin deficiency or leptin resistance in the ARC. Interestingly, possibly because *ob*/*ob* mice lacked endogenous leptin when weaned, but they received it from their heterozygous mothers during lactation, the remodeling of their synapses only occurred after weaning and was reversed by exogenous leptin injection (Cristino et al. 2013a, b) (Fig. 5). Recent findings from Morello et al. (2016) showed a CB1 and OX-1R interaction also in the regulation of POMC neurons, the master subset of the ARC anorexigenic hypothalamic neurons which act to reduce appetite and body weight (Schwartz et al. 2000; Cone 2005). Indeed, ablation of POMC neurons, or loss of α-MSH production, leads to obesity (Yaswen et al. 1999; Balthasar et al. 2004). Manyfold anatomical evidence, including our previous study (Cristino et al. 2013a, b), reveals that POMC neurons send projections to OX-A and regulate OX-A expression (López et al. 2007). OX-A neurons, in turn, send inputs back to POMC neurons (Chemelli et al. 1999) underlying a neuronal circuit perfectly organized to ensure food seeking accompanied by alertness. Notably, CB1 agonism at POMC neurons acutely enhances feeding in a dose-dependent manner (Gómez et al. 2002; Koch et al. 2015) without affecting α-MSH release at low doses (Koch et al. 2015). Furthermore, α-MSH, at doses effective at reducing food intake, does

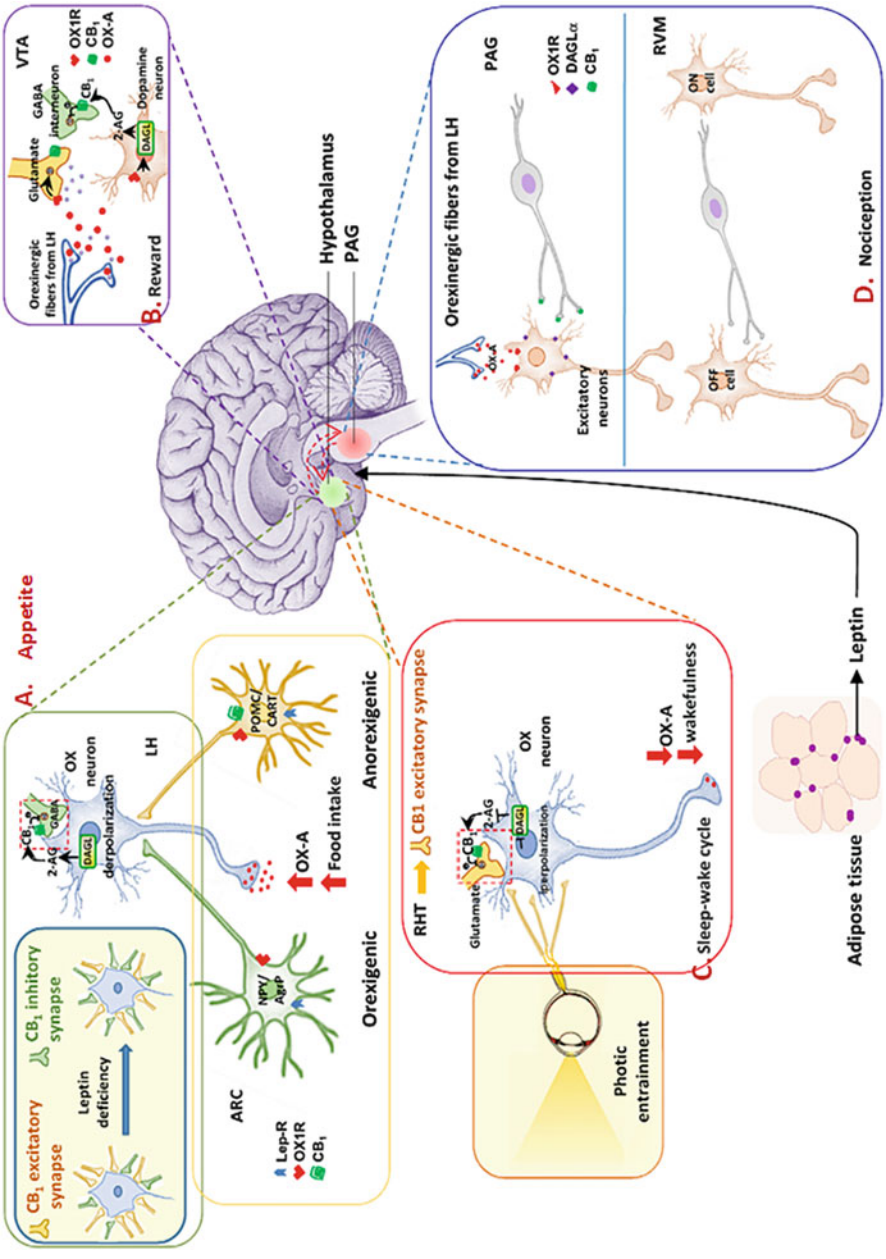

Fig. 5 (continued)

not alter hypothalamic endocannabinoid levels (Matias et al. 2008). In this scenario Morello et al. (2016) found that prolonged OX-1R activation by OX-A promotes appetite by blunting POMC production and α-MSH release as a consequence of OX-A-induced potentiation of 2-AG synthesis and CB1 activation at POMC neurons of mice under satiety state. This pathway relies its mechanism on the potentiation of ERK1/2-mediated Ser-727 phosphorylation/inhibition of STAT3 which affects negatively the expression of the *Pomc* gene (Fig. 6). To further support the occurrence of this autocrine mechanism, the authors also verified its existence in cultured POMC primary neurons. Several preclinical and clinical observations showing an association between obesity and ECS dysregulation in both central and peripheral tissues (Matias and Di Marzo 2007; Bermudez-Silva et al. 2010), as well as the occurrence of a positive correlation between the plasma endocannabinoid levels and markers of obesity or metabolic disorders, have been reported (Engeli et al. 2005; Cota 2007; Di Marzo et al. 2009; Abdulnour et al. 2014). The finding that an endogenously produced peptide such as OX-A can modulate the function of CB1 might shed light on the hyperphagic action of endocannabinoids and on the functional consequences of the inverse relationship between leptin and endocannabinoid levels occurring during obesity also in humans (Monteleone et al. 2005; Nicholson et al. 2015). Indeed, during obesity, the aberrant activation of OX-A-mediated endocannabinoid biosynthesis triggered by deficits in leptin signaling at POMC neurons causes a vicious circle with inhibition of POMC synthesis, hyperphagia, further body weight gain, hypertension, and dysmetabolism, such as hepatosteatosis (Morello et al. 2016; Imperatore et al. 2017). The discovery of this leptin/orexin/endocannabinoid/α-MSH signaling loop and its impact on hyperphagia, obesity, and fatty liver now call for investigations on potential synergies between OX-1R antagonists, CB_1R antagonists, and MC4R agonists for the reduction of body weight. This, in turn, might pave the way

Fig. 5 (continued) Scheme of the leptin-mediated interaction between cannabinoid and orexinergic systems in the control of food intake, sleep/wake cycle, nociception, and reward. Overview of the proposed anatomical pathways, synaptic receptor distribution, and mechanism of endocannabinoid-/orexin-modulated functions. **A**. Appetite. A leptin-driven synaptic rewiring occurs at OX-A neurons. In obese mice, the CB1-positive inputs to OX-A neurons shift from predominantly excitatory to inhibitory drive with consequent disinhibition of OX-A release to LH target areas and increase in food intake. **B**. Reward and seeking behavior. The OX-A-mediated activation of the GqCPR(OX-1R)-PLC-DAGLα-2AG pathway at VTA leads to DSI at CB1-positive inputs to DA neurons and consequent disinhibition of DA release and decrease on reward and seeking behavior. **C**. Wakefulness. OX-A neurons are entrained by a blue light-mediated activation of the retinohypothalamic pathway (RHT). This phenomenon induces a 2-AG synthesis at the OX-A target neurons and consequent DSE-mediated inhibition of OX-A neurons. These effects reduce both OX-A release and wakefulness. **D**. Analgesia. Incoming nociceptive signals are under the control of descending excitatory projections from vlPAG neurons to RVM. These projections undergo disinhibition by glutamate release and 2-AG-mediated DSI, respectively, at OX-A- and CB1-positive inputs to vlPAG output neurons to ON and OFF cells in the RVM. Hypothalamic leptin signal deficiency potentiates OX-A release to PAG and facilitates disinhibition of descending antinociceptive pathway and analgesia

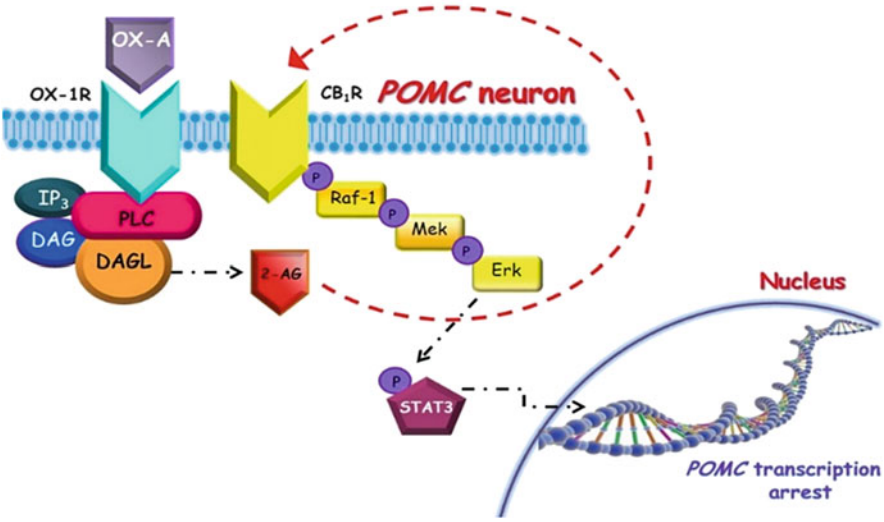

Fig. 6 Scheme of the molecular pathway underlying OX-A-mediated CB1 activation at POMC neurons. OX-A-mediated CB_1R activation via 2-AG production contributes to increase the OX-A potency to phosphorylate ERK1/2 ($pERK1/2^{Thr202/Tyr204}$) in POMC neurons concurrently with enhancement of STAT3 phosphorylation ($pSTAT3^{Ser727}$). These effects result in the lowering of POMC production and POMC-derived α-MSH levels

to new safer and more efficacious polypharmacological or multi-target treatments against metabolic disorders (Kälin et al. 2015).

6.2 *Reward, Arousal, and Seeking Behavior*

Orexin and endocannabinoids have been shown to be involved in reward processes, drug-seeking behavior, and addiction in animal models and human (España 2012; Oleson et al. 2012; Baimel et al. 2015; Hernandez and Cheer 2015). Orexinergic neurons project to the VTA and NAcc, the master regions of the brain critical for motivation and reward behavior (Wise and Rompre 1989; España 2012) whose activation is associated with preferences for cues linked with drug and food rewards (Harris et al. 2005). In particular, in the VTA, orexin directly activates dopaminergic neurons through OX-1R (Nakamura et al. 2000) or affects glutamate release onto dopaminergic neurons, inducing excitatory synaptic plasticity (Borgland et al. 2010; Baimel and Borgland 2012) and causing DA release in VTA target regions, such as the NAcc (Vittoz and Berridge 2006; Narita et al. 2006). More recently, orexin was also linked to cannabinoid-induced reward. Chiou and collaborators have proposed another cellular mechanism through which orexin can increase VTA dopaminergic activity by triggering endocannabinoid synthesis (Chiou et al. 2013). Endocannabinoid production and release have been found to occur at VTA-DA

neurons (Alger 2002; Melis et al. 2004). Electrophysiological studies demonstrated that activation of OX-1R in VTA-DA neurons initiates a Gq/11-coupled PLC-DAGL pathway leading to the biosynthesis of 2-AG, which, retrogradely, activates CB1 at both inhibitory and excitatory inputs to DA neurons, thus regulating burst firing (French 1997; Wu and French 2000) and neurotransmitter release (Chen et al. 1990; Cheer et al. 2007; Oleson et al. 2012) of VTA-DA neurons. In resting conditions, around 50% of DA neurons are innervated by inhibitory GABAergic inputs (Grace and Bunney 1984). The retrograde activation of CB1 on GABA inputs results in increase of VTA-DA firing and DA release to target areas (Overton and Clark 1997; Zweifel et al. 2009; Mátyás et al. 2008; Chiou et al. 2013). However, although in normal conditions the final endocannabinoid-mediated modulation of DA neuron activity depends on the functional balance between inhibitory and excitatory inputs to these neurons, acute restraint stress activates orexinergic neurons leading to downstream release of OX-A to the VTA and consequent activation of OX-1R of DA neurons. This event triggers the synthesis of 2-AG, which, through a retrograde inhibition of GABA release, induces disinhibition of VTA-DA neurons and initiation of seeking behavior. The opposite scenario occurs in the DR, where the OX-B-mediated inhibition of glutamate release is due to OX-2R-induced enhancement of 2-AG release and activation of CB1 at excitatory inputs and depression of glutamate-mediated synaptic currents of DR serotonergic neurons, as demonstrated by patch-clamp study in male rats (Haj-Dahmane and Shen 2005) (Fig. 5). This effect was mimicked by WIN55,212-2 and abolished by AM-251, confirming the involvement of CB1 receptors. The inhibition of glutamate release was also counteracted by inhibition of G-protein signaling in postsynaptic neurons by GDPβS, a non-hydrolyzable analog of GDP. These results were the first to suggest an orexin/endocannabinoid interaction in the regulation of DR serotonergic neurons by orexin-induced postsynaptic release of ECs. Since serotonergic neurons are activated during wakefulness, the inhibitory action of OX-B, an arousal-increasing neuropeptide, on these neurons therefore seems paradoxical. The authors, however, speculated that this negative feedback induced by endocannabinoid release is necessary for preventing excessive neuronal excitation, therefore ensuring a stable firing (Haj-Dahmane and Shen 2005). Accordingly, the orexin and endocannabinoid interaction is important in the physiological regulation of arousal. In agreement with the role of endocannabinoids and orexins in the regulation of anxiety-like responses, the overlapping distribution of both their receptors has been described in the VTA, NAcc, PFCx, septal nuclei, and amygdaloid nuclei (Maldonado et al. 2006; Aston-Jones et al. 2010; Plaza-Zabala et al. 2012; Flores et al. 2015), besides the DR and LC. Therefore, it can be speculated that orexins exert orexigenic functions by controlling both appetite and reward circuits through stimulation of endocannabinoid release. This is of special relevance to the observation that orexins, via mesolimbic circuits, promote ingestion of highly salient substances (e.g., high-fat diet, drugs of abuse), at least in part, via direct projections onto VTA-DA neurons (Petrovich et al. 2012; Cason and Aston-Jones 2013), which promote NMDA receptor-mediated excitatory postsynaptic potentials at DA neurons and DA release into the NA and PFCx (Borgland

et al. 2006; Vittoz and Berridge 2006; España et al. 2010). However, release of orexin and glutamate is required for long-term potentiation of DA signaling that underlies cue-induced reinstatement (seeking) of rewards. Notably, given that both OX-A and OX-B are co-released with dynorphin and glutamate, it has been proposed that both the orexins potentiate the glutamate-mediated long-term modifications that underlie natural reward and addiction to drugs (Mahler et al. 2013). By contrast, lowering the orexins vs. dynorphin ratio suppresses reward responses (Muschamp et al. 2014) by inhibiting DA release to the NAcc and drug, food, or sucrose seeking (Abizaid et al. 2006; España et al. 2010; Sharf et al. 2010; Smith and Aston-Jones 2012; Srinivasan et al. 2012).

6.3 Sleep/Wakefulness

It has been observed that orexinergic neurons are active during wakefulness (Modirrousta et al. 2005), while MCH neurons are active in REMS (Verret et al. 2003). Notably, OX knockout (KO) mice exhibit a narcoleptic-like phenotype, with sudden transitions from wakefulness into REMS (Chemelli et al. 1999). Furthermore, it is known that eCBs have been implicated in sleep regulation because of their strong hypnogenic properties (Pérez-Morales et al. 2012). Indeed, THC, the primary psychoactive agent in marijuana and hashish, tends to generate a distorted sense of time (Tinklenberg et al. 1976). The CB1 agonist CP55940 attenuates light-induced clock-phase advance in hamsters (Sanford et al. 2008), and endocannabinoids, both 2-AG and AEA, show a circadian variation in the brain (Valenti et al. 2004). Interaction between the orexinergic and endocannabinoid systems in the sleep regulation has been described by Pérez-Morales et al. (2013) starting from evidence indicating that WIN55-212-2 depolarizes MCH neurons and hyperpolarizes OX-A neurons in in vitro LH slices (Huang et al. 2007). By injection of 2-AG in the LH of rats, Pérez-Morales and colleagues found that 2-AG increases REMS through a CB1 activation and increases c-Fos expression in MCH neurons, without affecting c-Fos expression in OX-A neurons. Furthermore, Cristino et al. (2013a, b) studied the orexinergic and endocannabinoid interaction in wakefulness. They found the endocannabinoid biosynthesis in the LH of mice induced by a light-modulated excitation of the retinohypothalamic tract (RHT). By electron and confocal microscopy, the authors found CB1 expression at RHT projections to OX-A neurons and identified the retinal ganglion cells/OX-A light-mediated circuit activated by blue light pulse by inducing c-*Fos* expression in the target OX-A neurons (Fig. 5). This activation was paralleled by 2-AG synthesis in a manner prevented by antagonism of mGluR5 receptors which were, in turn, found at OX-A neurons. Collectively, these data suggest that orexinergic and endocannabinoid interaction, in specialized neuronal pathways, regulates different aspects of sleep/wake cycle including the REMS onset and the blue light entrainment of circadian functions.

6.4 Nociception

One common function for both orexin and endocannabinoid systems is the modulation of pain perception at spinal and supraspinal levels. The antinociceptive descending PAG-RVM pathway is crucial in the regulation of pain. PAG contains glutamatergic neurons receiving inhibitory GABAergic inputs from the local interneurons. PAG-mediated control of pain occurs concomitantly with the modulation of pain-responding neurons of the RVM: the ON cells, which are activated, and the OFF cells, which are inhibited, by nociceptive stimuli. In the ventrolateral PAG (vlPAG), activation of excitatory output neurons projecting monosynaptically to OFF cells in the rostral ventromedial medulla (RVM) causes antinociceptive responses via OFF cell stimulation and ON cell inhibition in the RVM, which send inhibitory projections to the dorsal horn of the spinal cord (Behbehani and Zemlan 1990). By morphological approach based on the injection of the retrograde tracing CTB-Alexa488 in the RVM of mice, Cristino and colleagues revealed the occurrence of monosynaptic projections from vlPAG neurons to the OFF cells in the RVM. More importantly, by high-resolution electron microscopy, the authors found that these OX-A neurons targeted by RHT express DAGLα at the cytoplasmic side of the membrane, near to OX-1R at postsynaptic side of excitatory OX-A-positive inputs, and are innervated by CB1-positive inhibitory inputs coming from local GABAergic interneurons (Cristino et al. 2016) (Fig. 5). This study provided the anatomical contribution to understand the OX-1R-mediated PLC-DAGL-2-AG disinhibition of the PAG-RVM descending antinociceptive pathway found by Ho et al. (2011). Indeed, Ho and colleagues were the first to demonstrate that 2-AG can be generated by OX-A-mediated activation of OX-1R in the rat PAG by using O-7460 (a DAGLα inhibitor) during patch-clamp recording of vlPAG slices. They found that, unlike OX-B, OX-A depressed IPSCs in PAG slices in a manner dependent on OX-1R, CB1, PLC, and DAGL activity (Ho et al. 2011) and was mimicked by a MAGL inhibitor. These data suggest that activation of OX-1R at postsynaptic site, as well as activation of mGluR5 and M1/M3 mAChRs, initiates the OX-1R-mediated PLC-DAGL-2-AG disinhibition mechanism in the PAG, thereby contributing to analgesia induced by intra-vlPAG injection of OX-A during the rat hot plate test (Chiou et al. 2013). More recently, Lee et al. found that 30-min restraint stress in mice was able to induce analgesia during hot plate test in a manner prevented by intra-vlPAG injection or intraperitoneal injection of SB334867 or AM251, but not TCS-OX2-29 or naloxone. They found that, during stress, orexins lead to endocannabinoid generation in the vlPAG by engaging the OX-1R-mediated PLC-DAGLα-2-AG pathway, thereby inducing the establishment of stress-induced analgesia (Lee et al. 2016). Accordingly, Cristino et al. found that reduced-pain sensitivity and enhanced OFF-decreased ON cell activity occur in *ob*/*ob* mice wherein the leptin deficiency strongly enhances OX-A release to vlPAG, among other LH targets. In *ob*/*ob* mice, these alterations result in (i) increased OX-1R-mediated PLC-DAGL-2-AG disinhibition of vlPAG output neurons by DSI of the neighboring CB1-expressing GABAergic inputs; (ii) subsequent increase of OFF

and decrease of ON cell activity in the RVM, as assessed by patch clamp and in vivo electrophysiology; and (iii) analgesia, in both healthy and neuropathic mice. Notably, in HFD obese mice, analgesia was only unmasked following leptin receptor antagonism because of the leptin insensitivity of these mice. All these effects were mimicked by i.c.v. OX-A injection in lean mice and were markedly reduced by treatment with AM251 or SB in *ob*/*ob* mice at a dose inactive in wt mice (Cristino et al. 2013a, b). According to the orexin/endocannabinoid synergistic effect in the control of analgesia, Kargar et al. found that intra-LC microinjection of OX-A exerted antinociceptive function in the rat formalin test (a model of inflammatory pain), in a manner prevented by intra-LC microinjection of either SB334867 or AM251 (Kargar et al. 2015).

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