

Phospholipase Cascades in OX₁ Orexin Receptor Signaling

Maria Jäntti

Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki
Finland

Academic dissertation

To be presented with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Walter Auditorium, Agnes Sjöbergin katu 2, Helsinki, on May 23rd, 2014, at 12 o'clock noon.

Supervisor: Jyrki Kukkonen, Professor of Cell and Molecular Biology
University of Helsinki, Finland

Reviewers: Mika Scheinin, Professor of Pharmacology
University of Turku, Finland

Raimo K. Tuominen, Professor of Pharmacology and
Toxicology
University of Helsinki, Finland

Opponent: Harpal S. Randeva, Associate Professor of Medicine
University of Warwick, UK

ISBN: 978-952-10-9902-1 (paperback)

ISBN: 978-952-10-9903-8 (PDF)

ISSN: 1799-7372

Unigrafia Oy,
Helsinki, Finland 2014

Table of Contents

List of original publications	6
Abstract	7
Abbreviations	9
1. Introduction.....	11
2. Review of the Literature.....	12
2.1 G-protein-coupled receptors and their main effectors	12
2.1.1 G-protein-coupled receptors	12
2.1.2 Heterotrimeric G-proteins	12
2.1.3 Phospholipase C and Ca ²⁺ signaling	14
2.1.4 Adenylyl cyclases	14
2.2 Orexins and orexin receptors	15
2.2.1 Orexins	15
2.2.2 Orexin receptors.....	16
2.3 Orexin receptor signaling	17
2.3.1 Ca ²⁺ in orexin receptor signaling	17
2.3.2 Adenylyl cyclases as effectors of orexin receptors.....	18
2.3.3 Activation of protein kinases by orexin receptors	18
2.3.4 Lipid mediators in orexin receptor signaling.....	19
2.4 Orexin physiology	22
2.4.1 Orexins and their receptors in the CNS.....	22
2.4.2 Regulation of sleep/wakefulness	23
2.4.3 Feeding and metabolism.....	25
2.4.4 Thermoregulation.....	26
2.4.5 Reward and addiction.....	27
2.4.6 Synaptic plasticity, learning, and memory	28
2.4.7 Pain modulation	29
2.4.8 Functions in the gastrointestinal tract	30
2.4.9 Regulation of endocrine secretion	31
2.4.10 Functions in white adipose tissue	33
2.5 Endocannabinoids.....	34
2.5.1 Receptors and their signaling	34
2.5.2 Physiology of endocannabinoids	35
2.6 Interaction of orexin and cannabinoid systems	39
2.6.1 Evidence from electrophysiological studies.....	39
2.6.2 Evidence from behavioral experiments.....	40
2.6.3 Evidence from molecular studies.....	41
3. Aims of the study	43

4. Materials and methods	44
4.1 Cell culture (I–IV)	44
4.2 Transfection and transduction (I–IV)	44
4.3 Chemicals	44
4.4 Phospholipase D assay (I)	45
4.5 Arachidonic acid and 2-arachidonoylglycerol release (II)	45
4.6 Lipid extraction (I–II)	45
4.6.1 Extraction from cells (I)	46
4.6.2 Extraction from supernatant (II)	46
4.7 Thin-layer chromatography (I–II)	46
4.8 ³H-overflow from cells labeled with ³H-arachidonic acid and ³H-oleic acid (II)	47
4.9 Phospholipase C assay (I–II)	47
4.10 Adenylyl cyclase assay (I–II)	47
4.11 Ca²⁺ measurements (II)	48
4.12 ERK phosphorylation assay (III)	48
4.13 Luciferase assay (III)	49
4.14 Cell–cell communication assay (II–III)	49
4.15 Receptor binding	50
4.16 Bioluminescence resonance energy transfer (BRET²) assay (IV)	51
4.17 Data analysis and statistical procedures (I–IV)	51
5. Results and Discussion	53
5.1 Phospholipase D activation (Paper I)	53
5.2 AA and 2-AG release (Paper II)	56
5.3 Cellular actions of 2-AG from orexin receptor signaling	59
5.3.1 Paracrine signaling (Papers II and III)	59
5.3.2 Autocrine signaling	60
5.4. The role of Ca²⁺ in lipid signaling cascades	63
5.5. Heteromerization of OX₁, OX₂, and CB₁ receptors	64
5.5 Comments on interactions between CB₁ and OX₁ receptors	65
6. Conclusions	67
7. Future perspectives	68
8. Acknowledgements	70
9. References	72

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

- I. Maria H. Jääntti, Jaana Putula, Pentti Somerharju, Michael A. Frohman and Jyrki P. Kukkonen (2012). OX₁ orexin receptor activates phospholipase D via PKC δ . *Br J Pharmacol* **165**: 1109-23.
- II. Pauli M. Turunen, Maria H. Jääntti, and Jyrki P. Kukkonen (2012). OX₁ orexin receptor signaling via arachidonic acid and endocannabinoid release. *Mol Pharmacol* **82**: 156-67.
- III. Maria H. Jääntti, Jaana Putula, Pauli M. Turunen, Johnny Näsman, Sami Reijonen, Christer Lindqvist and Jyrki P. Kukkonen (2013). Autocrine endocannabinoid signaling through CB₁ receptors potentiates OX₁ orexin receptor signaling. *Mol Pharmacol* **83**: 621-32.
- IV. Maria H. Jääntti, Ilona Mandrika and Jyrki P. Kukkonen (2014). Human orexin receptors form constitutive homo- and heteromeric complexes with each other and with human CB₁ cannabinoid receptors. *Biochem Biophys Res Commun* **445**: 486-90.

The original papers are reprinted with the permission of the copyright holders.

Abstract

Orexins (orexin-A and -B) are neuropeptides with multiple physiological functions, among which regulation of wakefulness and appetite are the best known. Neurons producing orexins are localized in the lateral hypothalamus, from where they send projections to many parts of the brain. Orexins exert their functions by activating two G-protein-coupled receptors, OX₁ and OX₂. Orexin receptor expression was first reported in the brain, but they are also expressed outside of the central nervous system (CNS). Upon activation, OX₁ and OX₂ receptors can couple to heterotrimeric G-proteins of different families, including G_q, G_s and G_{i/o}. Orexin receptor activation can evoke increases in intracellular Ca²⁺ concentrations via multiple mechanisms, including activation of phospholipase C and increased Ca²⁺ influx, and it also regulates adenylyl cyclase activity, both positively and negatively. Multiple kinases have also been reported in their signaling cascades, including protein kinases C and D, extracellular signal-regulated kinase, p38, Src, and phosphatidylinositol-3-kinase.

Previous studies have suggested interactions between the orexin and endocannabinoid systems. The endocannabinoid system consists of endocannabinoids, which are neuromodulatory lipids produced on demand by neurons, their effectors (CB₁ and CB₂ cannabinoid receptors, as well as other receptors and channels), and finally the enzymes that degrade them. The cannabinoid and orexin systems have several overlapping functions, such as regulation of pain transmission, appetite, learning, and reward. Evidence for the existence of interactions between these systems has been gained from physiological, but also molecular studies. Even heteromerization of CB₁ and OX₁ receptors has been reported.

In this thesis, the signaling of the OX₁ receptor was further investigated, with special emphasis on lipid mediators. Recombinant cells (mainly CHO cells) were employed as model systems. We were able to directly demonstrate the activation of phospholipase D upon OX₁ stimulation, and consequently add PLD to the signaling cascades mediating orexin responses. Phospholipase D activation was mediated by a novel protein kinase C isoform, most likely protein kinase C δ . OX₁ receptor activation also leads to the release of two other messengers: arachidonic acid and the endocannabinoid 2-arachidonoylglycerol. In this thesis, the release of these messengers and the pathways leading to their production, upon orexin receptor activation, were investigated in detail for the first time. Powerful arachidonic acid release by cytosolic phospholipase A₂ (cPLA₂) was observed in recombinant CHO cells. In contrast, 2-arachidonoylglycerol was released by a cascade involving activation of phospholipase C and diacylglycerol lipase; this was observed in CHO, neuro-2a, and HEK293 cells. By utilizing CHO cells in an artificial cell-cell communication assay, we saw that the released 2-arachidonoylglycerol can act as a paracrine messenger, activating neighboring cells expressing CB₁ cannabinoid receptors. 2-Arachidonoylglycerol similarly also acted as an autocrine messenger, and co-signaling of OX₁ and CB₁ receptors upon orexin stimulation of the receptor-co-expressing cells via the "2-arachidonoylglycerol loop" led to the potentiation of ERK

activation. This implies that the significance of the previously reported OX₁–CB₁ interaction is more likely to originate from functional than physical interaction of the receptors. However, the idea of heteromerization between OX₁ and CB₁ receptors is interesting, and in the final study of the present series, we utilized the bioluminescence resonance energy transfer (BRET) method to investigate constitutive homo- and heteromerization between OX₁, OX₂, and CB₁ receptors. According to our results, all receptor combinations readily form heteromeric complexes when expressed in CHO cells.

Abbreviations

AA	arachidonic acid
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
2-AG	2-arachidonoyl glycerol
AKAP	A-kinase anchoring proteins
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPK	adenosine monophosphate-activated protein kinase
ARC	arachidonate-regulated Ca^{2+} channel
ARF	adenosine diphosphate-ribosylation factor
AVP	arginine vasopressin
BAT	brown adipose tissue
BRET	bioluminescence resonance energy transfer
CaMK	Ca^{2+} /calmodulin-dependent kinase
CHO	Chinese hamster ovary
CNG	cyclic-nucleotide-gated channel
CRF	corticotropin releasing factor
cPLA ₂	cytosolic/ Ca^{2+} -dependent phospholipase A ₂
CPP	conditioned place preference
CRAC	Ca^{2+} release-activated channels
CREB	cAMP response element-binding protein
CSF	cerebrospinal fluid
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DORA	dual orexin receptor antagonist
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAAH	fatty acid amide hydrolase
FRET	fluorescence resonance energy transfer
GABA	γ -amino butyric acid
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLUT	glucose transporter
GPCR	G-protein-coupled receptor
HBM	Hepes-buffered medium
HCN	hyperpolarization-activated channel
HEK	human embryonic kidney
iBAT	interscapular brown adipose tissue
i.c.v.	intracerebroventricular
IgG	immunoglobulin G
IL	interleukin
IP ₃	inositol trisphosphate
i.v.	intravenous
LPA	lysophosphatidic acid
LTD	long-term depression
LTP	long-term potentiation
MAFP	methyl arachidonoyl fluorophosphonate
MAGL	monoacylglycerol lipase
MAPKKK	mitogen-activated protein kinase kinase kinase
MCH	melanin-concentrating hormone

mTOR	mammalian target of rapamycin
NMDA	<i>N</i> -methyl-D-aspartate
NSCC	non-selective cation channel
NPY	neuropeptide Y
PA	phosphatidic acid
PAP	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PI3K	phosphatidylinositol 3-kinase
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PLD	phospholipase D
PP	pyrrophenone
PPAR	peroxisome proliferator-activated receptor
PPO	prepro-orexin
REM	rapid eye movement
RGS	regulator of G-protein signaling
Rluc	<i>Renilla</i> luciferase
ROC	receptor-operated channel
RTK	receptor tyrosine kinase
S-BSA	stripped bovine serum albumin
SOC	store-operated channel
STIM	stromal interaction molecule
TLC	thin layer chromatography
TRP	transient receptor potential
TRPV	transient receptor potential vanilloid
UCP	uncoupling protein
WAT	white adipose tissue

Chemicals are listed in Appendix I

Brain anatomical abbreviations

ARN	arcuate nucleus
DR	dorsal raphe
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
LH	lateral hypothalamus
PAG	periaqueductal gray
PH	posterior hypothalamus
PPT	pedunculopontine nucleus
PVN	paraventricular nucleus
TMN	tuberomammillary nucleus
VLPO	ventrolateral preoptic area
VTA	ventral tegmental area

1. Introduction

Cells are able to communicate with each other by various means. This communication is referred to as signaling, and it is fundamental to the function of cells and tissues. These signals can be relayed by different transmitters, for example nucleotides, lipids, amino acid derivatives, biogenic amines, or polypeptides, which cells secrete and which then relay the signal to the same cells that secrete them (autocrine signaling), neighboring cells (paracrine signaling), or cells that can be far from the producing cells, in which case the signaling molecules reach their target by being carried in blood or hemolymph (endocrine signaling).

When these signaling molecules reach their targets, they bind to their receptors, which can be on the cell membrane or inside the cells (nuclear receptors). Molecules that bind to certain receptors and activate them are called agonists, while receptor inhibitors are called antagonists. Receptor activation leads to the activation of different signaling cascades inside the cell. This is called intracellular signaling.

The cell membrane receptors can be divided into three major groups based on their structure and function. Ionotropic receptors are protein complexes that function as ion channels, opening upon agonist binding. Enzyme receptors, on the other hand, are receptors that, from the cytosolic side, act as enzymes once activated. The third class of cell membrane receptors is G-protein-coupled receptors (GPCRs).

GPCRs are the largest family of cell membrane receptors, and they are a target for about 40 % of all currently used pharmaceuticals. Therefore, understanding the biology of these receptors and the signaling cascades they activate is fundamental for the discovery and development of new therapeutic drugs.

Orexin receptors are GPCRs activated by the neuropeptides orexin-A and -B. They participate in the regulation of several physiological functions including wakefulness, appetite, pain and addiction. The lack of orexin signaling causes narcolepsy with cataplexy, a disorder where a patient falls asleep at inappropriate times and loses muscle control. Because orexins are so central for the regulation of wakefulness, they are seen to have potential for development of treatments for sleep-related problems.

The intracellular signaling pathways activated by orexin receptors are very diverse. This thesis concentrates on expanding our knowledge of those signaling pathways. The interactions of orexin receptors with cannabinoid receptors (another GPCR subfamily with many physiological functions) are also investigated.

2. Review of the Literature

2.1 G-protein-coupled receptors and their main effectors

2.1.1 G-protein-coupled receptors

G-protein-coupled receptors (GPCRs) constitute the largest family of cell membrane receptors, with over 800 genes in the human genome (reviewed in Civelli *et al.*, 2013). They are a heterogeneous group of receptors, divided into five families according to the phylogenetic GRAFS classification system: Glutamate, Rhodopsin, Adhesion, Frizzled, and Secretin (Schiøth and Fredriksson, 2005). GPCRs are also very interesting from the point of view of the pharmaceutical industry, and about 40% of existing therapeutic drugs act through targeting GPCRs (reviewed in Lin, 2013).

The GPCRs respond to a huge variety of stimuli, ranging from messengers secreted by cells (e.g. lipids, biogenic amines, polypeptides, amino acids) to external stimuli such as photons or organic compounds, acting as olfactants or pheromones (reviewed in Kobilka, 2007). Despite the variability of their ligands, they all share certain structural features. GPCRs have 7 transmembrane domains of α -helical conformation, spanning the plasma membrane, connected by 3 intracellular and 3 extracellular loop regions. The N-terminus is extracellular and the C-terminus is intracellular. The binding site for ligands varies according to the type of ligand, but the N-terminal parts and transmembrane regions of the receptors have been shown to be important for ligand binding (reviewed in Kobilka, 2007).

2.1.2 Heterotrimeric G-proteins

Upon activation, GPCRs interact with intracellular heterotrimeric G-proteins (Figure 1). These G-proteins are GTPases, which are formed of three subunits: α , β , and γ . The classical scheme of signal transduction is that when the heterotrimeric G-protein interacts with the activated GPCR, it releases GDP, making the nucleotide-binding site available for GTP. The GPCR thus acts as a guanine nucleotide exchange factor (GEF) for the G-protein. The GTP binding site is in the α -subunit of the G-protein, and when GTP is bound, the $\beta\gamma$ -complex dissociates from the α -subunit. The GTP-bound α -subunit can then interact with and activate its targets. The $\beta\gamma$ -subunit is then also free to activate its target proteins, such as various ion channels and kinases (Milligan and Kostenis, 2006). The inactivation of the $G\alpha$ -subunit is regulated by a group of proteins called regulators of G-protein signaling (RGS), which accelerate GTP hydrolysis. After GTP hydrolysis and expulsion of the phosphate, the $G\alpha$ -subunit returns to its inactive, GDP-bound state and reunites with the $G\beta\gamma$ -subunit, and the cycle can start again (Hamm, 1998; Hewavitharana and Wedegaertner, 2012). It is, however, noteworthy that this classical scheme of heterotrimeric G-protein

dissociation has been challenged, and there is evidence that dissociation of $G\alpha$ - and $G\beta\gamma$ -subunits does not always occur (Rebois *et al.*, 1997; Gales *et al.*, 2006).

The effectors of dissociated G-protein subunits, as well as the nature of the interaction (activation or inhibition), depend on the G-protein type. Heterotrimeric G-proteins are divided into four subfamilies: G_s , $G_{i/o}$, G_q , and $G_{12/13}$. This division is according to their α -subunits ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$), and the typical members of these subfamilies have certain classical responses upon receptor activation: $G\alpha_s$ activates adenylyl cyclase (AC), whereas $G\alpha_i$ inhibits it. $G\alpha_q$, on the other hand, activates phospholipase C β (PLC β), and $G\alpha_{12/13}$ is known to regulate many proteins, of which RGS-RhoGEFs (RGS domain-containing Rho GEFs) are the best known (Hewavitharana and Wedegaertner, 2012).

In addition to activating a variety of signaling cascades via G-proteins, GPCRs can also activate signaling pathways independent of G-proteins. The best-known proteins that interact with GPCRs are GPCR kinases and arrestins (reviewed in Magalhaes *et al.*, 2012). Through this ability to signal both via G-proteins and independently of them, the signaling cascades activated by GPCRs become extremely variable.

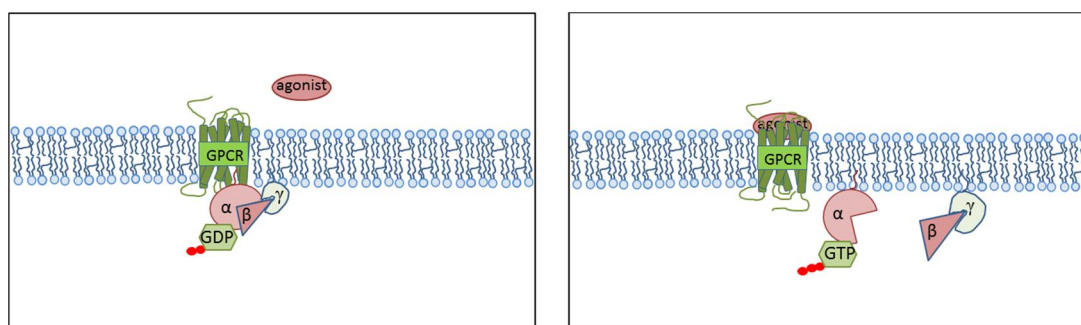


Figure 1. A classical scheme of activation of a heterotrimeric G-protein by a GPCR upon agonist binding. GPCR, G-protein-coupled receptor; GDP, guanosine diphosphate; GTP guanosine triphosphate.

Table 1. Main effectors for typical members of different heterotrimeric G-protein families.

Heterotrimeric families	G-protein	Main effectors
$G\alpha_{q/11}$		PLC \uparrow
$G\alpha_s$		AC \uparrow
$G\alpha_{i/o}$		AC \downarrow
$G\alpha_{12/13}$		PLC and PLD \uparrow

AC, adenylyl cyclase; PLC, phospholipase C; PLD, phospholipase D. Arrows pointing upwards represent increasing activity, whereas arrows pointing downwards represent decreasing activity.

2.1.3 Phospholipase C and Ca²⁺ signaling

Classically, coupling to G_q proteins results in the activation of phospholipase Cβ (PLCβ), which hydrolyzes phosphatidylinositols, especially phosphatidylinositol-4,5-bisphosphate (PIP₂). Hydrolyzation of PIP₂ produces inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), both of which are important second messengers (reviewed in Berridge, 2009; Yang *et al.*, 2013b). IP₃ activates IP₃ receptors in the endoplasmic reticulum (ER), which leads to the release of Ca²⁺ into the cytosol, and hence an increase in the cytoplasmic Ca²⁺ concentration (Exton, 1996). Depletion of ER Ca²⁺ stores is detected by stromal interaction molecule 1 (STIM1), which then leads to the opening of Ca²⁺ release-activated Ca²⁺ (CRAC) channels, now known to consist of Orai1 subunits (reviewed in Berridge, 2009; Fahrner *et al.*, 2013).

Ca²⁺ is a fundamental second messenger. It regulates an array of functions, from the secretion of transmitters in synapses and contraction of smooth muscle to fertilization, proliferation, and cell metabolism. Because Ca²⁺ is involved in controlling so many and so variable functions, it is considered one of the most important second messengers (reviewed in Berridge, 2009). The importance of Ca²⁺ as a signaling molecule is reflected in the wide range of channels and pumps that regulate the intracellular Ca²⁺ concentration. The temporal and spatial dynamics of the increase in Ca²⁺ determine the target regulation by Ca²⁺ (Bootman *et al.*, 2001).

2.1.4 Adenylyl cyclases

Another classical second messenger is cAMP, which was actually the first intracellular second messenger to be discovered (reviewed in Antoni, 2000). Levels of cAMP in cells are regulated by adenylyl cyclases (ACs), which convert ATP to cAMP. Cyclic nucleotide phosphodiesterases, on the other hand, hydrolyze cAMP to AMP. In mammals, there are nine membrane-bound AC isoforms, which can be divided into four families based on their sequences (reviewed in Halls and Cooper, 2011). The classical pathway for AC activation is that a GPCR activates a G_s protein and the Gα_s subunit activates the effector, AC. Alternatively, if the GPCR couples to G_i, the Gα_i subunit inhibits AC activity (reviewed in Antoni, 2000). However, also other factors regulate the activity of ACs. The intracellular Ca²⁺ concentration can modulate AC activity: this can occur via the Ca²⁺-calmodulin complex, calcineurin, Ca²⁺-calmodulin-activated kinase (CaMK), or protein kinase C (PKC). The end result of the modulation (inhibition or activation) depends on the AC subtype (reviewed in Halls and Cooper, 2011).

There are many targets for cAMP in cells. The most classical and well-characterized target for cAMP is activation of the cAMP-dependent kinase, protein kinase A (PKA). When cAMP levels in the cell increase, PKA is activated, its catalytic subunit moves into the nucleus, and it activates by phosphorylation the transcription factor cAMP-responsive element-binding protein (CREB). Phosphorylated CREB regulates the expression of various genes, most of them related to cell survival and growth (Mayr and Montminy, 2001). CREB is, however, only one

of the many PKA targets. A-kinase anchoring proteins (AKAP) bind PKA, but also other proteins, and scaffold its targets to multiprotein signaling complexes. New AKAPs and their binding partners are continuously being discovered (reviewed in Esseltine and Scott, 2013). In addition to PKA-mediated pathways, cAMP also directly regulates the function of HCN and CNG channels and exchange proteins directly activated by cAMP (EPACs), which act as GEFs for the small G-proteins Rap1 and Rap2 (reviewed in Schmidt *et al.*, 2013). Physiologically, cAMP is essential for almost all functions imaginable, and cAMP signaling has been verified in practically all tissues.

2.2 Orexins and orexin receptors

2.2.1 Orexins

In 1998, two groups independently reported novel neuropeptides found in the lateral hypothalamus of the rat. One of the groups called these peptides hypocretin-1 and -2 (de Lecea *et al.*, 1998), while the other group called them orexin-A and -B and their receptors OX₁ and OX₂ (Sakurai *et al.*, 1998). These groups were, in fact, describing the same peptides, and both names (hypocretins and orexins) are still in use. However, the Nomenclature Committee of the International Union of Basic and Clinical Pharmacology (IUPHAR) recommends the use of orexin nomenclature for the peptides and receptors, and hypocretin for the genes (Gotter *et al.*, 2012). Thus, the peptides are prepro-orexin (PPO), orexin-A and orexin-B, and the receptors are OX₁ and OX₂ receptors, while the rodent genes encoding PPO and the receptors are *Hcrt*, *Hcrtr1* and *Hcrtr2* (all capital letters for the corresponding human genes), respectively. Both orexin-A (33 amino acids) and -B (28 amino acids) are cleaved and modified from the precursor PPO (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998; Figure 2). In mammals, the peptide sequences for orexin-A and -B are highly conserved: orexin-A is identical in rats, mice, pigs, dogs, and humans, whereas in orexin-B, there are only slight changes in one or two amino acids between these species (reviewed in Kukkonen, 2013b). Orexins are also expressed and have been shown to function in teleost fish (Alvarez and Sutcliffe, 2002; Kaslin *et al.*, 2004; Yokobori *et al.*, 2011). The orexin system therefore seems to have evolved quite early in vertebrate evolution.

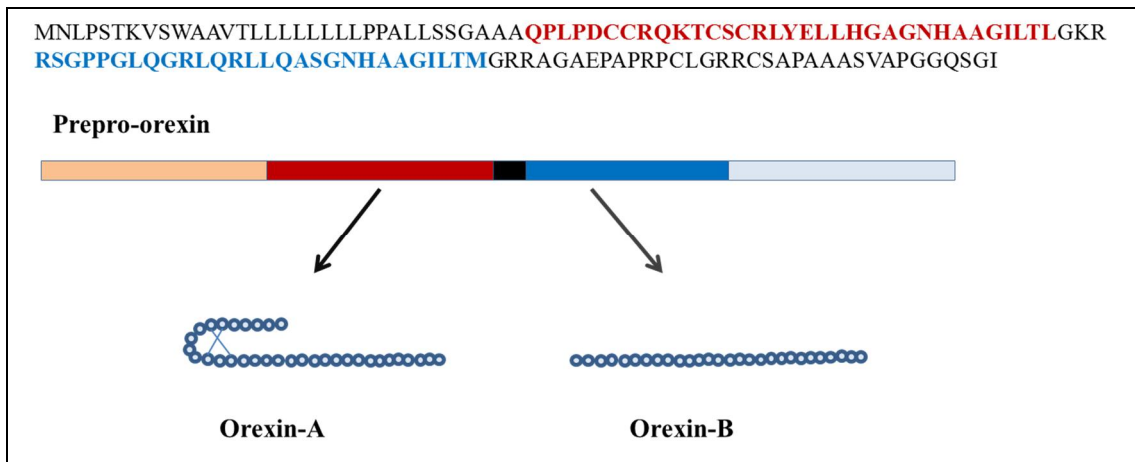


Figure 2. Orexin-A and -B are both cleaved from prepro-orexin. Human prepro-orexin amino acid sequence with orexin-A in red and orexin-B in blue.

2.2.2 Orexin receptors

Orexins act via OX₁ and OX₂ orexin receptors, which are GPCRs of the rhodopsin family (Sakurai *et al.*, 1998). According to the original study, the affinity of orexins for the two receptors is different: OX₂ receptor bound both orexin-A and -B with equal affinity, whereas OX₁ bound and was activated 10 times more potently by orexin-A than by orexin-B (Sakurai *et al.*, 1998). However, more recent findings have questioned this (reviewed in Kukkonen, 2013b).

Orexin receptors and orexins were initially found in the hypothalamus (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998). The orexin-producing neurons have their cell bodies in the lateral hypothalamus, from where they send projections to many regions in the brain (Peyron *et al.*, 1998). The regions of the brain that express orexin receptors are discussed in more detail in 2.4.1. It has subsequently been shown that orexin receptors are also expressed in tissues outside of the central nervous system (CNS), such as the gastrointestinal tract, white and brown adipose tissue, adrenal gland, and pancreas (reviewed in Kukkonen, 2013b).

Orexin receptors were first reported to couple to G_q proteins (Sakurai *et al.*, 1998), but later it became clear that they actually also couple to G_s and G_{i/o} proteins (Karteris *et al.*, 2001; Bernard *et al.*, 2002; Karteris *et al.*, 2005; reviewed in Kukkonen and Leonard, 2013). In addition to coupling to G-proteins, orexin receptors, like other GPCRs, are able to activate signaling cascades independent of G-protein coupling (reviewed in Kukkonen and Leonard, 2013).

2.3 Orexin receptor signaling

Orexin receptors activate versatile signaling cascades. A simplified overview of these signaling cascades is presented in Figure 3.

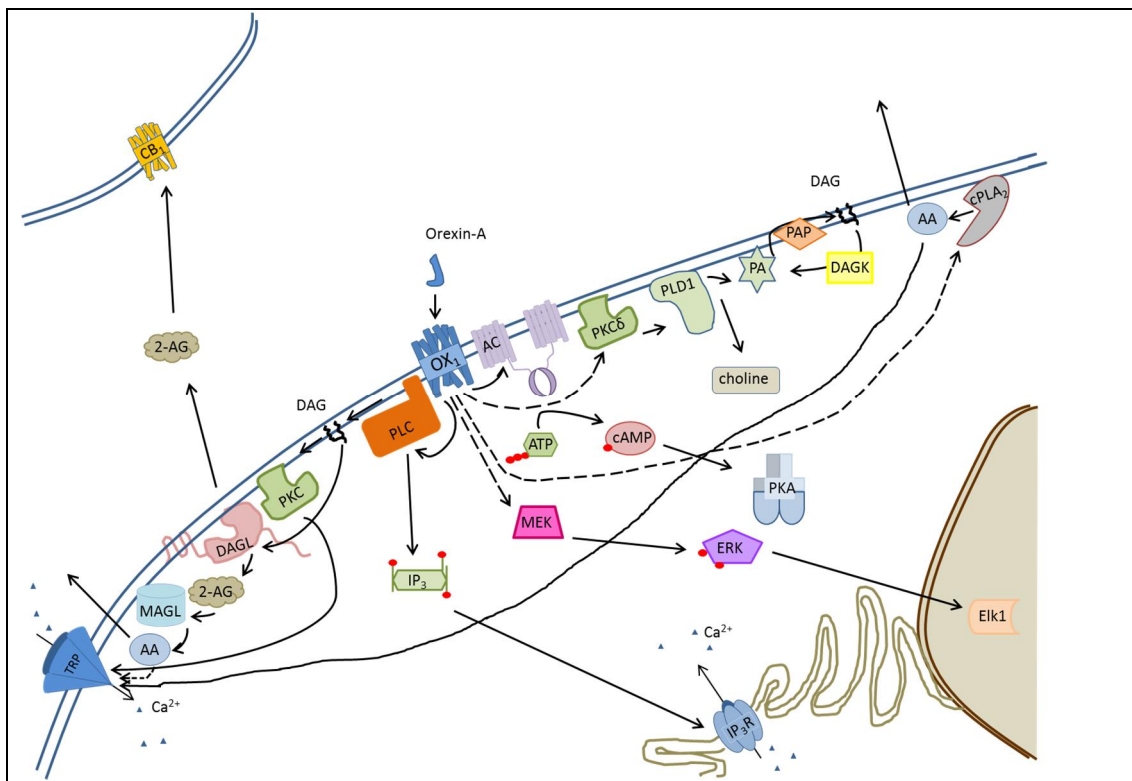


Figure 3. A simplified scheme of signaling cascades regulated by OX₁ orexin receptors. AA, arachidonic acid; AC, adenylyl cyclase; 2-AG, 2-arachidonoylglycerol; cAMP, cyclic adenosine monophosphate; CB₁, cannabinoid receptor 1; cPLA₂, Ca²⁺-dependent/cytosolic phospholipase A₂; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DAGL, diacylglycerol lipase; ERK, extracellular signal-regulated kinase; Elk1, ETS domain containing protein; IP₃, inositol trisphosphate; IP₃R, inositol trisphosphate receptor, MAGL, monoacylglycerol lipase; MEK, extracellular signal-regulated kinase kinase; OX₁, orexin receptor 1; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; TRP, transient receptor potential channel.

2.3.1 Ca²⁺ in orexin receptor signaling

Ca²⁺ signaling was among the first functional responses reported for orexin receptors upon stimulation with orexin-A. When the orphan GPCR, GFAN72, was deorphanized and named an orexin receptor, this was done by transfecting CHO cells with the cDNA encoding this receptor, activating it with possible ligands, and measuring the Ca²⁺ responses (Sakurai *et al.*, 1998). Upon orexin stimulation, the concentration of intracellular Ca²⁺ increases: this was suggested to be due to G_q

coupling of orexin receptors (Sakurai *et al.*, 1998). However, orexin receptor activation-induced Ca^{2+} signaling is more complicated than this.

It has been shown that the increase in intracellular Ca^{2+} upon orexin receptor activation is dependent on the extracellular Ca^{2+} concentration (Smart *et al.*, 1999; Lund *et al.*, 2000). Orexin receptors are thought to regulate receptor-operated calcium channels (ROCs), which include non-selective cation channels [NSCCs, such as transient receptor potential (TRP) channels] and the hyperpolarization-activated (HCN) and cyclic-nucleotide-gated (CNG) channels (Yang and Ferguson, 2002, 2003; Yang *et al.*, 2003; Larsson *et al.*, 2005; Li *et al.*, 2010). Orexin receptor activation can also lead to an increase in the intracellular Ca^{2+} concentration by the reverse-mode action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) (Louhivuori *et al.*, 2010). In neurons and other excitable cells, orexin receptors also activate L- and N-type voltage-gated Ca^{2+} channels (Uramura *et al.*, 2001; Kohlmeier *et al.*, 2008; Xia *et al.*, 2009; Wu *et al.*, 2013). The rise in the intracellular Ca^{2+} concentration is therefore dependent on many different mechanisms, including ROCs, SOCs, and IP_3 -mediated Ca^{2+} release.

2.3.2 Adenylyl cyclases as effectors of orexin receptors

Orexin receptors have been shown to regulate AC activity (Malendowicz *et al.*, 1999; Mazzocchi *et al.*, 2001; Randeva *et al.*, 2001; Holmqvist *et al.*, 2005; Karteris *et al.*, 2005; Tang *et al.*, 2008). Both positive and negative regulation has been detected in recombinant cells, and various signaling cascades activated by orexin receptors lead to modulation of AC activity, including direct activation via $\text{G}\alpha_s$, direct inhibition via $\text{G}\alpha_i$, and indirect and conditional activation via $\text{G}\alpha_q$ -mediated activation of the PLC–PKC cascade (Holmqvist *et al.*, 2005). The physiological relevance of orexin receptor-induced modulation of AC activity has mostly been studied in adrenocortical cells, both *in vitro* and *in vivo*. Orexin receptor-induced AC–PKA activation has been shown to regulate the secretion of corticosteroids from the adrenal gland (Malendowicz *et al.*, 1999; Mazzocchi *et al.*, 2001).

2.3.3 Activation of protein kinases by orexin receptors

Orexin receptors utilize several kinases in their signaling. These kinases include protein kinase C (PKC) (Uramura *et al.*, 2001), extracellular signal-regulated kinase 1 and 2 (ERK1 and -2) (Milasta *et al.*, 2005; Ammoun *et al.*, 2006a), p38 (Ammoun *et al.*, 2006a; Sellayah *et al.*, 2011), Src (Ammoun *et al.*, 2006a; Voisin *et al.*, 2008), protein kinase D (PKD) (Peltonen *et al.*, 2010), AMP-activated kinase (AMPK) (Wu *et al.*, 2013), and phosphoinositide-3-kinase (PI3K) (Ammoun *et al.*, 2006a; Skrzypski *et al.*, 2011).

The kinases that orexin receptors are known to activate, differ according to the cell type. Interestingly, orexin receptors activate kinases that are related to cell survival, growth, proliferation, and motility, such as ERK and PI3K (Mendoza *et al.*,

2011). On the other hand, orexin receptors are also known to activate p38, which is a kinase known to mediate apoptosis, but which is also involved in cell cycle progression (Thornton and Rincon, 2009). Orexin receptor-induced p38 activation has been shown to cause apoptotic cell death in CHO cells (Ammoun *et al.*, 2006b), but differentiation in other cell lines such as HIB1b preadipocytes, mesenchymal C3H10T1/2 cells (Sellayah *et al.*, 2011) and in human adrenal cell line H293R (Ramanjaneya *et al.*, 2008; Ramanjaneya *et al.*, 2009). Orexins regulate synaptic plasticity, possibly partially via kinase cascades (Selbach *et al.*, 2004; Borgland *et al.*, 2006; Chen *et al.*, 2008; Selbach *et al.*, 2010; Yang *et al.*, 2013a).

PKCs are serine/threonine kinases divided into three groups: classical (cPKC), novel (nPKC), and atypical (aPKC). These PKC subtypes differ from each other in their activation, and partially also in their substrates. Orexin receptors can activate at least cPKCs and nPKCs. These subtypes are activated by DAG, which is an important second messenger in orexin receptor signaling. Upon activation by orexin receptors, PKCs are indicated to regulate, for example, NSCCs (Xia *et al.*, 2009), L- and N-type Ca^{2+} channels (Uramura *et al.*, 2001), phospholipase D (Paper I), inwardly rectifying K^+ channels (Nakajima and Nakajima, 2010), ERK, and CREB (Ammoun *et al.*, 2006a; Guo and Feng, 2012).

2.3.4 Lipid mediators in orexin receptor signaling

Phospholipases hydrolyze cell membrane glycerophospholipids. The glycerophospholipid substrates and the cleavage sites where phospholipases act differ between phospholipases, and a wide range of products is thus formed. Phospholipase-produced lipid mediators are important for orexin receptor signaling (reviewed in: Kukkonen, 2013a). Orexin receptors activate phospholipases C, A_2 and D (Lund *et al.*, 2000; Johansson *et al.*, 2007, 2008; Turunen *et al.*, 2010; Paper I; Paper II).

Phospholipase C

As mentioned in 2.2.2, orexin receptors couple to heterotrimeric G_q proteins, which likely signal further to $\text{PLC}\beta$. There are six subtypes of phospholipase C (PLC): β , γ , δ , ϵ , ζ and η , and all of these subtypes share the requirement for Ca^{2+} for their activation (reviewed in Yang *et al.*, 2013b). In addition to G_q coupling, orexin receptor activation is capable of leading to a rise in the intracellular Ca^{2+} concentration, the activation of different kinases, including PI3K, and the activation of small G-proteins of the Ras family. Through these pathways, orexin receptors could be able to activate all subtypes of PLCs, but it is still unclear which subtypes are relevant for orexin receptor signaling (Johansson *et al.*, 2007; reviewed in: Kukkonen, 2013a). The PLC activity leads to production of DAG, which can be further phosphorylated to phosphatidic acid (PA), both of which are important lipid mediators with many targets.

Phospholipase A₂

The first discovered PLA₂ was sPLA₂, found in the venom of cobras and rattlesnakes. Similar types of secreted enzymes were then found in the porcine pancreas (reviewed in Dennis *et al.*, 2011). In 1991, two groups also managed to clone (Clark *et al.*, 1991) and purify (Kramer *et al.*, 1991) a Ca²⁺-dependent cytosolic PLA₂ from human monoblasts. Phospholipase A₂ (PLA₂) enzymes can be divided into secreted (sPLA₂), cytosolic (cPLA₂), Ca²⁺-independent (iPLA₂), and lipoprotein-associated (Lp-PLA₂) groups (reviewed in Dennis *et al.*, 2011). All of these groups contain many members. PLA₂ enzymes hydrolyze different glycerophospholipids at the *sn*-2 position, therefore releasing a free fatty acid. In most cases, the released fatty acid is arachidonic acid (AA), a precursor for the synthesis of prostaglandins and leukotrienes, among others, but also a signaling molecule by itself. Orexin receptor activation leads to strong AA release by cPLA₂ activation in recombinant CHO cells (Turunen *et al.*, 2010; Paper II).

Free AA can regulate the activity of different cation channels, for example the highly Ca²⁺-selective AA-regulated channels (ARCs) (Mignen and Shuttleworth, 2000). These channels resemble CRAC channels, and are also composed of Orail and 3 subunits, but function independently of the depletion of internal Ca²⁺ stores. Like CRACs, ARCs are also activated by STIM1, but the activation mechanism is different. ARC activation, by receptor-induced AA generation, already occurs at low (physiologically relevant) agonist concentrations and generates oscillatory Ca²⁺ signals (reviewed in Shuttleworth, 2009). AA also modulates the activity of many other channels, such as various K⁺ and Na⁺ channels, L- and N-type Ca²⁺ channels, and TRP channels (reviewed in Meves, 2008). The effect of AA on these channels is mainly inhibitory, while AA or its metabolites activate TRP channels, such as the vanilloid TRPs, TRPV1, and TRPV4 (reviewed in Meves, 2008; Kukkonen, 2011).

Phospholipase D

Phospholipase D1 and D2 (PLD1 and PLD2) are the classical mammalian PLD enzymes (reviewed in Jenkins and Frohman, 2005). There are also other PLDs that are structurally different from the classical PLDs, such as PLD3 and NAPE-PLD (Wang and Ueda, 2009; Osisami *et al.*, 2012). Yet another different PLD enzyme is mitoPLD, which, as its name implies, resides in mitochondrial membranes, where it hydrolyzes cardiolipin to produce PA to stimulate mitochondrial fusion (reviewed in Gao and Frohman, 2012). PLD1 and PLD2 utilize phosphatidylcholine (PC) as a substrate to produce PA and free choline. They are regulated quite differently, although they both require PIP₂ for their activity. PLD1 is activated by various factors, such as the tyrosine kinase Src, small G-proteins of Rho and Arf families, and PKC, whereas PLD2 is constitutively active, and can be activated by Arf only in special circumstances (reviewed in Frohman *et al.*, 1999).

Orexin receptors activate PLD1 via nPKC (likely PKC δ) (Paper I). This activation is very powerful, but it has so far only been studied in CHO cells, and its physiological relevance thus remains to be shown. PA has dozens of different protein targets, and therefore the variety of possible downstream signaling pathways is large (Jang *et al.*, 2012). PA activates many kinases, including phosphatidylinositol-4-phosphate 5-kinase, which generates PIP₂, mitogen-activated protein kinase kinase kinase (MAPKKK) Raf1, and protein kinase C ζ . Some phosphatases and the small G-proteins, Arf1 and Arf6, are also activated by PA. One of the most studied PA targets is the mammalian target of rapamycin complex 1 (mTORC1). These targets of PA are involved in the regulation of various cellular functions, such as growth, proliferation, vesicle trafficking, endocytosis, exocytosis, cell migration, and the regulation of apoptosis (reviewed in Jang *et al.*, 2012). In addition, released PA can be easily converted to other lipid mediators, i.e. to DAG by phosphatidic acid phosphohydrolase (PAP) or to lyso-PA (LPA) by PA-PLA₁ or PA-PLA₂, which further increases the complexity of the signaling network.

Endocannabinoid production

Endocannabinoids are lipid mediators that have various functions in the CNS, but also in the peripheral tissues (reviewed in Fonseca *et al.*, 2013). Their functions and physiology are further discussed in section 2.5.

The endocannabinoid system includes the endocannabinoids, their synthesizing enzymes, their receptors (CB₁ and CB₂), and the enzymes that degrade them. The best-characterized endocannabinoids are anandamide (Devane *et al.*, 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura *et al.*, 1995). Both molecules are derivatives of AA, but their generation pathways are different. Anandamide is released from *N*-arachidonoyl-phosphatidylethanolamine by an enzyme called NAPE-PLD. 2-AG, on the other hand, is derived from PLC activation, which produces DAG. This is then hydrolyzed by DAG lipase (DAGL) to 2-AG and a free fatty acid. There are also alternative pathways for endocannabinoid production, but these are thought to be of lesser importance. Endocannabinoids are degraded via different pathways. Anandamide is hydrolyzed by fatty acid amide hydrolase (FAAH), and 2-AG is hydrolyzed by monoacylglycerol lipase (MAGL). They can also be oxidized by cyclooxygenases (COX) or lipoxygenases (LOX), which act on the arachidonate moiety of endocannabinoids (reviewed in Fonseca *et al.*, 2013).

One recently discovered lipid mediator for orexin receptor signaling is 2-AG (Haj-Dahmane and Shen, 2005; Ho *et al.*, 2011; Turunen *et al.*, 2012). Orexin receptor activation leads to the release of 2-AG via the activation of PLC and DAGL.

2.4 Orexin physiology

Orexins are involved in the regulation of several physiological functions, from the best-known effects on appetite and sleep/wakefulness to roles in reward, stress and pain modulation (Sakurai *et al.*, 1998; Chemelli *et al.*, 1999; Lin *et al.*, 1999; Sutcliffe and de Lecea, 1999; Ida *et al.*, 2000b; Bingham *et al.*, 2001; Harris *et al.*, 2005). They have also been shown to regulate plasma corticosterone levels, blood pressure, overall metabolism, and the function and development of brown adipose tissue (BAT) (Lubkin and Stricker-Krongrad, 1998; Malendowicz *et al.*, 1999; Samson *et al.*, 1999; Sellayah *et al.*, 2011; Tupone *et al.*, 2011). Orexins and their receptors were first found in the hypothalamus (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998), but their expression is not restricted there. Orexin receptors are also expressed outside the CNS in various tissues, including the gastro-intestinal tract, pancreas, pituitary gland, adrenal gland, the reproductive system of males, and brown and white adipose tissue (reviewed in Kukkonen, 2013b; Leonard and Kukkonen, 2013). Functions of the orexinergic system are often divided into functions within the CNS and in the periphery. However, it is not always easy to make a distinction between the central and peripheral functions.

2.4.1 Orexins and their receptors in the CNS

Orexin neurons refer to neurons that produce orexin peptides. Orexin neurons are situated in the lateral hypothalamic area, from which they send projections to multiple brain regions (Figure 4). Many of these are involved, for example, in maintaining wakefulness, but regions involved in learning and memory, reward, and feeding also receive projections from orexin neurons. The brain regions where orexin neurons send projections have been investigated mostly in rats by immunostaining of the orexin-containing neurons (Peyron *et al.*, 1998; Nambu *et al.*, 1999) and by determining the expression of OX₁ and OX₂ mRNA in the target areas (Trivedi *et al.*, 1998; Lu *et al.*, 2000; Zhang *et al.*, 2011). These areas include the locus coeruleus (LC), dorsal raphe (DR), tuberomammillary nucleus (TMN), ventral tegmental area (VTA), laterodorsal tegmental nucleus (LDT), arcuate nucleus (ARN), paraventricular thalamic nucleus (PVN), pedunculopontine nucleus (PPT), amygdala, hippocampus, and cerebellum (Peyron *et al.*, 1998; Trivedi *et al.*, 1998; Hagan *et al.*, 1999; Nambu *et al.*, 1999; Lu *et al.*, 2000; reviewed in Sakurai, 2007; Zhang *et al.*, 2011; Tsujino and Sakurai, 2013). The expression of OX₁ and OX₂ receptors partly overlaps, but there are also differences in the expression patterns (Trivedi *et al.*, 1998; Lu *et al.*, 2000), which might reflect partially different functions of the receptor subtypes.

Orexin neurons are innervated by neurons from various parts of the brain. For example, neurons of the ARN (Broberger *et al.*, 1998), amygdala, basal forebrain, preoptic area (PO) (Sakurai *et al.*, 2005), DR, lateral parabrachial nucleus, periaqueductal gray (PAG), lateral and posterior hypothalamus (LH and PH), and dorsomedial nucleus (Yoshida *et al.*, 2006) send projections to orexin neurons. In

addition, orexin neurons are regulated by homeostatic signals. They are inhibited by glucose (Burdakov *et al.*, 2006; Williams *et al.*, 2008) and activated by some amino acids (Karnani *et al.*, 2011).

The brain regions connected to orexin neurons either up- or downstream reflect the physiological functions of the orexinergic system. Next, some of the functions of orexins are introduced, starting from the best-known and most obvious effect, the regulation of wakefulness.

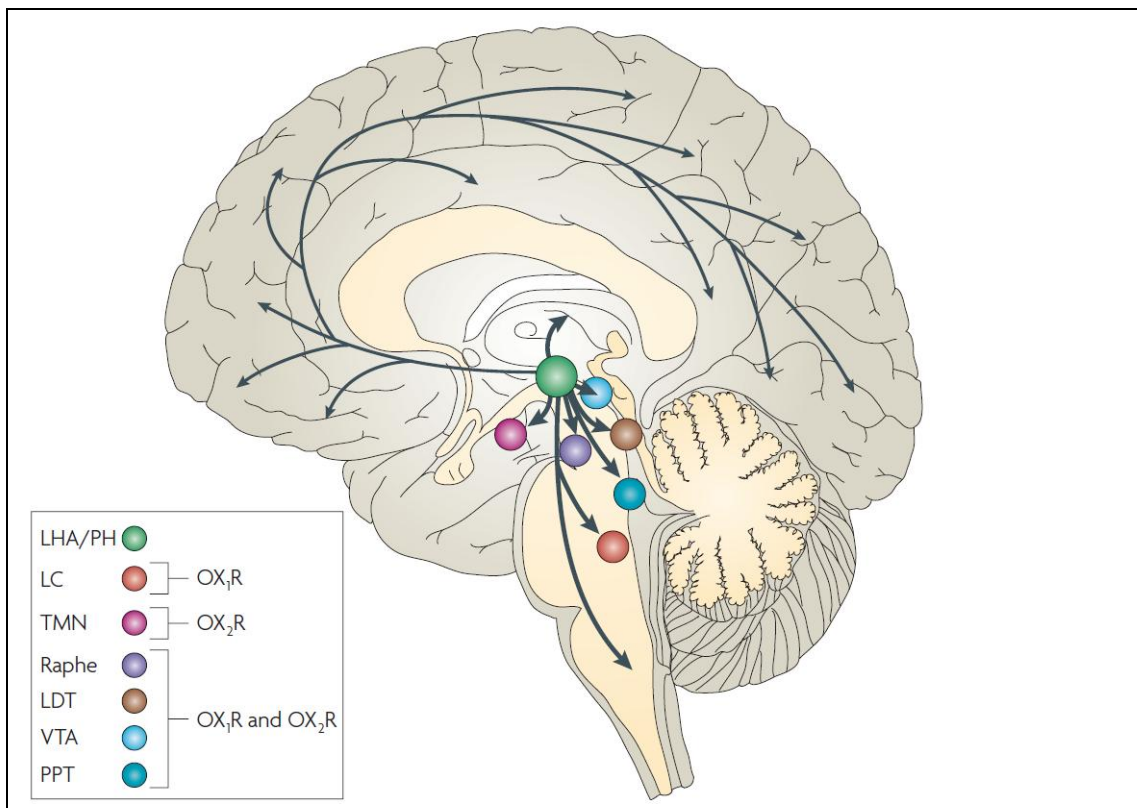


Figure 4. Orexin neuron projections and orexin receptor expression in the human brain. The arrows represent orexin neuron projections from the hypothalamus to other regions of the brain. LHA/PH, Lateral hypothalamic area/posterior hypothalamus; LC, locus coeruleus; TMN, tuberomammillary nucleus; LDT, laterodorsal tegmental nucleus; VTA, ventral tegmental area; PPT, pedunculopontine nucleus. From (Sakurai, 2007) with permission of Nature Publishing Group.

2.4.2 Regulation of sleep/wakefulness

Soon after the discovery of orexins and orexin receptors, their involvement in sleep/wakefulness became apparent, as it was found that the lack of orexins or their receptors causes narcolepsy. This was first described in dogs and mice (Chemelli *et al.*, 1999; Lin *et al.*, 1999), and then in human narcoleptic patients. Human narcoleptic patients have been reported to have dramatically diminished numbers (80–85% fewer than in healthy subjects) of orexin neurons (Peyron *et al.*, 2000;

Thannickal *et al.*, 2000), but even more importantly, reduced levels of orexin-A in their cerebrospinal fluid (CSF) (Nishino *et al.*, 2001). Reduced CSF levels of orexin-A are now used as one diagnostic criterion for narcolepsy (Mignot *et al.*, 2002). Narcolepsy with cataplexy is a condition where a patient suffers from daytime sleepiness and sleep attacks during inappropriate times, and exhibits fragmented sleep during the night. Cataplexy denotes paralysis of the skeletal muscles for a short period of time, often, but not always, caused by strong emotions (Adie, 1926; reviewed in Sakurai, 2013).

Orexin neurons are connected to various brain regions involved in the regulation of sleep and wakefulness (reviewed in Alexandre *et al.*, 2013). Orexins activate the neurons in areas such as the DR, TMN and basal forebrain, all of which are known to be important for the maintenance of wakefulness (Eggermann *et al.*, 2001). On the other hand, orexin neurons are inhibited during sleep by neurons projecting from the ventrolateral preoptic area (VLPO) and DR, (Yoshida *et al.*, 2006; reviewed in Tsujino and Sakurai, 2013). Therefore it is not surprising that orexin neurons are important for maintaining wakefulness: this has indeed been considered their main function. On the other hand, they are involved in sustaining a normal sleep pattern.

There are numerous animal models with no orexin signaling. These models range from single orexin receptor-knockout mice to PPO-ataxin-3 knockin rats and mice, where the orexin neurons are postnatally destroyed (reviewed in De la Herran-Arita and Drucker-Colin, 2012; Kukkonen, 2013b). OX₂/OX₁-double-knockout and PPO-deficient mice show similar severe narcoleptic symptoms, whereas OX₂ knockout mice are clearly narcoleptic, but with less severe symptoms (Willie *et al.*, 2001). In dogs, however, malfunctioning of OX₂ is enough to produce dramatic narcoleptic symptoms (Lin *et al.*, 1999; Hungs *et al.*, 2001). Thus, there appear to be species-specific differences. OX₁ knockout mice display only mild fragmentation of sleep (Willie *et al.*, 2001). The current view, however, is that while the OX₂ receptor is more important for maintaining wakefulness, the OX₁ receptor is also needed (Tsujino and Sakurai, 2013).

Orexins have a balancing effect on the regulation of sleep/wakefulness. Through negative and positive feedback loops, between orexin neurons and sleep-promoting and wakefulness-promoting regions of the brain, orexins regulate the transitions between these two states. When orexin action is absent, the transitions become more random, causing symptoms of narcolepsy (reviewed in Sakurai, 2007; Tsujino and Sakurai, 2013).

Besides narcolepsy, other less severe sleep-related problems have been associated with the orexin system. For example, obstructive sleep apnea has been suggested to cause damage to neurons mediating wakefulness, including orexin neurons, and narcoleptic patients quite often suffer from obstructive sleep apnea (Wang *et al.*, 2013b). There have been studies indicating decreases in PPO, OX₂ mRNA, and orexin neurons with age in rodents (Terao *et al.*, 2002; Porkka-Heiskanen *et al.*, 2004; Kessler *et al.*, 2011). These changes might contribute to the changes in sleep with aging, such as the typical decrease in the duration of sleep bouts and

daytime sleepiness (Porkka-Heiskanen *et al.*, 2004). These findings underline the importance of the orexin system in controlling wakefulness.

Due to its central role in the regulation of wakefulness, the orexin system is also a target for drug discovery to develop medications for sleep disorders. Antagonists of orexin receptors could be useful in treating insomnia, and dual orexin receptor antagonists (DORAs), such as suvorexant, have especially been under investigation. According to one study, blocking OX₂ alone could promote a more favorable sleep architecture, since it increases the amount of non-rapid eye movement (non-REM) sleep, whereas DORAs increase the amount of REM sleep (Betschart *et al.*, 2013).

2.4.3 Feeding and metabolism

Orexins were named based on their capacity to trigger a feeding response when centrally administered to rats (Sakurai *et al.*, 1998; Sakurai, 1999); the Greek word *orexis* means appetite. Orexin neurons localize in the lateral hypothalamus, which is a center for appetite regulation. In addition, they send projections to other brain regions regulating appetite, overall metabolism, and thermoregulation. These regions include the LH, posterior hypothalamus (PH), ARN, TMN, and PVN (Peyron *et al.*, 1998; Trivedi *et al.*, 1998). Orexins have been shown to activate AMPK, a key sensor of the energy status, in the NPY neurons of ARN (Wu *et al.*, 2013). These neurons produce neuropeptide Y (NPY), a peptide known for its orexigenic effect (Stanley and Leibowitz, 1984). An interaction between orexin neurons and NPY neurons has also been suggested in other studies, and there is evidence that the orexin-induced feeding response is mediated by NPY signaling (Dube *et al.*, 2000; Ida *et al.*, 2000a; Jain *et al.*, 2000; Yamanaka *et al.*, 2000; Wu *et al.*, 2013).

In addition to appetite regulation, i.c.v. administration of orexin-A to mice or anesthetized rats increases whole body energy consumption, as indicated by an increase in O₂ consumption (Lubkin and Stricker-Krongrad, 1998; Wang *et al.*, 2001, 2003). Central administration of orexin-A also indirectly increases energy consumption through the induction of spontaneous physical activity (SPA) (Jones *et al.*, 2001; Kotz *et al.*, 2002; Kiwaki *et al.*, 2004). Upon fasting, the expression of PPO and OX₁ mRNA increases (Cai *et al.*, 1999; Lu *et al.*, 2000), and the effect of fasting on PPO mRNA expression is reversed by injection of the satiety hormone leptin (Lopez *et al.*, 2000). The ability of orexin neurons to sense and respond to dietary amino acids and glucose also suggests that they play a central role in overall energy balance regulation (Williams *et al.*, 2008; Karnani *et al.*, 2011).

Different kinds of animal models have been employed to explore the role of orexins in the regulation of feeding and metabolism. PPO-overexpressing mice are resistant to diet-induced obesity (Funato *et al.*, 2009), and mice lacking orexin neurons show hypophagia, but are still obese (Hara *et al.*, 2001). In orexin-ataxin-3 mice and PPO-knockout mice, the genetic background and gender of the mice also influenced their tendency to gain weight. Female mutant animals gained more weight

compared to wild-type (wt) females (Fujiki *et al.*, 2006). Even though PPO-knockout mice have been reported to be hypophagic and gain more weight than wt mice, the effects of orexin neuron ablation have been reported to be more severe. This is not surprising, since the orexin neurons express other peptides besides orexins, and the ablation of orexin neurons also leads to ablation of these other expressed peptides (Hara *et al.*, 2005). In addition, the neurons in this model are destroyed in adult mice, thus reducing the ability for compensatory mechanisms to take place. It appears that even though orexin induces a feeding response, its effect on overall metabolism counteracts this and results in resistance to weight gain. It is probable that orexins are more important in regulating overall metabolism than in regulating the feeding response.

2.4.4 Thermoregulation

One function of orexins closely linked to their ability to regulate overall metabolism and energy consumption is thermoregulation (Yoshimichi *et al.*, 2001). Orexins increase the body temperature, but the mechanism of this has been debated. One regulator of body temperature in mammals is brown adipose tissue (BAT). Characteristic features of BAT are multilocular lipid droplets, a high content of mitochondria, and especially the expression of uncoupling protein 1 (UCP1) (reviewed in Bartelt and Heeren, 2014). UCP1, also known as thermogenin, functions by uncoupling the mitochondrial electron transport, required for ATP production, and releasing the energy as heat. This is called non-shivering thermogenesis, and it can be induced in response to cold or excess caloric intake (so-called diet-induced thermogenesis). By this mechanism, BAT can affect the overall metabolism, and protect against obesity induced by high caloric intake (reviewed in Cannon and Nedergaard, 2010).

In one of the first studies on the ability of orexins to induce hyperthermia, orexins were shown to up-regulate the expression of UCP3 mRNA in muscle tissue, whereas no changes were detected in the expression of UCP1 mRNA in BAT or UCP2 mRNA in white adipose tissue (WAT). According to this study, the thermoregulatory action of orexins was independent of peripheral thermogenesis by BAT (Yoshimichi *et al.*, 2001). Another study, however, showed that orexins induce hyperthermia by activating sympathetic nerve firing to interscapular BAT (Monda *et al.*, 2001). The importance of BAT in orexin-induced hyperthermia has gained more support from later studies (Monda *et al.*, 2004; Berthoud *et al.*, 2005; Yasuda *et al.*, 2005; Tupone *et al.*, 2011; reviewed in Madden *et al.*, 2012). Orexins not only increase BAT thermogenesis, but they are also essential for the development of BAT (Sellayah *et al.*, 2011). All of these studies have been conducted in rats. BAT is known to be important for thermoregulation in rodents, but its role in humans has been debated. Recently, however, studies have concentrated on BAT in humans, and the possible therapeutic properties of inducing BAT development in adults. The amount of BAT is inversely proportional with overall adiposity and body mass index

in humans (reviewed in Kajimura and Saito, 2014). The capacity of orexins to resist weight gain and adiposity (Funato *et al.*, 2009) could at least partly be due to their role in regulating the development and function of BAT.

2.4.5 Reward and addiction

The VTA, nucleus accumbens (NAc) and amygdala are parts of the CNS tightly involved in reward and addiction. Orexin neurons have projections to all these structures, and also receive inputs from them (Peyron *et al.*, 1998; Sakurai *et al.*, 2005; Yoshida *et al.*, 2006). Numerous studies have focused on orexins in drug addiction and reward. This has been investigated with a number of different drugs and substances of abuse, including opioids such as morphine (Georgescu *et al.*, 2003; Harris *et al.*, 2005; Narita *et al.*, 2006; Sharf *et al.*, 2008) and heroin (Smith and Aston-Jones, 2012), central stimulants such as cocaine (Boutrel *et al.*, 2005; Borgland *et al.*, 2006; Espana *et al.*, 2010; Espana *et al.*, 2011; Hollander *et al.*, 2012; Yeoh *et al.*, 2012) and amphetamine (Quarta *et al.*, 2010; Hutcheson *et al.*, 2011), cannabis (Rotter *et al.*, 2012), ethanol (Lawrence *et al.*, 2006; Shoblock *et al.*, 2011; Brown *et al.*, 2013)}, and nicotine (Pasumarthi *et al.*, 2006; Hollander *et al.*, 2008; Plaza-Zabala *et al.*, 2010; Plaza-Zabala *et al.*, 2013). Food can also function as a rewarding stimulus and activate the same pathways as addictive drugs (Harris *et al.*, 2005; Kelley *et al.*, 2005; Choi *et al.*, 2012; Kay *et al.*, 2014). Therefore, it is difficult to distinguish between the actions orexins in appetite regulation and reward.

The results of studies on addiction and relapse have been variable, and have depended on the drugs employed and the experimental designs. Orexins were first shown to have a role in morphine dependence and subsequently also in the reinstatement of cocaine-seeking (Georgescu *et al.*, 2003; Boutrel *et al.*, 2005). However, in one of the first papers on the role of orexins in reward-seeking, besides morphine and cocaine, food was also used as a rewarding stimulus for rats (Harris *et al.*, 2005). Conditioned place preference (CPP) is a widely used method in addiction research. In CPP experiments animals are taught to associate one area (compartment of a cage) with a stimulus, such as a certain drug, while another area is associated with the absence of the stimulus (only the vehicle of the drug). When the training session for associating the areas with different stimuli is over, the animal is allowed to freely move between these areas. If the animal spends more time in the area associated with the drug as compared to the control area, this is called CPP (Prus *et al.*, 2009). All of the investigated rewarding stimuli (morphine, cocaine and food) led to similar outcomes in this study. There was a positive correlation between CPP and c-Fos activation in orexin neurons. Injection of an OX₁ antagonist, SB-334867, decreased CPP. On the other hand, an extinguished CPP was reinstated by activating orexin neurons. These results indicate that orexins are involved in reward seeking, drug addiction and relapse (Harris *et al.*, 2005). PPO-knockout mice were reported not to develop morphine-induced CPP, which supports the idea that orexins are involved in reward seeking and addiction (Narita *et al.*, 2006). In another study,

however, PPO-knockout mice did not show altered CPP or locomotor responses to morphine (Sharf *et al.*, 2010). Despite the discrepancy between these studies, most of the evidence supports the involvement of orexins in reward and addiction. Some investigators have even suggested the orexin system as a target for pharmaceutical drugs for treating addiction (Boutrel *et al.*, 2013; Xu *et al.*, 2013).

2.4.6 Synaptic plasticity, learning, and memory

Addiction involves complex neural adaptations, and thus requires synaptic plasticity in parts of the brain involved in reward processing and addiction (reviewed in Kauer and Malenka, 2007). Addiction can therefore be thought of as one (pathological) form of learning and memory. As discussed above, orexins are involved in many aspects of addiction, but they have also been shown to be involved in other types of learning (van den Pol *et al.*, 2002; Selbach *et al.*, 2004; Akbari *et al.*, 2011; Yang *et al.*, 2013a).

For learning to occur, synapses have to be able to weaken or strengthen according to their activity. This is called synaptic plasticity, and the forms of these activity-dependent changes in synaptic strength are called long-term potentiation (LTP) and long-term depression (LTD) (Bliss and Lomo, 1973; Alger and Teyler, 1976; Dunwiddie and Lynch, 1978; reviewed in Kauer and Malenka, 2007). LTP and LTD were first reported to occur in the hippocampus, but were soon shown to also take place elsewhere in the brain (Kirkwood *et al.*, 1993). There are different mechanisms of LTP and LTD, but probably the most studied and understood is *N*-methyl-D-aspartate receptor (NMDAR, a subfamily of ionotropic glutamate receptors)-dependent LTP. This type of LTP requires that the postsynaptic membrane is strongly depolarized at the same time as the NMDARs are activated by glutamate. This leads to an increase in the intracellular Ca^{2+} concentration in the postsynaptic neuron, and through activation of various signaling cascades, this ultimately leads to an increase in the number of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA receptors, another family of ionotropic glutamate receptors) in the postsynaptic cell membrane, while the number of NMDARs remains unaltered. These changes are followed by structural changes in the synapse, such as enlargement of dendritic spines and increased postsynaptic densities (reviewed in Kauer and Malenka, 2007).

Orexin-A has been shown to enhance synaptic strength in VTA dopamine neurons (Borgland *et al.*, 2006). First, orexin-A induced a rapid enhancement of synaptic strength, probably by translocation of NMDARs to the postsynaptic membrane from an intracellular compartment. This was followed 3–4 hours later by increased numbers of AMPARs on the postsynaptic membrane. The OX_1 antagonist SB334867 blocked these responses (Borgland *et al.*, 2006). In CA-1 hippocampal neurons, orexin-A has been reported to induce LTD (Doreulee *et al.*, 2009), and in cortical neuron cultures, incubation with either orexin-A or -B reduced the expression of NMDAR and AMPAR subunit mRNA (Yamada *et al.*, 2008). These results

indicate that orexins could produce both LTP and LTD, and therefore mediate learning effects.

Many behavioral studies support the idea that orexins are important for learning and memory. I.c.v. injection of orexin-A improved active and passive avoidance learning in mice and rats (Jaeger *et al.*, 2002; Telegdy and Adamik, 2002). The OX₁ antagonist SB334867 has been shown to impair performance in memory tests, such as the Morris water maze, and especially to influence memory acquisition and consolidation (Akbari *et al.*, 2006, 2007; Akbari *et al.*, 2008). SB334867 has also been shown to impair motor learning in the cerebellum (Chen *et al.*, 2013) and threat learning in the LC (Sears *et al.*, 2013).

The involvement of orexins in learning has also been investigated using OX₁ receptor-knockout mice and orexin-ataxin-3 mice. OX₁-knockouts showed deficits in the formation of emotional memory, as assessed by their performance in fear-conditioning tests (Soya *et al.*, 2013). Orexin-ataxin-3 mice had impaired long-term social memory, which was partially restored by administration of exogenous orexin-A (Yang *et al.*, 2013a). A decreased number of orexin neurons and reduced CSF orexin-A levels have been reported in humans suffering from Alzheimer's disease (Fronczek *et al.*, 2012). This disease is characterized by cognitive decline, but it is also accompanied by daytime sleepiness and other sleep problems resembling symptoms of narcolepsy.

2.4.7 Pain modulation

Reward circuits of the CNS overlap with nociceptive circuits (Borsook *et al.*, 2007). Brain regions such as the amygdala, NAc, VTA, periaqueductal grey (PAG) and lateral prefrontal cortex have been shown to be involved in both reward and pain processing circuits. Furthermore, chronic pain has been shown to alter the reward circuitry (Borsook *et al.*, 2007). Considering the role of orexins in reward and drug addiction, it is not surprising that orexins are also involved in pain processing.

Orexin neurons send projections to brain regions involved in pain processing, but also to the spinal cord, which is important for pain transmission (van den Pol, 1999; Bingham *et al.*, 2001). In the first studies on the role of orexins in pain modulation, it was shown that intravenous (i.v.) injection of orexin-A caused analgesia in hyperalgesic rats and mice (Bingham *et al.*, 2001). This effect was blocked by an orexin receptor antagonist, but not by the opioid receptor antagonist naloxone. This suggests that the orexin system modulates nociception independently of the opioid system (Bingham *et al.*, 2001). In another study intrathecal injection of orexin-A produced an analgesic effect, which was abolished by pre-treatment with SB-334867. This implies that the analgesic effects of orexin-A in the spinal cord are mediated by OX₁ receptors. Orexin-B did not have similar effects (Yamamoto *et al.*, 2002). In rat models of neuropathic pain, orexin-A showed analgesic effects and reduced allodynia symptoms. These effects apply to situations of post-operative pain (Cheng *et al.*, 2003) and sciatic nerve injury-induced pain (Yamamoto *et al.*, 2003;

Suyama *et al.*, 2004), as well as diabetic neuropathic pain models (Kajiyama *et al.*, 2005).

Some studies have implied that primary headaches such as cluster headache and migraine could be connected with the orexin system (Bartsch *et al.*, 2004; Holland *et al.*, 2005; Sarchielli *et al.*, 2008). Certain variants of the OX₁ gene increase the susceptibility to migraine (Rainero *et al.*, 2011). One of the factors causing migraine headaches is trigeminal nerve activation and subsequent dilation of cranial blood vessels. Orexin-A inhibits this vasodilatation, and might in this way relieve the migraine pain (Holland *et al.*, 2005).

Orexin-A modulates different kinds of pain on the spinal cord level, but also in various brain regions, which include the periaqueductal grey (PAG) (Azhdari Zarmehri *et al.*, 2011; Ho *et al.*, 2011), the oral part of the pontine reticular nucleus (PnO) (Watson *et al.*, 2010), the lateral paragigantocellular nucleus (Erami *et al.*, 2012), VTA, and NAc (Azhdari-Zarmehri *et al.*, 2013a; Sadeghi *et al.*, 2013). In the PAG, paragigantocellularis lateralis, and PnO, orexin-A injections had analgesic effects in hot plate and formalin tests (Azhdari-Zarmehri *et al.*, 2013b). In the VTA and NAc, the OX₁ receptor antagonist SB334867 inhibited analgesic effects induced by carbachol, suggesting that orexin receptors lie downstream of cholinergic neurons producing analgesia. There is also evidence that OX₁ receptors might have a role in morphine-induced analgesia, because SB334867 has also decreased morphine-induced antinociception (Azhdari-Zarmehri *et al.*, 2013b).

2.4.8 Functions in the gastrointestinal tract

The expression of PPO, OX₁, and OX₂ mRNA in the small intestine and pancreas of rats was verified relatively soon after the discovery of orexins in the hypothalamus (Kirchgessner and Liu, 1999). OX₁, OX₂, orexin-A and orexin-B were also histochemically detected in different parts of the gastrointestinal tract of the rat, mouse and guinea pig. Orexins increase the mobility of isolated guinea pig ileum segments, and the small intestine of the mouse *in situ* (Kirchgessner and Liu, 1999; Satoh *et al.*, 2001). Orexins were also shown to increase gastric acid secretion when administered centrally (Takahashi *et al.*, 1999).

Further studies have confirmed that orexins increase both gastric acid and also duodenal bicarbonate secretion in rats (Flemström *et al.*, 2003; Yamada *et al.*, 2005; Bengtsson *et al.*, 2007). These effects of orexin-A were abolished by pretreatment with SB334867, suggesting that they were mediated by OX₁ receptors (Yamada *et al.*, 2005; Bengtsson *et al.*, 2007). The effects of orexins on duodenal secretion are dependent on the feeding status of the animals; orexins increase the duodenal bicarbonate secretion in fed rats, but not in overnight-fasted rats (Flemström *et al.*, 2003; Bengtsson *et al.*, 2007).

2.4.9 Regulation of endocrine secretion

Orexin receptors are expressed in many organs that have endocrine functions. The functions of orexins in these tissues have been extensively investigated, but the direct actions, in particular, are still somewhat unclear. In addition, physiological concentrations of orexins in peripheral tissues are difficult to measure, and the source of orexins in these tissues is not yet resolved (reviewed in Kukkonen, 2013b). There is, however, a considerable amount of evidence that orexins are involved in the regulation of secretion of certain hormones.

The expression of both orexins and their receptors has been reported in the pituitary glands of many species, including rats and humans (Date *et al.*, 2000; Blanco *et al.*, 2001). The expression levels of orexin receptors in the pituitary vary according to hormonal changes, for example during the oestrus cycle in pigs (Kaminski *et al.*, 2010) and following gonadectomy in rats (Jöhren *et al.*, 2003). I.c.v. injection of orexin-A has been shown to lead to an increase in plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels in rats (Al-Barazanji *et al.*, 2001). In the same study, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) mRNA expression was also reported to increase in the hypothalamic parvocellular paraventricular nucleus following orexin-A injection (Al-Barazanji *et al.*, 2001). These results were supported by other studies, one of which also implicated that the regulation of CRF release by orexins is mediated by NPY (Russell *et al.*, 2001), whereas another study demonstrated orexin-mediated stimulation of CRF and ACTH secretion in the absence of NPY activity (Moreno *et al.*, 2005).

In cultured cells, the effects of orexins are somewhat contradictory. In ovine somatotropes, orexins increased growth hormone release, induced by growth hormone-releasing hormone (Chen and Xu, 2003), whereas in porcine cultured anterior pituitary cells, orexin-B inhibited growth hormone but increased luteinizing hormone release (Barb and Matteri, 2005). Orexin-induced luteinizing hormone secretion was also detected in cultured pituitary cells of immature but not mature female rats (Martynska *et al.*, 2011). Furthermore, orexins were shown to reduce the oxytocin and vasopressin release induced by epinephrine, histamine, or serotonin in rat neurohypophyseal cell cultures (Kis *et al.*, 2011; Ocsko *et al.*, 2012).

In narcoleptic humans, ACTH and corticoid secretion was reported to be less regular than in healthy subjects (Kok *et al.*, 2002). The timing of growth hormone release in narcoleptic humans has also been shown to differ from healthy subjects. Narcoleptic patients secreted approximately 50% of their daily growth hormone during the day, whereas healthy subjects secrete only about 25%, and most of the secretion occurs during the night (Overeem *et al.*, 2003). However, in these reports, other possible factors affecting the hormone secretion cannot be ruled out. Therefore the involvement of orexins in these phenomena is difficult to evaluate. It can be speculated that orexins affect hormone release from the pituitary gland, but the results of the studies so far conducted have been inconsistent, perhaps due to differences in the species and cell types investigated, or the methods employed.

As described above, orexins might regulate hormone secretion from the pituitary gland, including ACTH secretion. The target of ACTH is the adrenal cortex, and orexins affect the function and secretion of the adrenal cortex via this pathway, but there is also evidence of direct effects on the adrenal gland. The expression of either one or both orexin receptors has been reported in the adrenal glands of many species, including rats (Lopez *et al.*, 1999; Malendowicz *et al.*, 1999), humans (Karteris *et al.*, 2001; Mazzocchi *et al.*, 2001; Randeva *et al.*, 2001), pigs (Nanmoku *et al.*, 2002), and cows (Kawada *et al.*, 2003). In cultured rat adrenocortical cells, orexins stimulated corticosterone secretion (Malendowicz *et al.*, 1999), and chronic exposure to systemically administered orexins increased aldosterone and corticosterone secretion in female rats (Malendowicz *et al.*, 2001). In another study, i.c.v. injections of both orexin-A and -B induced corticosterone production in male rats, whereas intraperitoneal injections did not (Jaszberenyi *et al.*, 2000). In human adrenocortical cells, orexin-A induced corticosterone and cortisol secretion, but the receptor mediating this was suggested to be OX₂ in one study (Randeva *et al.*, 2001) and OX₁ in another (Ziolkowska *et al.*, 2005).

Orexins up-regulate the expression of the steroidogenic acute regulatory protein in human adrenal H295R cells (Ramanjaneya *et al.*, 2008). In the same cell line, mRNA for steroidogenic enzymes, such as CYP11B1 and CYP11B2, was also increased following treatment with orexin-A and -B, although orexin-B was less potent (Wenzel *et al.*, 2009). Thus, according to studies performed with primary cells and cell lines, it appears that orexins can directly modulate the endocrine activity of adrenal cells.

Both PPO and OX₁ mRNA have been reported to increase during fasting (Cai *et al.*, 1999; Lu *et al.*, 2000), which is a hypoglycemic situation, and glucose has been shown to inhibit orexin neurons (Williams *et al.*, 2008). Therefore, it would make sense that orexins would be involved in the regulation of blood glucose levels by regulating the secretion of insulin or glucagon from the pancreas. There have been studies demonstrating that central orexins regulate pancreatic exocrine secretion via activation of the vagal efferent nerve (Miyasaka *et al.*, 2002; Wu *et al.*, 2004), and it could therefore be possible that they also affect endocrine secretion. The expression of orexins and their receptors has also been shown in the pancreas of rats and humans (Kirchgessner, 2002; Nakabayashi *et al.*, 2003; Ehrstrom *et al.*, 2005; Adeghate *et al.*, 2010; Adeghate and Hameed, 2011). The effects of orexins on the endocrine secretion of the pancreas are far from clear. There has been one study showing that orexins increase glucagon, and decrease glucose-induced insulin secretion in isolated pancreatic islets and in fasted rats (Ouedraogo *et al.*, 2003), while two other groups have reported inhibition of glucagon secretion in perfused rat pancreas, isolated rat pancreatic islets, and an endocrine pancreatic cancer cell line (InR1-G9) (Göncz *et al.*, 2008), and stimulation of insulin secretion from rat pancreatic islets (Nowak *et al.*, 2005).

OX₁ mRNA expression has been reported in the ovaries and testes of rats (Jöhren *et al.*, 2001), and in the testes of sheep (Zhang *et al.*, 2005). Both orexin receptors are expressed in the human testis, penis, epididymis and seminal vesicle

(Karteris *et al.*, 2004). Orexin-A induced testosterone secretion from rat testis both *in vitro* and *in vivo* (Barreiro *et al.*, 2004), and orexin-A-induced testosterone secretion was also reported in the testis of the South American camelid, alpaca (Liguori *et al.*, 2012).

2.4.10 Functions in white adipose tissue

Both OX₁ and OX₂ are expressed in human white adipose tissue (WAT) (Digby *et al.*, 2006). When isolated adipocytes from human omental and subcutaneous (s.c.) adipose tissue were treated with orexins, the expression of peroxisome proliferator-activated receptor γ (PPAR γ) was increased, whereas hormone-sensitive lipase activity as well as glycerol release were decreased (Digby *et al.*, 2006).

In the 3T3-L1 preadipocyte cell line, orexin-A increased proliferation (Zwirska-Korczala *et al.*, 2007) but not differentiation to mature adipocytes (Skrzypski *et al.*, 2012). Orexin-A also increased glucose uptake by translocating the glucose transporter GLUT4 to the plasma membrane, and increased the accumulation of triglycerides in differentiated 3T3-L1 adipocytes, and primary adipocytes isolated from rats (Skrzypski *et al.*, 2011). GLUT4 expression was increased in differentiated 3T3 L1 adipocytes, whereas in 3T3-L1 preadipocytes, the expression of PPAR γ was increased following treatment with orexin-A (Shen *et al.*, 2013). In the same study, OX₁ receptor levels were decreased in the omental adipose tissue of obese rats, and there was a negative correlation between OX₁ expression levels and body fat mass, plasma cholesterol, and triacylglycerol levels, and the fasting insulin level (Shen *et al.*, 2013).

It is interesting that while the direct effects of orexins in adipocytes inhibit lipolysis and increase lipogenesis (Digby *et al.*, 2006; Skrzypski *et al.*, 2011), *i.c.v.* injection of orexin-A has been shown to stimulate lipolysis in WAT via autonomic nerve activity (Shen *et al.*, 2008). This effect was, however, dependent on the dose; a small dose of orexin-A decreased lipolysis, while a higher dose increased it (Shen *et al.*, 2008).

The regulation of WAT by orexins is more complicated than simply their direct effects on adipocytes. Even though it appears that orexins increase the amount of WAT by increasing their proliferation and lipogenesis, the net effect of orexins is a decrease in adiposity and resistance to weight gain, as judged by the phenotypes of, for example, PPO-overexpressing mice, or orexin neuron-ablated mice (Hara *et al.*, 2001; Funato *et al.*, 2009).

2.5 Endocannabinoids

Endocannabinoids, such as 2-AG and anandamide, are lipid mediators that function in the CNS, but also in the periphery. Their synthesis and degradation are discussed in section 2.3.4. This section concentrates on their receptors and physiological functions.

2.5.1 Receptors and their signaling

The plant *Cannabis sativa* has been used for medication and recreational purposes for hundreds of years, but the main psychoactive compound, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), was identified and its chemical structure revealed in the 1960s (reviewed in Fonseca *et al.*, 2013). After this discovery, it took almost 30 years to resolve the mechanism by which Δ^9 -THC exerts its effects in animals.

In 1984, Howlett *et al.* (1984) reported that Δ^8 -THC and Δ^9 -THC mediated inhibition of AC activity in neuroblastoma cells. At the time, receptors for cannabinoids had not yet been found, but the effect of cannabinoids on AC was sensitive to pertussis toxin, indicating the involvement of G_i proteins (Howlett *et al.*, 1986). The search for cannabinoid receptors continued, and the ^3H -labeled analgesic cannabinoid CP55,940 was used to determine the pharmacology and binding kinetics of the putative cannabinoid receptors (Devane *et al.*, 1988), as well as the localization of cannabinoid binding sites in the brain (Herkenham *et al.*, 1990). In 1990, the cloning and expression of a cDNA that encoded a GPCR, which fulfilled the criteria for being a cannabinoid receptor, was reported (Matsuda *et al.*, 1990). Later, another receptor for cannabinoids was cloned from macrophages in the spleen (Munro *et al.*, 1993). These cannabinoid receptors are rhodopsin-like GPCRs called CB_1 and CB_2 , respectively. CB_1 is mainly expressed in the CNS, while CB_2 is the peripheral receptor and mainly found in cells of the immune system (reviewed in Kano *et al.*, 2009). Despite this rough division into central and peripheral cannabinoid receptors, CB_1 is not restricted to the CNS, but is also expressed in many peripheral locations (reviewed in Pagotto *et al.*, 2006). The endogenous ligand anandamide was found around the same time as CB_1 (Devane *et al.*, 1992), followed by the discovery of 2-AG, another endogenous ligand, a few years later (Sugiura *et al.*, 1995). These and other endogenous cannabinoid receptor agonists are called endocannabinoids.

Coupling to G_i proteins was implied for cannabinoid receptors even before the receptors had been identified (Howlett *et al.*, 1986). Both CB_1 and CB_2 receptors couple to G_i , and have been shown to inhibit AC (Howlett, 1984; Kaminski *et al.*, 1992). Coupling to other G-proteins, such as G_s and G_q , has also been suggested for CB_1 (Glass and Felder, 1997; Lauckner *et al.*, 2005; Chen *et al.*, 2010). Cannabinoid receptors utilize various pathways in their signaling, in addition to inhibiting AC. CB_1 and CB_2 activate ERK1 and ERK2, PKC and PI3K (Bouaboula *et al.*, 1995; Bouaboula *et al.*, 1996; Sanchez *et al.*, 2003). CB_1 activates inwardly rectifying and A-type K^+ channels (Henry and Chavkin, 1995; Mackie *et al.*, 1995; Mu *et al.*, 1999) and inhibits N- and P/Q-type Ca^{2+} channels and M- and D-type K^+ currents (Pan *et al.*, 1996; Twitchell *et al.*, 1997; Mu *et al.*, 1999; Schweitzer, 2000). It has been

suggested that cannabinoid receptors show constitutive activity, i.e. signaling activity in the absence of an agonist (Bouaboula *et al.*, 1997; reviewed in Console-Bram *et al.*, 2012). This view has, however, been challenged (Savinainen *et al.*, 2003).

CB₁ receptors are one of the most abundant GPCRs in the CNS. They are expressed throughout the brain, especially in the presynaptic locations (reviewed in Kano *et al.*, 2009). Endocannabinoids are involved in so-called retrograde synaptic transmission, where activation of the postsynaptic neuron leads to the release of endocannabinoids into the synaptic cleft. This released endocannabinoid then binds to CB₁ receptors in the presynaptic neuron and inhibits further neurotransmitter release. The released endocannabinoids often also affect other nearby synapses in a paracrine manner. The end result, inhibition or activation of action potentials, depends on the nature of the synapse: CB₁ receptors have been shown to regulate the release of several neurotransmitters, such as glutamate, GABA, acetylcholine, norepinephrine, dopamine, and serotonin (reviewed in Kano *et al.*, 2009).

CB₁ and CB₂ are not the only targets for endocannabinoids. Anandamide is known to activate TRPV1, and both anandamide and 2-AG can activate PPARs and GPR55 (reviewed in Kano *et al.*, 2009; Fonseca *et al.*, 2013).

2.5.2 Physiology of endocannabinoids

Endocannabinoids are involved in numerous physiological functions. This is due to the wide expression of CB₁ in the brain, especially in the cerebral cortex, hippocampus, and amygdala, and because they act as retrograde transmitters in a wide array of synapses (reviewed in Kano *et al.*, 2009). In addition to functions in the CNS, endocannabinoid signaling is also found in the periphery of the body, where it is involved in the regulation of overall metabolism, endocrine organs, and the immune system (reviewed in Pagotto *et al.*, 2006). The functions regulated by endocannabinoids are diverse and have been extensively studied, but only the most central functions will be briefly described here. There are many excellent reviews on the different aspects of endocannabinoid physiology.

Synaptic plasticity, learning, and memory

The hippocampus is a part of the brain involved in learning and memory, and both LTP and LTD were first reported to occur in hippocampal neurons (Bliss and Lomo, 1973; Alger and Teyler, 1976; Dunwiddie and Lynch, 1978). As mentioned above, hippocampal neurons express high levels of CB₁, and through their action in retrograde synaptic transmission, endocannabinoids are involved in learning. They can produce short-term inhibition of the synapse, but also of LTD and LTP (Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001; Carlson *et al.*, 2002; Chevaleyre and Castillo, 2003).

Studies on CB₁-knockout mice suggest that endocannabinoids are involved in memory processes by facilitating memory extinction and forgetting processes (Varvel and Lichtman, 2002). CB₁-knockout mice have enhanced memory, especially fear

memory (Reibaud *et al.*, 1999; Jacob *et al.*, 2012). The enhancement of fear memory could be explained by the crosstalk between glucocorticoids and endocannabinoids (reviewed in Akirav, 2013). Endocannabinoids negatively regulate the hypothalamic-pituitary-adrenal axis, thus inhibiting the release of corticosterone, a typical stress hormone (Patel *et al.*, 2004). Fearful stimuli induce stress responses in animals, and endocannabinoid release attenuates this response, therefore also affecting the memory process. Knocking out the CB₁ receptor leads to dysregulation of the hypothalamic-pituitary-adrenal axis (Cota *et al.*, 2007). Typical features of post-traumatic stress disorder are vivid memories and nightmares about a traumatic event. Some studies suggest that cannabinoids could be used to treat this disorder, because they have been reported to decrease these symptoms (reviewed in Akirav, 2013). The endocannabinoid system has been shown to mediate the extinction of aversive memories (Laricchiuta *et al.*, 2013).

Pain modulation

Cannabinoids have been used to treat pain, and they have also been shown to be effective in decreasing nausea and vomiting (reviewed in Sharkey *et al.*, 2014). These features make them useful for treating, for example, cancer patients suffering from chemotherapy-induced nausea. Cannabinoids suppress spinal and thalamic nociceptive neuron activity, thereby mediating analgesia (Zogopoulos *et al.*, 2013). CB₁ and CB₂ receptors, but also TRPV1 channels, mediate these effects. Interestingly, anandamide has been shown to produce analgesic effects in the spinal cord of rats suffering from neuropathic pain, by both CB₁ and TRPV1 activation (Starowicz *et al.*, 2012). TRPV1 is activated by painful stimuli, such as noxious heat and capsaicin, and anandamide at least partly mediates its analgesic effects by desensitizing TRPV1 (Starowicz *et al.*, 2012).

In human fetal astrocytes, the interleukin-1 β (IL-1 β)-induced production of inflammatory mediators was inhibited by WIN55,212-2, a synthetic cannabinoid, which activates both CB₁ and CB₂ receptors, suggesting a mechanism by which cannabinoid receptors attenuate inflammation in the brain (Sheng *et al.*, 2005). In addition to inhibiting the production of inflammatory mediators, anandamide induces the release of IL-6 from mouse astrocytes (Molina-Holgado *et al.*, 1998). IL-6 can function as a pro-inflammatory cytokine, but it also has anti-inflammatory effects (reviewed in Petersen and Pedersen, 2005). Endocannabinoids are effective in the treatment of inflammatory pain, and the inhibition of enzymes metabolizing them, mainly FAAH, has been studied as one possible strategy for treating chronic inflammatory pain disorders (Naidu *et al.*, 2010).

The endocannabinoids also mediate their analgesic action by modulating pain perception in the brain. The brain regions involved in the analgesic effects of cannabinoids were studied by local injections of WIN55,212-2, and measuring the effect of thermal noxious stimuli in the tail-flick test (Martin *et al.*, 1995). According to this study, cannabinoids mediate analgesia via the dorsolateral PAG and DR

(Martin *et al.*, 1995). Subsequently, regions such as the superior colliculus, amygdala, the noradrenergic A5 region of the brainstem, and lateral posterior and submedial nuclei of the thalamus have been added to the list of brain regions mediating cannabinoid-induced analgesia (Martin *et al.*, 1999). The involvement of the PAG in cannabinoid-mediated analgesia has been supported by other studies (Lichtman *et al.*, 1996; Walker *et al.*, 1999; Galdino *et al.*, 2014). Electrical activation of PAG neurons resulted in the release of anandamide, and subcutaneous injections of formalin, a painful stimulus, also produced this effect (Walker *et al.*, 1999). Interestingly, aerobic exercise-induced anti-nociception is also mediated, at least in part, via the endocannabinoid system in the PAG. CB₁ receptor expression and activity in the PAG was reported to increase in response to aerobic exercise. Plasma endocannabinoid levels also increased after exercise, implicating both the central and peripheral action of endocannabinoids in aerobic exercise-induced analgesia (Galdino *et al.*, 2014).

Neuroprotection

Partly because of their anti-inflammatory effects, endocannabinoids are neuroprotective. Anandamide protects rat cerebral cortical neurons from ischemia both *in vitro* and *in vivo* (Nagayama *et al.*, 1999; Sinor *et al.*, 2000). In traumatic brain injury, reactive oxygen species (ROS), massive glutamate release, and inflammatory agents cause damage to the brain cells (reviewed in Mechoulam *et al.*, 2002). Endocannabinoids have been shown to inhibit the release of ROS and inflammatory agents in human astrocytes, but also inhibit the release of tumor necrosis factor α (TNF α) in murine peritoneal macrophages (Gallily *et al.*, 2000; Sheng *et al.*, 2005). Endocannabinoids also inhibit glutamate release (Shen *et al.*, 1996). One study demonstrated that 2-AG levels are increased after closed head injury in mice, and that 2-AG inhibited the activation of nuclear factor- κ B in mice, thereby inhibiting the release of pro-inflammatory cytokines (Panikashvili *et al.*, 2005). This effect was attenuated by the CB₁ antagonist SR141716, and 2-AG did not produce these effects in CB₁-knockout mice. These mice also showed impaired recovery after closed head injury when compared with wt mice (Panikashvili *et al.*, 2005). In addition to these effects, endocannabinoids have been shown to counteract endothelin-1, a potent vasoconstrictor, thus restoring blood flow to the damaged brain tissue, as (reviewed in Mechoulam *et al.*, 2002). Endocannabinoid signaling via CB₁ mediates regeneration and neural progenitor cell proliferation after excitotoxic injury (Aguado *et al.*, 2007). Therefore, endocannabinoids are involved in all aspects of neuroprotection following brain injuries.

Feeding, reward, and metabolism

Endocannabinoids increase appetite (Di Marzo *et al.*, 2001) and hedonic aspects of food. CB₁ expression increases upon fasting, and injections of anandamide have been

reported to cause overeating in rats (Williams and Kirkham, 1999; Burdyga *et al.*, 2004). Endocannabinoids increased the intake of highly palatable food, even in non-deprived rats (Higgs *et al.*, 2003; Shinohara *et al.*, 2009). The increase in highly palatable food consumption was prevented by the CB₁ inverse agonists/antagonists AM251 (DiPatrizio and Simansky, 2008) and SR141716 (also known as rimonabant) (Mathes *et al.*, 2008). There have been studies, however, implicating that SR141716 decreases overall food intake, and not selectively the intake of highly palatable food (Freedland *et al.*, 2000; Verty *et al.*, 2004). Endocannabinoids appear to regulate the hedonic and rewarding aspects of feeding, rather than just feeding when nutrition is needed. A recent study reported that an increase in the plasma 2-AG concentration was associated with hedonic eating in humans (Monteleone *et al.*, 2012). Endocannabinoids are involved in reward circuits (reviewed in Gardner, 2005), and they have been shown to directly modulate the activity of dopaminergic neurons in the VTA (Pillolla *et al.*, 2007). The reward circuits are also essential for addiction. There is evidence of endocannabinoid involvement in addiction to substances such as ethanol (Arnone *et al.*, 1997), cocaine (De Vries *et al.*, 2001), morphine (Gonzalez *et al.*, 2003), and nicotine (De Vries *et al.*, 2005).

Endocannabinoids increase appetite, but they also regulate energy metabolism both centrally and peripherally (reviewed in Di Marzo and Matias, 2005; O'Keefe *et al.*, 2014). CB₁-knockout mice are lean and resistant to obesity due to their decreased food intake, but also because of metabolic factors (Cota *et al.*, 2003). Even when fed a high-fat diet, CB₁-knockout mice did not become obese and their plasma insulin and leptin levels remained low (Ravinet Trillou *et al.*, 2004). The use of SR141716 has been associated with a similar phenotype: when mice with diet-induced obesity were treated with orally administered SR141716, it led to a decrease in their body weight, especially their adiposity (Jbilo *et al.*, 2005). The morphology and function of the adipocytes in these mice also changed towards that of lean mice. Energy expenditure and lipolysis in adipocytes were increased, and glucose homeostasis was improved. Obesity is often accompanied by inflammation, caused by pro-inflammatory factors released within the adipose tissue. SR141716 also decreased the release of pro-inflammatory factors from adipocytes (Jbilo *et al.*, 2005). In addition, an increased tendency towards conversion to brown adipocytes was reported in cultured adipocytes derived from CB₁-knockout mice (Wagner *et al.*, 2011).

Blocking the action of CB₁ also has possibly beneficial metabolic effects in muscle cells. Injections of the CB₁ agonist HU210 induced a reduced uptake of glucose in skeletal muscles of wt mice, and this effect was prevented with AM251 (Song *et al.*, 2011). In cultured myotubes from lean and obese humans, AM251 increased the expression of genes that are important for fatty acid oxidation (Cavuoto *et al.*, 2007). Because antagonism of CB₁ action has been shown to be beneficial for weight loss and to counteract the metabolic consequences of obesity, such as insulin resistance, SR141716 was used as an anti-obesity drug for humans. However, it was withdrawn from the market because of serious side effects, such as depression. Therefore, a CB₁ blocker that would not cross the blood–brain barrier, but would still

have the desired effects in the periphery, could be ideal as an anti-obesity drug (reviewed in O'Keefe *et al.*, 2014).

2.6 Interaction of orexin and cannabinoid systems

The orexin and cannabinoid systems of the brain share many functions. They are both involved in pain modulation, the regulation of appetite, reward, memory, and the regulation of overall metabolism. Some studies have suggested interactions between these two systems.

2.6.1 Evidence from electrophysiological studies

The first electrophysiological evidence for interactions between orexin and cannabinoid systems came from a patch-clamp study on a slice preparation of the DR from male rats (Haj-Dahmane and Shen, 2005). In the serotonergic neurons of the DR, orexin-B induced a depression of glutamate-mediated synaptic currents. This effect was mimicked by WIN55,212-2 and abolished by AM-251, suggesting 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 interpreted to suggest that orexin-B regulates the activity of DR serotonergic neurons by the postsynaptic release of endocannabinoids. These endocannabinoids then inhibit glutamate release to serotonergic neurons presynaptically. Serotonergic neurons are activated during wakefulness, and an inhibitory action of orexin-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 excitation of the neurons and therefore for stable firing (Haj-Dahmane and Shen, 2005). According to this study, the interaction of orexins and endocannabinoids is important in the physiological regulation of arousal.

Another group utilized the patch clamp approach to investigate the actions of cannabinoid agonists and antagonists on melanin concentrating hormone (MCH) neurons and orexin neurons in mouse hypothalamic slices (Huang *et al.*, 2007). WIN55,212-2 caused depolarization and an increase in spike frequency in MCH neurons, while in orexin neurons it caused hyperpolarization and reduced spontaneous firing. Both of these effects, the activation of MCH neurons and inhibition of orexin neurons, were prevented by AM-251. Furthermore, the effects on MCH neurons were inhibited by bicuculline, a GABA_A antagonist, whereas the ionotropic glutamate antagonists AP-5 and CNQX abolished the effects on orexin neurons. These results were interpreted to indicate that MCH neurons are innervated by GABAergic neurons and orexin neurons by glutamatergic neurons, and the release of endocannabinoids from MCH and orexin neurons and their retrograde synaptic effects therefore result in the effects described. The activation of MCH neurons might contribute to the appetite- and weight-increasing effects of cannabinoids, while their inhibitory actions

on orexin neurons could, according to the authors, explain how cannabinoids decrease cognitive arousal (Huang *et al.*, 2007).

One common function for the orexin and endocannabinoid systems is the modulation of pain. The PAG is a brain region that initiates inhibition of nociception (Ho *et al.*, 2011). Patch-clamp measurements of rat brain slices containing PAG neurons were used to study the mechanism of orexin-A -induced antinociception. Orexin-A depressed GABAergic inhibitory postsynaptic currents, and the effect was blocked by SB334867, but also by AM-251, the DAGL inhibitor THL, and the PLC inhibitor U73122. The effect of orexin-A was mimicked by WIN55,212-2, and the MAGL inhibitor URB602 enhanced the effect. Therefore, it seems that orexins mediate their antinociceptive effect in the rat ventrolateral PAG by evoking production of 2-AG, which acts as a retrograde messenger inhibiting GABA release from nearby neurons. By this mechanism, orexin-A causes disinhibition in the ventrolateral PAG, thus allowing initiation of the descending pain inhibition. Behavioral data were also collected to support this hypothesis. Orexin-A was shown to reduce hot-plate nociceptive responses in rats when injected into the ventrolateral PAG, and both SB334867 and AM-251 blocked this effect to a similar extent (Ho *et al.*, 2011).

Cristino *et al.* (2013) investigated obesity-associated changes in the neural circuits of the LH. The studies employed leptin-knockout *ob/ob* mice and normal mice made obese with a high-fat diet. In lean mice, orexin neurons were mostly innervated by glutamatergic neurons, as also reported previously (Huang *et al.*, 2007). However, in *ob/ob* mice, as well as in mice made obese by a high-fat diet, orexin neurons were mostly innervated by GABAergic neurons, implying synaptic remodeling. This would explain the switch of retrograde endocannabinoid signaling from orexin neurons from inhibition of excitation to inhibition of inhibition, the end result now being further excitation of orexin neurons when inhibition would be needed. This, then, would lead to overeating. In this study, the mechanism of this synaptic remodeling was also investigated. The cause for this change was the lack of leptin signaling in the ARN, from where most of the neurons that innervate orexin neurons originate. The *ob/ob* mice lacked endogenous leptin altogether, but they received it from their heterozygous mothers during lactation; therefore, the remodeling of their synapses only occurred after weaning. The mice made obese by diet also had deficient leptin signaling in the ARN, because they were leptin-resistant. Leptin resistance is typical in obesity (Cristino *et al.*, 2013).

2.6.2 Evidence from behavioral experiments

Crespo *et al.* (2008) studied the interaction of the endocannabinoid and orexin systems in regulating appetite in rats. They used i.c.v. injections of orexin to pre-fed rats, and reported a dose-dependent increase in their short-term feeding behavior. They also tested the effect of the CB₁ inverse agonist SR141716 when given alone, and observed a decrease in feeding behavior. Finally, the combined effect of orexin

and SR141716 was tested. Intraperitoneal injection of SR141716, ten minutes prior to i.c.v. orexin injection, blocked orexin-induced feeding already at doses that did not reduce feeding when used alone (Crespo *et al.*, 2008). Therefore, it can be speculated that orexins exert their orexic functions via the release of endocannabinoids, and this is due to direct activation of appetite controlling circuits (for example, by inhibiting the inhibitory GABAergic neurons), but also by increasing the motivation to feed via reward circuits. Both orexins and endocannabinoids have been shown to be involved in both of these functions (see also **2.4.3**, **2.4.5**, **2.5.2**).

Because both orexins and endocannabinoids are involved in reward and addiction, their crosstalk in the VTA, a central dopaminergic nucleus associated with reward, was investigated by Taslimi *et al.* (Taslimi *et al.*, 2011). In this study, injections of the cholinergic agonist carbachol into the LHA were used to induce CPP in rats. Injections of SB334867 and AM-251 into the VTA prior to carbachol treatment inhibited carbachol-induced CPP. However, when applied into the VTA together, the antagonists did not produce an additive effect, but inhibited CPP to a similar degree as when applied alone (Taslimi *et al.*, 2011). The authors speculated that there could be cross-talk between these systems in the VTA, and it is indeed a tempting hypothesis. However, more evidence would be needed to verify this.

2.6.3 Evidence from molecular studies

In addition to the physiological evidence for interactions between the brain cannabinoid and orexin systems, investigations have also been conducted in recombinant cells that have supported the idea. Actually, the first study suggesting an interaction between these systems was carried out with recombinant CHO cells that expressed both CB₁ and OX₁ receptors (Hilaret *et al.*, 2003). The authors reported potentiation of OX₁ signaling to ERK when the receptors were co-expressed. The potentiation was attenuated by the CB₁ antagonist SR141716 and by pertussis toxin, indicating a requirement for CB₁ signaling. OX₁ signaling via G_q was not altered by SR141716, as assessed by IP production, and ERK activation evoked by the CB₁ agonist CP55,940 was similar in CHO cells expressing only CB₁ and in cells expressing both receptors: OX₁ did not have an effect on CB₁ signaling to ERK. The authors suggested, based on the co-localization of the receptors, that the potentiation of OX₁ signaling to ERK was due to heteromerization of OX₁ and CB₁ receptors (Hilaret *et al.*, 2003).

Other studies have subsequently been performed to examine the heteromerization of CB₁ and OX₁ receptors. Ellis *et al.* (2006) used HEK-293 cells expressing OX₁ or CB₁ receptors, and reported internalization of OX₁ receptors upon orexin-A exposure, whereas CB₁ receptors showed spontaneous internalization. When the receptors were co-expressed, both showed this spontaneous internalization. SR141716 treatment brought both of the receptors to the cell surface, and also reduced the potency of OX₁ signaling to ERK (Ellis *et al.*, 2006).

Ward *et al.* (2011) examined the direct physical interactions of CB₁ and OX₁ receptors with FRET, biotinylation and co-immunoprecipitation. The formation of heteromeric and homomeric complexes of the receptors in these test systems was reported, and it appears that not only dimers, but also larger oligomers such as tetramers are formed. When expressed alone, CB₁ receptors were internalized when exposed to the agonist WIN55,212-2. When expressed together with OX₁, CB₁ receptors were also internalized when the cells were exposed to orexin-A, and the maximally effective orexin-A concentration caused equal CB₁ receptor internalization as WIN55,212-2. Orexin-A was ten times more potent in causing the internalization of OX₁-CB₁ heteromeres than of OX₁ homomeres, and this was suggested to be due to higher affinity of orexin-A to OX₁-CB₁ complexes than to OX₁-OX₁ complexes (Ward *et al.*, 2011).

Although the reports described above have presented strong evidence of the formation of heteromeric complexes between OX₁ and CB₁ receptors, we wished to consider an alternative explanation for some of the reported observations. In two papers included in this thesis we investigated the production of endocannabinoids upon OX₁ stimulation and their effect on signaling in cells expressing both OX₁ and CB₁ receptors (Paper II, Paper III). The results of these studies are presented in more detail later (5.2, 5.3, 5.5).

3. Aims of the study

This thesis concentrates on lipid mediators in OX₁ orexin receptor signaling. The aim of the research was to expand the knowledge on the signaling cascades utilized by OX₁ receptors, and finally also to investigate the interactions between OX₁ and CB₁ receptors. More specifically, the aims of this thesis research were:

- 1) To investigate and verify PLD activation in OX₁ signaling, and to determine the signaling cascade leading to this activation.
- 2) To investigate whether OX₁ receptor activation evokes release of endocannabinoids, thus possibly adding a new lipid mediator to OX₁ signaling.
- 3) To determine whether the released 2-arachidonoylglycerol has paracrine or autocrine actions, and whether previously reported potentiation of OX₁ receptor signaling upon coexpression with CB₁ receptors could be due to the autocrine actions of released endocannabinoids.
- 4) To examine the capacity of OX₁, CB₁, and OX₂ receptors to form constitutive homo- and heteromeric complexes.

4. Materials and methods

4.1 Cell culture (I–IV)

Chinese hamster ovary (CHO) cells were grown in Ham's F12 culture medium supplemented with 100 U/ml penicillin, 80 U/ml streptomycin, and 10% fetal calf serum. Recombinant cell-lines stably expressing OX₁ or CB₁ receptors were used. For CHO-hOX₁, 400 mg/ml geneticin, and for CHO-hCB₁, 0.25 mg/ml phleomycin was also included in the culture medium. Neuro-2a-hOX₁, PC12-hOX₁, and HEK293 cells were grown in Dulbecco's Modified Eagle's medium supplemented with 100 U/ml penicillin, 80 U/ml streptomycin, and 10% fetal calf serum. The growth medium of PC12-hOX₁ cells was also supplemented with 5% horse serum. All cells were cultured on plastic cell culture dishes (56 cm² bottom area) in a humidified air-ventilated incubator with 5% CO₂ at 37 °C. Insect Sf9 cells were cultured to produce baculovirus stocks for transductions. They were grown in suspension in spinner flasks in Grace's insect cell medium supplemented with 8% fetal bovine serum, 50 µg/ml streptomycin, 50 U/ml penicillin, and 0.02% pluronic F68 at 27 °C.

4.2 Transfection and transduction (I–IV)

For the transfections, the cells were cultured on 96-, 48-, 24- or 6-well plates, depending on the experiment. Transfections were used in all studies (I–IV). The amount of vector DNA varied according to the size of the culture plate well, but the ratio of DNA and the FugeneHD transfection reagent was kept constant. For 2 µg DNA, 5 µl of FugeneHD was used. The amounts of FugeneHD and DNA were linearly related to the number of cells in the wells.

Baculoviral transduction was used for Western blot analysis of ERK phosphorylation (Paper III), where the expression of both OX₁ and CB₁ receptors in the same cells was needed. Baculoviral transduction was chosen because its transduction efficacy was close to 100%. The baculovirus expression vectors contained C-terminally GFP-tagged hOX₁ or hCB₁ receptors, or only GFP under the cytomegalovirus promoter. CHO cells were transduced by first centrifuging high-titer virus stocks, removing their growth medium and resuspending the viruses in Ham's F12. This medium was then placed on the cells 24 h before the experiment. For some experiments, the virus-containing medium was removed from the cells 5 h after transduction, the cells were washed with PBS, and new medium was added.

4.3 Chemicals

The chemicals used in the experiments are listed in Appendix I (Tables 1 and 2).

4.4 Phospholipase D assay (I)

PLD cleaves PC to PA and choline by hydrolysis. Water is normally added (as in each hydrolysis), but PLD prefers primary alcohols, such as 1-butanol by 1000-fold, if available. In this reaction, the products are choline and a phosphatidylalcohol instead of PA. PA can be quickly metabolized, and it is also made by other enzyme cascades, and the PA level may therefore be a poor measure of PLD activity, while phosphatidylalcohol is solely formed by PLD. Phosphatidylalcohols are also dead-end products, allowing cumulative detection (reviewed in Morris *et al.*, 1997).

For the PLD assay, cells on 6-well plates were labeled with ^{14}C -oleic acid by mixing the label in fetal calf serum and adding this to serum-free medium. The normal cell medium was then replaced with this labeling medium 16 h prior to the experiment. After labeling, the medium was replaced with normal Ham's F12, and the cells were allowed to settle for 60 min. After this, the medium was removed and replaced by medium containing the tested inhibitors (if used), and the cells were incubated for a further 30 min (except for GGTI-2133, which was added 16 h prior to the experiment together with the ^{14}C -oleic acid label). All incubations were carried out at 37 °C. The preincubation medium (with inhibitors) was then removed and replaced by the activation medium [Ham's F12 or Hepes buffered medium (HBM) with 0.3% (v/v) 1-butanol] containing inhibitors (if used) and stimulators. Cells were stimulated for 30 min at 37 °C, after which the reaction was terminated by replacing the medium with 300 μl of ice-cold methanol and placing the plate on ice. The cells were then scraped off and collected in Eppendorf tubes. The wells were then washed with another 300 μl of methanol, which was combined in the same tubes with the first set. Lipids were next extracted from these samples before continuing to TLC separation (4.7).

4.5 Arachidonic acid and 2-arachidonoylglycerol release (II)

For the measurement of AA and 2-AG release, the cells (CHO-hOX₁, neuro-2a-hOX₁, PC12-hOX₁, and HEK-293 transiently transfected to express hOX₁) were grown on 6-well plates. Cells were labeled with ^{14}C -AA as described for ^{14}C -oleic acid (4.4) 16 h prior to the experiment. The experiment was started by washing the cells twice with HBM supplemented with stripped bovine serum albumin (S-BSA; 2.4 mg/ml). The cells were then stimulated with orexin-A for 7 min at 37 °C, after which the supernatant (1000 μl) was collected in Eppendorf tubes and centrifuged briefly in order to remove detached cells. Next, 800 μl of the supernatant was collected for lipid extraction.

4.6 Lipid extraction (I–II)

Lipid extraction was used in both the PLD assay and AA and 2-AG release assays. Modified Bligh and Dyer protocols were used (Bligh and Dyer, 1959). For the PLD

assay (4.4), the lipids were extracted from the cells, whereas for the AA and 2-AG release assay (4.5) they were extracted from the supernatant.

4.6.1 Extraction from cells (I)

For the PLD assay, the cells were scraped from the wells into methanol and collected in Eppendorf tubes in 600 μ l of methanol (scraping volume plus washing volume). Next, 500 μ l of chloroform was added to the samples and they were vortex-mixed and incubated for 15 min at room temperature (RT). After this, 400 μ l of water was added to the samples, they were vortex-mixed again and centrifuged (5 min, 13500 g, RT). The upper phase was then removed and the lower phase containing the lipids was dried under a stream of nitrogen.

4.6.2 Extraction from supernatant (II)

For the AA and 2-AG release assay, supernatants from the centrifuged samples (800 μ l) were collected in Kimax tubes, followed by the addition of 2 ml of methanol and 1 ml of chloroform. The tubes were thoroughly shaken, and 1 ml of water and 1 ml of chloroform were then added. The tubes were shaken again and then centrifuged (5 min, 500 g, RT). The lower phase was collected and dried under a stream of nitrogen.

4.7 Thin-layer chromatography (I–II)

The dried lipid extracts were dissolved in 19:1 chloroform:methanol (PLD assay) or pure chloroform (AA and 2AG assay) and small amounts of non-labeled lipid standards (PtdBut for the PLD assay, and AA and 2-AG for the AA and 2-AG release assay) were added to the samples or applied separately on the TLC plate. The samples were applied on TLC plates that had been dried at 110 °C for 1 h. For the PLD assay, plates were pre-treated with 1% (w/v) K⁺ oxalate in 2:3 (v/v) methanol:water before drying. The PLD assay plates were developed in an unlined chromatography chamber with an organic phase of ethyl acetate:isooctane:acetic acid:water (11:5:2:10). For the AA and 2-AG release assay, the plates were developed in ethyl acetate:methanol (90:10) in a chromatography chamber lined with filter paper. An imaging plate (BAS-MS, Fujifilm, Tokyo, Japan) was exposed overnight with the vacuum-dried TLC plates. The exposed plate was scanned with a FLA 5100 scanner (Fujifilm) and the band intensities were quantitated with Nikon NIS-Elements AR (Nikon, Tokyo, Japan). The amount of the ¹⁴C-label in the bands was also measured by scraping off the bands, humified with water, into scintillation tubes, adding scintillation cocktail (Hisafe 3, Wallac-PerkinElmer, Turku, Finland), and measuring with a liquid scintillation counter (Wallac 1414). The bands were identified with the help of the non-labeled lipid standards made visible on the TLC plate with iodine vapor.

4.8 ³H-overflow from cells labeled with ³H-arachidonic acid and ³H-oleic acid (II)

In study II, the release of radiolabeled lipid products from cultured cells was first measured without distinguishing between the different lipid species. This was done by seeding the cells on 24-well plates and labeling them 16 h prior to the experiment with either ³H-AA or ³H-OA. The following day, the cells were washed twice with HBM supplemented with S-BSA (2.4 mg/ml), and then stimulated with orexin-A in 250 µl HBM with S-BSA for 7 min at 37 °C. Next, 200 µl of this stimulation medium was collected in Eppendorf tubes on ice and centrifuged (1.5 min, 16 000 g, +4 °C). After this, 100 µl of each sample was transferred into a scintillation tube and counted (4.7).

4.9 Phospholipase C assay (I–II)

PLC activity was measured by determining the cumulative amount of inositol phosphates produced in cells upon orexin receptor activation. The cells were grown on 24-well plates and labeled with ³H-inositol 20 h prior to the experiment by adding the label into the growth medium. Before the experiment, the cells were washed with HBM. HBM containing 10 mM LiCl (inhibitor of inositol monophosphatase) was added and the cells were incubated at 37 °C for 10 min. After this, the cells were stimulated with orexin-A for another 20–30 min at 37 °C. The reactions were stopped by rapidly replacing the medium with 200 µl of 0.4 M ice-cold perchloric acid, followed by freezing of the plate. After thawing of the samples, 100 µl of neutralizing medium (0.36 M KOH, 0.3 M KHCO₃) was added, and the insoluble fragments were spun down (10 min, 1100 g, +4 °C). Anion-exchange chromatography was used to isolate inositol phosphates from the supernatant. The amount of inositol phosphates was determined by liquid scintillation counting (4.7).

4.10 Adenylyl cyclase assay (I–II)

AC activity was determined in papers I, II and III in different formats (96- and 24-well plates) and with some variation with regard to the amounts of inhibitor or activation solutions and activation times. Therefore, the method is presented here without these details. Cells were labeled with ³H-adenosine 2 h prior to the experiment, washed with PBS, and incubated in Ham's F12 containing 500 µM IBMX (1-methyl-3-isobutylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase) for 10 min at 37 °C. Activators were then added for 10 min. The reaction was stopped by replacing the activation medium with 0.33 M perchloric acid and freezing the cells. The thawed samples were centrifuged (10 min, 1100 g, RT) and the supernatants were placed in chromatography columns to separate the ³H-ATP+³H-ADP from ³H-cAMP fractions with sequential Dowex/alumina

chromatography. The amount of radioactivity in the fractions was determined by liquid scintillation counting (4.7).

4.11 Ca²⁺ measurements (II)

For Ca²⁺ measurements, the cells were grown on glass coverslips coated with polyethylene imine to reduce detachment of the cells during the experiment. The cells were loaded with the Ca²⁺ indicator fura-2 acetoxymethyl ester (4 μM) in HBM with 1 mM probenecid for 20 min at 37 °C. The coverslips were then rinsed with HBM and placed into an imaging chamber with constant perfusion at 35 °C. When inhibitors were used, the cells were pre-incubated with them, and the inhibitors were also included in the perfusion medium throughout the experiment. Ca²⁺ responses were measured by exciting the cells with light rapidly alternating between 340 and 380 nm and collecting the emitted light through a 400-nm dichroic mirror and a 450-nm long-pass filter. The measurements were performed with a Nikon TE 2000 fluorescence microscope and an Andor iXon 885 electron-multiplying charge-coupled device (EM-CCD) camera under the control of Nikon NIS-Elements software.

4.12 ERK phosphorylation assay (III)

Cho-hCB₁ cells were grown on 6-well plates and transduced with recombinant baculovirus vectors with hOX₁-GFP or only GFP (negative control). Five hours later, the virus-containing transduction medium was removed, cells were washed with PBS and left overnight in serum-free Ham's F12 medium. Inhibitors were added to the cells in the serum-free medium 30 min prior to stimulation. The cells were stimulated with orexin-A or HU210 for 10 min and then washed with ice-cold PBS on ice and rapidly lysed with 100 μl of lysis buffer [50 mM Hepes, 150 mM NaCl (pH 7.5) + 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Na¹-pyrophosphate, 1 mM Na¹-orthovanadate, 10 mM NaF, 250 mM *p*-nitrophenol phosphate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and PhosSTOP phosphatase inhibitor cocktail (Roche)]. The samples were collected in Eppendorf tubes, briefly centrifuged (2 min, 13 500 g, RT) and the supernatant was collected. From the supernatant, a sample with 10 μg of protein was collected, boiled in Laemmli buffer, and separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel by electrophoresis. After separation, the proteins were transferred from the gel to a nitrocellulose membrane. The membrane was then washed with PBS and blocked with Odyssey blocking buffer (LI-COR bioscience, Lincoln, NE, USA):PBS (50:50) for 1 h. After blocking, the membrane was incubated with primary antibodies [rabbit anti-active MAPK IgG (Promega) and mouse p44/42 MAPK (ERK1/2) IgG (Cell Signaling Technology, Danvers, MA) in a 50:50 mixture of Odyssey blocking buffer and PBS] overnight at 4 °C with gentle shaking. On the next day, the membrane was washed with PBS (3 times 10 min) and incubated with secondary antibodies (in a 50:50 mixture of Odyssey blocking buffer and PBS) for 60 min at RT, protected from light. The secondary antibodies were Dylight 800-

conjugated goat anti-rabbit IgG and 680-conjugated goat anti-mouse IgG antibodies (LI-COR). After the incubation, the membrane was kept protected from light and washed with PBS (3 times 10 min). The bands from the membrane were then detected with an Odyssey Infrared Imaging system (LI-COR) and the band intensities were quantitated with Nikon NIS-Elements software.

4.13 Luciferase assay (III)

The activation of ERK can be detected by following it downstream to the transcription factor Elk-1. The pSG-GalElk-1 plasmid codes for a fusion protein of the DNA-binding domain of the yeast transcription factor Gal4 and the transactivation domain of Elk-1 (Kortenjann M, 1994). In our ERK luciferase assay, this fusion plasmid was transfected to the cells together with pGL3 G5 E4 Δ38, which contains five binding domains for Gal4 that control the expression of firefly luciferase (Kamano *et al.*, 1995). When ERK is activated, it phosphorylates Elk-1, which then leads to the expression of firefly luciferase. Firefly luciferase activity is measured to determine the activity of ERK. The cells are also transfected with the pRL-TK plasmid, which contains *Renilla reniformis* luciferase under the control of thymidine kinase promoter from herpes simplex virus. *Renilla* luciferase activity is used for normalizing the firefly luciferase signal.

The cells were grown on 96-well plates, transfected with the plasmids described above, accompanied with receptor plasmids for hOX₁, hCB₁ or M₁. If no receptor plasmids were used, an empty vector was used to keep the DNA amounts equal. Five hours after the transfection, the cells were washed with phosphate buffered saline (PBS) and left in serum-free medium overnight. On the following day, inhibitors were added in serum-free medium, and the cells were stimulated 30 min later with orexin-A, HU210 or 2-AG for 5 h. After the stimulation, the cells were lysed with Passive Lysis Buffer (from the Dual Luciferase Assay Kit; Promega) for 15 min, and the samples were collected and briefly centrifuged. The luminescence from both luciferases (activated by adding their substrates; Dual Luciferase Assay Kit) was sequentially measured from the supernatants with a Glomax 20/20 luminometer (Promega) according to the manufacturer's instructions.

4.14 Cell–cell communication assay (II–III)

The capacity of the 2-AG released after OX₁ activation to activate nearby cells expressing CB₁ receptors was investigated in papers II and III by two different means (Figure 5). Modulation of AC activity and activation of Elk-1 were measured in an artificial cell–cell communication assay.

CB₁ receptors couple to G_i proteins, which inhibit AC activity and therefore cAMP production. In the reporter assay, CHO-hCB₁ cells were labeled with ³H-adenosine for 2 h, washed with PBS and detached with PBS containing EDTA. Detached cells were spun down and resuspended in Ham's F12 medium containing 500 μM IBMX. After 10 min of incubation at 37 °C, the cells were placed on top of

CHO-hOX₁ cells grown on a 24-well plate. The plates were then gently centrifuged (3 min, 100 g, RT) and were left at 37 °C for 10 min to settle. The cells were then stimulated with forskolin combined with orexin-A and HU210 for 7 min. The rest of the experiment was performed as described for the AC assay (4.10). In this experiment, the measured cAMP signal came from the CHO-hCB₁ cells, because only these were labeled with tritium.

A similar principle was used in studying Elk1 activation. CHO-hCB₁ cells were transfected as described previously for the luciferase assay. Five hours after the transfection, CHO-hCB₁ cells were detached and mixed with detached CHO-hOX₁ cells. Both cell types were then plated on a 24-well plate in normal growth medium. About 24 h after plating the cells, the medium was replaced with serum-free medium and the cells were serum-starved overnight. The following day, the cells were treated as described for the luciferase assay (4.13).

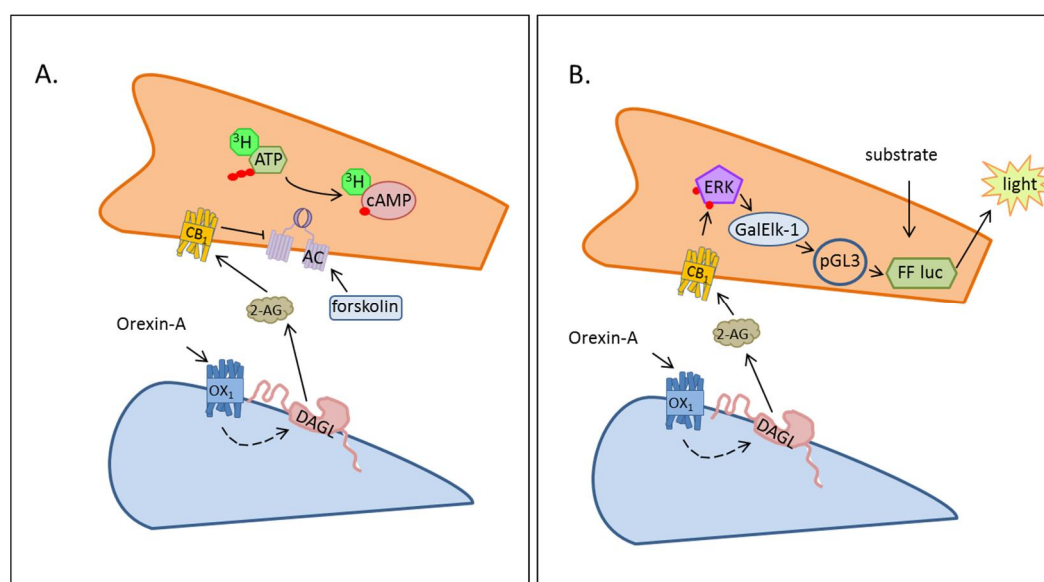


Figure 5. A schematic illustration of the cell–cell communication assay. OX₁ activation-induced release of 2-arachidonoylglycerol (2-AG) leads to activation of CB₁ receptors in the neighboring cells. Activated CB₁ receptors then modulate adenylyl cyclase (AC) activity (A) and activate the extracellular signal-regulated kinase (ERK) pathway (B). ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAGL, diacylglycerol lipase; FF luc, firefly luciferase; GalElk1, fusion plasmid of Gal4 and Elk1 transcription factor; pGL3, plasmid with Gal4 binding sites, controls FF luc expression.

4.15 Receptor binding

Expression levels of recombinant receptors were determined with radioligand binding assays. ¹²⁵I-orexin-A was used for OX₁ receptors and ³H-SR141716 for CB₁ receptors. For OX₁ expression studies, CHO-hOX₁ cells (transfected with constructs used in the Elk-1 luciferase assay) or CHO-hCB₁ cells transfected with OX₁ (and constructs used in the Elk-1 luciferase assay to make the results comparable) were

used. Twenty-four hours after the transfection, the cells were detached, spun down, and resuspended in HBM containing 0.1% (w/v) S-BSA. The cells were then placed in low-protein-binding Eppendorf tubes with ^{125}I -orexin-A for 10 min. Nonspecific binding was determined by preincubating the cells with 10 μM SB-334867 before adding ^{125}I -orexin-A. The cells were then rapidly spun down (30 s, 14 000 g, +2 °C), the pellet was washed superficially with ice-cold HBM and then resuspended in HBM, and the radioactivity measured with a Wallac Wizard 1480 gamma counter. To determine the expression levels of CB_1 receptors, the cells were plated on 48-well plates coated with polyethylene imine. Similar transfections as for OX_1 expression studies were carried out, and the cells were used 24 h after transfection. The cells were washed with HBM, and incubated in HBM containing 0.1 % (w/v) S-BSA and ^3H -SR141716. The nonspecific binding was determined with 10 μM HU210. After a 60-min incubation at RT, the plate was placed on ice and the cells were washed twice with ice-cold HBM. The cells were then lysed with NaOH and collected in scintillation tubes for scintillation counting (4.7).

4.16 Bioluminescence resonance energy transfer (BRET²) assay (IV)

Dimerization of OX_1 , OX_2 and CB_1 receptors was studied using the bioluminescence resonance energy transfer (BRET²) method. In this method, cells are transfected with fusion constructs of *Renilla* luciferase (Rluc) or green fluorescent protein 2 (GFP²) and the receptors of interest. When a receptor tagged with Rluc comes in close proximity to a GFP²-tagged receptor, and a substrate for Rluc is present, the light energy of Rluc may excite the nearby GFP², producing a signal. These signals can be measured, and the specific GFP² signal divided by the specific luminescence is called the BRET ratio. CHO control cells were grown on 6-well plates and transfected with different ratios of the two constructs. The amount of Rluc construct DNA was kept constant, while GFP² was varied. An empty vector was used to keep the total amount of DNA constant. After 24 h, the cells were detached with PBS containing EDTA, spun down and resuspended in HBM. The cell suspensions were dispensed on white 384-well plates and the plates placed in a Pherastar FS instrument (BMG, Labtech GmbH). The measurements were performed at RT, using the BRET² optic module. *Renilla* luciferase substrate coelenterazine 400a was added to the cells in a 5 μM final concentration with an automated dispenser.

4.17 Data analysis and statistical procedures (I–IV)

The results are presented as mean \pm S.E.M.; N refers to the number of batches of cells. The number of parallel samples differed between experiments. In WB (III) and TLC (II) experiments, it was two, and in AC, PLC (I-II) and the luciferase assay experiments (III), it was three or four. ^3H -overflow experiments (II) were conducted with six and Ca^{2+} -imaging experiments (II) with 30 or more parallel samples. Each experiment was repeated at least three times.

All pairwise comparisons were carried out with Student's paired or non-paired two-tailed *t*-tests with Bonferroni's correction (I–IV), except for the Ca²⁺-imaging experiments (II), where the χ^2 test was used for corresponding cell counts. The *F*-test was used when comparing different models for concentration–response curves (I). Statistical significance is indicated as follows: ns (not significant), **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Nonlinear curve-fitting and determination of EC₅₀ values were performed with Microsoft Excel (I–III).

5. Results and Discussion

5.1 Phospholipase D activation (Paper I)

There has been indirect evidence for PLD action upon OX₁ receptor activation (Johansson *et al.*, 2008). In paper I, we utilized the PtdBut generation assay (4.4) to directly show that PLD is potently activated upon OX₁ receptor stimulation. Orexin-A was a 10 times more potent activator than orexin-B, as expected for OX₁.

With the help of PLD1 and PLD2 inhibitors, we determined that the PLD isoform responsible for PtdBut generation was PLD1. The employed PLD2 inhibitor also showed some inhibition of the PtdBut response when used at a high concentration (1 μ M). This inhibition was stronger for 100 nM orexin-A than for 1 nM. At high orexin concentrations, PLD1 activation would produce significant amounts of PA, leading to the activation of PIP5K, and an increased amount of PIP₂. PIP₂ would then activate PLD2, offering an explanation for the finding. However, high concentrations of the PLD2 inhibitor also inhibit PLD1 activity (Scott *et al.*, 2009), and thus the finding may also be due to PLD1 inhibition.

Next, we aimed to determine what lies upstream of PLD1 activation. PLD1 can be activated by various factors. PIP₂ increases the activity of both PLD1 and PLD2, but more importantly, it is required for their membrane localization and as a cofactor (Brown *et al.*, 1993; Liscovitch *et al.*, 1994). PA, the product of PLD1, can activate PIP5K, which leads to PIP₂ production and therefore creates a positive feedback loop (Jenkins *et al.*, 1994). Many other factors regulate PLD activation. As described in 2.3.4, various PKC subtypes and small G-proteins of the ARF and Rho families can activate PLD1 (Henage *et al.*, 2006). In previous studies, OX₁ receptors in CHO cells have been shown to utilize nPKC but not cPKC for signaling to AC (Holmqvist *et al.*, 2005). Of the nPKC isoforms, CHO cells were reported to express PKC ϵ and δ , (Hill *et al.*, 2003; Holmqvist *et al.*, 2005), and to be able to activate both of them, as assessed from the translocation of recombinant isoforms (Holmqvist *et al.*, 2005). Holmqvist *et al.* (2005) suggested, however, that the isoform utilized in signaling to AC was PKC δ , but not PKC ϵ . Direct activation of PKC (likely both conventional and novel isoforms) with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) strongly activated PLD1 in CHO cells. We therefore used inhibitors of PKC subclasses to determine whether PKC was also involved in OX₁ signaling to PLD. Gö6976 (an inhibitor of cPKCs and PKD) and GF109203X (an inhibitor of both novel and classical PKCs) were used, but only GF109203X showed potent inhibition of both TPA and orexin responses, while Gö6976 showed only very weak inhibition when used at a high concentration (10 μ M).

We further studied the involvement of different PKC subclasses in PLD activation in CHO cells. For this, we utilized small peptide activators and inhibitors. For PKC regulation, these peptides utilize the PKC-binding domains of receptors for activated C kinase (RACKs). In some systems, the membrane permeation peptides alone have been reported to inhibit the action of PKC and PKA (Ekokoski *et al.*,

2010), but we did not observe this effect. An activator of cPKC (KAC1-1) did not produce any PLD stimulation, while activators of PKC ϵ (KAE1-1) and PKC δ (KAD1-1) activated PLD. Furthermore, the activity produced by KAE1-1 could be abolished by an inhibitor of PKC ϵ (KIE1-1), which had no effect, however, on PLD activation induced by orexin-A or TPA. Thus, PKC δ emerged as the more interesting of the nPKC isoforms. Unfortunately, a specific peptide inhibitor of PKC δ was not commercially available. Consequently, we used rottlerin as a PKC δ inhibitor, although we were aware that it is not a very selective or good inhibitor (Soltoff, 2007). Rottlerin produced a clear concentration-dependent inhibition of PLD activation, supporting the notion that PLD was activated by PKC δ .

Gö6976 also inhibits PKD, and according to one study, PKD1 and PKD3 are activated by OX₁ (Peltonen *et al.*, 2010). We tested with dominant-negative PKD constructs whether the small amount of inhibition by Gö6976 could be due to inhibition of PKD, but the dominant-negative constructs had no effect on PLD activation.

Small G-proteins of the Rho family can activate PLD1 (reviewed in Frohman *et al.*, 1999). Furthermore, many GPCRs, for example the M₃ muscarinic acetylcholine receptor, utilize Rho-family G-proteins to activate PLD (Schmidt *et al.*, 1996). Therefore, we tested whether inhibition of geranylgeranyl transferase with GGTI-2133, and subsequently the maturation and attachment of Rho family G-proteins to the cell membrane, would have an effect on OX₁ receptor-mediated PLD activation. The cells were incubated with GGTI-2133 24 h prior to the experiment to inhibit the isoprenylation of Rho family G-proteins in the early phase. Clear changes in the shape of the cells were observed after 24 h, indicating an effect by the inhibitor. Due to concerns about cell viability with longer incubations, the incubation time was kept at 24 h. No effect on PLD activity upon OX₁ stimulation was seen with a 100 nM concentration of GGTI-2133. A concentration of 1 μ M tended to evoke some, statistically non-significant inhibition, but to be certain that these G-proteins were not involved, we utilized more specific inhibitor constructs of Rho family G-proteins. None of these constructs showed any inhibition of the PLD response. Rho family G-proteins do not therefore appear to be involved in OX₁ receptor-induced PLD activation.

A major question is how the nPKC/PKC δ activation by orexin receptors takes place. nPKCs are Ca²⁺-insensitive, but are instead activated by DAG and phosphorylation of either serine/threonine or tyrosine residues (reviewed in Zhao *et al.*, 2012). An additional means of activating PKC δ is proteolytic cleavage by caspase-3, but this is mainly associated with apoptosis (Ghayur *et al.*, 1996; reviewed in Zhao *et al.*, 2012). We aimed to test whether other OX₁ signaling pathways, such as PLC or PLA₂, were involved in activating PLD. Both of these pathways could activate PKC, PLC by producing DAG and PLA₂ by producing AA (Shirai *et al.*, 1998). U-73122 was used to inhibit PLC and methyl arachidonyl fluorophosphonate (MAFP) to inhibit PLA₂, but neither of them had any effect on PLD activation by orexin-A. Therefore, the nPKC activation occurs via some other pathway. As mentioned above, PKC δ is activated by phosphorylation of many sites, including

various tyrosine residues (reviewed in Steinberg, 2004). Kinases involved in this include Src family kinases, and OX₁ receptors have been suggested to activate them (Ammoun *et al.*, 2006a; Voisin *et al.*, 2008). This could be one possible pathway for OX₁ receptors to activate PKC δ , but it has not yet been demonstrated.

Rhodopsin family GPCRs, such as M₃ muscarinic, H₁ histamine, and B₂ bradykinin receptors, have been reported to activate PLD via Rho and Arf family G-proteins in 1321N1 human astrocytoma cells (Rumenapp *et al.*, 1995; Schmidt *et al.*, 1996; Mitchell *et al.*, 1998). As mentioned in 2.2.2, OX₁ receptors also belong to the rhodopsin family of GPCRs, and like M₃ are strongly G_q-coupled, even though OX₁ can also couple to other heterotrimeric G-proteins. It is therefore rather interesting that while M₃ activates PLD via Rho and Arf, these small G-proteins do not appear to be involved in the OX₁-mediated activation of PLD. In one study, it was reported that M₃ receptors activated PLD via R-Ras in HEK-293 cells, and that the R-Ras activation was driven by cAMP via EPAC (Lopez De Jesus *et al.*, 2006). We tested the effect of increasing cAMP levels by activating AC with forskolin, but it had no effect on PLD; therefore, it appears that this mechanism is not involved in OX₁-mediated PLD activation in CHO cells. OX₁ receptors also only activate AC at such high orexin concentrations that involvement of cAMP would not have been plausible here. OX₁ receptors differ from the GPCRs mentioned above, because PLD activation appears to solely rely on PKC δ , while other GPCRs often utilize multiple pathways to activate PLD. These experiments have, however, been performed in different types of host cells, which probably also affects the signaling. The signaling of OX₁ receptors can vary according to the cells type, as is seen, for example, in Paper II, for 2-AG and AA production (5.2).

OX₁ receptors potentially activate PLD, i.e. in the same orexin-A concentration range as PLC in CHO cells, but the physiological significance of this activation is not clear. PA, the product of PLD activity, is a potent lipid messenger and also easily converted into other potent messengers, DAG or LPA. These lipids have a plethora of targets in cells. Probably one of the best studied PA targets is the mammalian target of the rapamycin (mTOR) complex, which is stabilized by PA, (reviewed in Foster, 2009). This complex is known to be anti-apoptotic and to promote cell survival and proliferation. Orexin-A has been shown to induce apoptosis (Rouet-Benzineb *et al.*, 2004; Ammoun *et al.*, 2006b), but also to enhance the proliferation of neurons in the dentate gyrus (Ito *et al.*, 2008) and 3T3-L1 preadipocytes (Skrzypski *et al.*, 2012). In 3T3-L1 preadipocytes, this proliferative effect was mediated by ERK signaling, but the activation of mTOR following orexin-A exposure has not been studied. Therefore, it would be interesting to investigate whether mTOR complexes are activated upon OX₁ activation, and whether they are involved in orexin-A-mediated cell proliferation.

In summary, OX₁ potentially activates PLD1 in CHO cells. This solely relies on an nPKC, probably PKC δ (Figure 6). The activation mechanism of this PKC is unclear, but it (or PLD) does not require AC, PLC, PLA₂, Rho family GTPase, G $\beta\gamma$, or PKD activity. The physiological significance of OX₁ signaling to PLD has not yet been investigated.

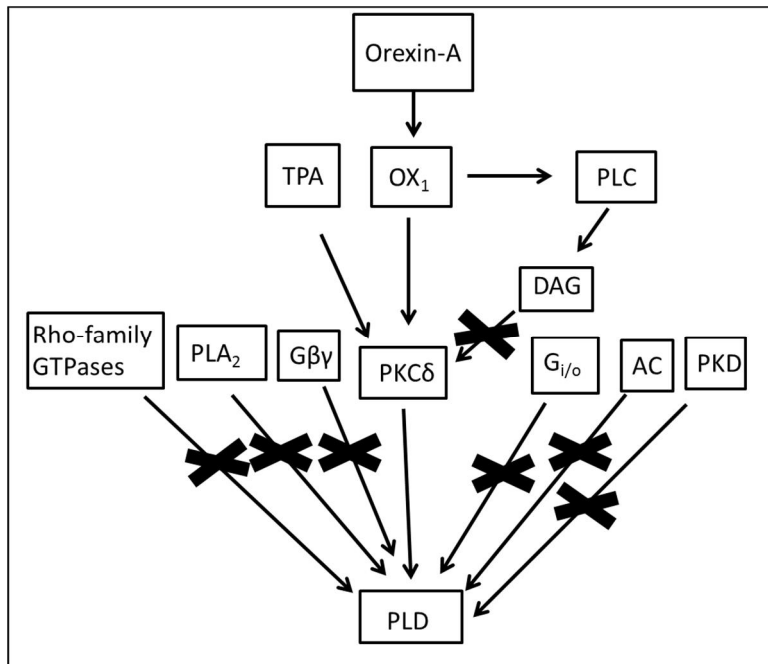


Figure 6. Investigated pathways for OX_1 induced phospholipase D (PLD) activation. Crosses on top of the arrows indicate that the pathway is not involved in OX_1 induced activation of PLD. AC, adenylyl cyclase; DAG, diacylglycerol; $PKC\delta$, protein kinase C δ ; PKD, protein kinase D; PLC, phospholipase C; PLD, phospholipase D.

5.2 AA and 2-AG release (Paper II)

Turunen *et al.* (2010) reported that when 3H -AA-labeled CHO-h OX_1 cells were stimulated with orexin-A, abundant release of the 3H -label into the medium was observed. This indicates the activation of a lipid-signaling cascade, which was interpreted as PLA_2 activity. However, the identity of the released lipid (or other molecular) species was not determined. In Paper II, we examined this issue further with more selective pharmacological inhibitors and by determining the identity of the released lipid species.

TLC separation of the lipid species released from ^{14}C -AA-labeled cells revealed two prominent ^{14}C -labeled bands, which migrated at the same speed as non-labeled reference standards of AA and 2-AG. There is one pathway mainly responsible for 2-AG production, as described in 2.3.4, and it includes PLC and DAGL. PLA_2 activation, on the other hand, is known to lead to AA release, and this pathway was probably also involved here. Indeed, when applied together, the inhibitors of DAGL and c $PLA_2\alpha/\zeta$, THL (Bisogno *et al.*, 2006) and pyrrophenone (PP) (Seno *et al.*, 2000; Ghomashchi *et al.*, 2001), fully blocked the release of both AA and 2-AG. THL alone entirely abolished the 2-AG release, but also a part of the AA release. PP inhibited a part of the AA release, while it had no effect on 2-AG release. We thus concluded that 2-AG release was solely dependent on DAGL activation, while both AA produced by c $PLA_2\alpha$ or ζ activity and AA formed by

hydrolysis of 2-AG contributed to the observed release of AA (Figure 7). We utilized these inhibitors in the previously used "³H-overflow" method (4.8)(Turunen *et al.*, 2010), and observed that the release of the ³H-label was completely abolished by co-application of THL and PP. Upon analysis of the concentration–response curves of orexin-A and the impacts of the inhibitors, in both TLC separation and ³H-overflow experiments, it appears that cPLA₂ (pEC₅₀ ≈ 9.3 from ³H-overflow, and pEC₅₀ ≈ 9.8 from TLC separation) is activated more potently than DAGL (pEC₅₀ ≈ 8.4 from ³H-overflow, and pEC₅₀ ≈ 8.3 from TLC separation).

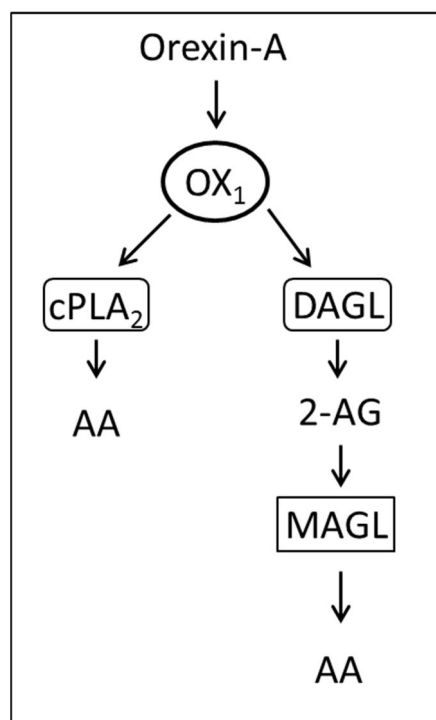


Figure 7. Pathways producing arachidonic acid (AA) upon activation of OX₁ orexin receptors by orexin-A in recombinant CHO-hOX₁ cells. 2-AG, 2-arachidonoylglycerol; cPLA₂, Ca²⁺-dependent/cytosolic phospholipase A₂; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase.

DAG, the substrate of DAGL, can be derived from either PLC action or from PLD-produced PA. The concentration–response curves for PLD1 and PLC are very similar (Paper I, Figure 8), and this does not therefore suggest either pathway. Therefore, we utilized a PLD1 inhibitor and the PLC inhibitor U73122 to determine which of these enzymes is involved. The PLD1 inhibitor showed only minor inhibition of ³H-overflow, while PLC inhibition blocked it completely. Unfortunately, the PLC inhibitor U73122 is not very specific (Taylor and Broad, 1998), but no better alternatives are available for inhibiting PLC. However, the PLD1 inhibitor used is both specific and potent (Paper I), and thus it appears reliable to conclude that the PLC pathway is the one producing DAG in this case. This is also reported to be the pathway producing 2-AG in the ventrolateral PAG of the rat brain (Ho *et al.*, 2011).

When CHO cells were labeled with ^3H -oleic acid instead of ^3H -AA, the ^3H -overflow induced by OX_1 activation was much smaller and less potent. PP had no effect on this ^3H -overflow, while THL inhibited it completely. The enzyme responsible for the effect in ^3H -OA-labeled cells therefore appears to be DAGL, while cPLA₂ is not involved. This would suggest that the cPLA₂ species involved in AA release is cPLA₂ α and not cPLA₂ ζ , as the α isoform is highly AA-specific (reviewed in Ghosh *et al.*, 2006). Oleic acid may also mostly end up in the *sn1* position of a phospholipid and might not therefore be released by cPLA₂, which mostly acts on fatty acids in the *sn2* position (reviewed in Ghosh *et al.*, 2006).

We also studied OX_1 activation in more neuron-like cells: neuro-2a-hOX₁, PC12-hOX₁, and HEK293 cells. In neuro-2a and HEK-293 cells, stimulation with 100 nM orexin-A induced the release of 2-AG and AA. In these cells, unlike CHO cells, the release of both AA and 2-AG was fully blocked with THL, indicating that the AA released from these cells was the product of 2-AG hydrolysis, and not of cPLA₂ activity. In PC-12 cells, we could not see any response to orexin-A, but this may have been masked by strong basal release of AA and 2-AG. It appears that the ability of OX_1 receptors to evoke 2-AG release is dependent on the cell type, as is AA release. The 2-AG release is, however, a signaling pathway also utilized by other cell types besides CHO cells.

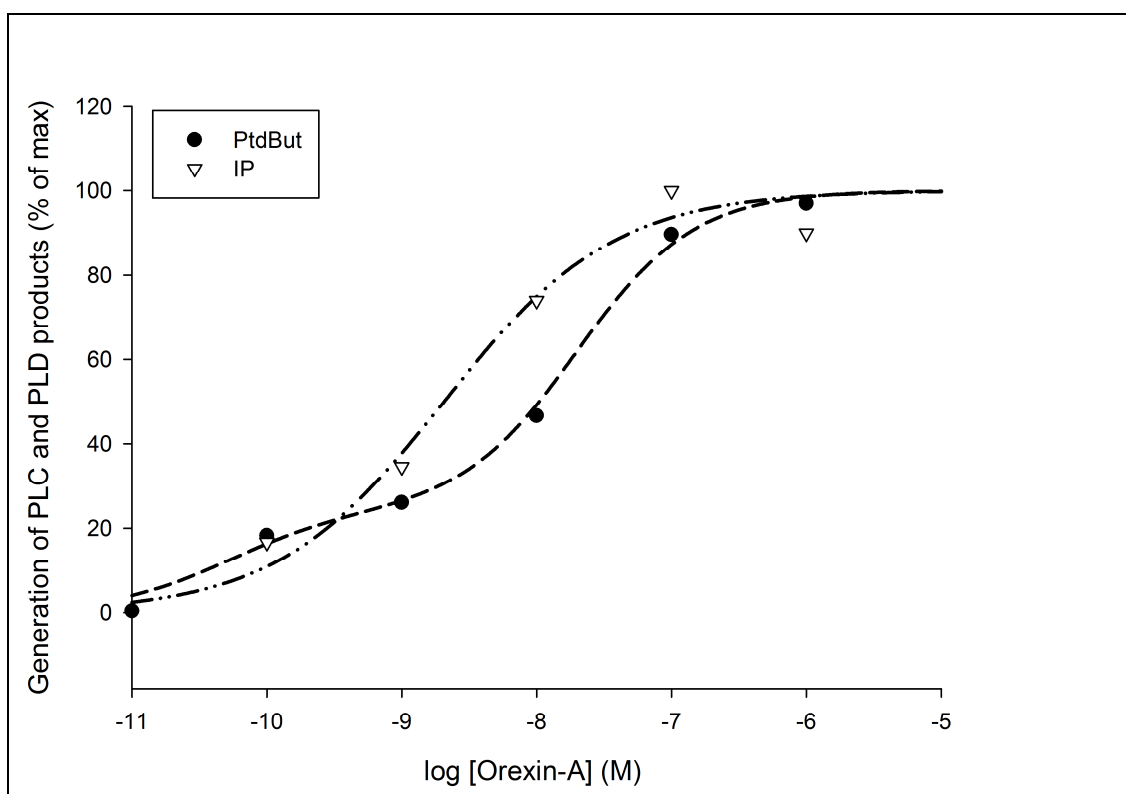


Figure 8. Activation of PLD and PLC. PLD activation is depicted by PtdBut generation, and PLC by IP production. Both enzymes are activated in the same orexin-A concentration range. Redrawn from Paper I.

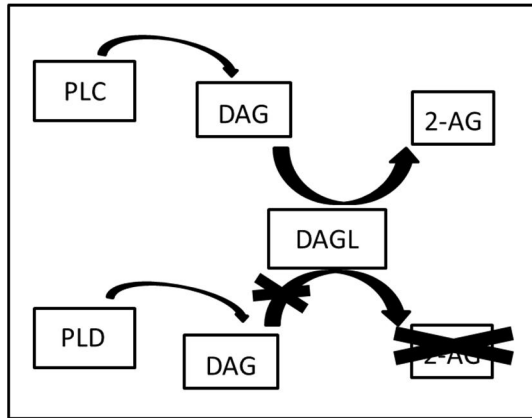


Figure 9. Pathway producing 2-arachidonoylglycerol (2-AG) upon OX₁ activation. Diacylglycerol (DAG) for 2-AG production comes solely from the action of phospholipase C.

5.3 Cellular actions of 2-AG from orexin receptor signaling

5.3.1 Paracrine signaling (Papers II and III)

We managed to show in Paper II that 2-AG was released into the extracellular medium from CHO-hOX₁ cells upon OX₁ stimulation. 2-AG should be able to act on CB₁ receptors, and we next wanted to examine whether this would occur in the classical paracrine manner (2.5.1) in our test system. For this purpose, we devised a cell–cell communication assay (4.14), modified from a method used to study nitric oxide production upon muscarinic receptor stimulation (Hu and el-Fakahany, 1993). We used CHO-hCB₁ cells to measure 2-AG release from CHO-hOX₁ cells by assessing the AC activity in CHO-hCB₁ cells. We first verified the ability of direct CB₁ stimulation to affect AC activity in CHO-hCB₁ cells. Exogenous 2-AG (and the synthetic agonist HU210) strongly and concentration-dependently inhibited AC activity (Paper II), as expected based on previous knowledge (Howlett, 1984). The effect was fully blocked by AM-251, verifying that the effect was solely mediated by CB₁ receptors.

We thus proceeded with the cell–cell communication assay. Briefly, the CHO-hCB₁ cells were labeled with ³H-adenine and plated together with CHO-hOX₁ cells. The cells were then stimulated with forskolin and orexin-A, and the cAMP responses were measured. Only the CHO-hCB₁ cells were labeled, and the responses measured would therefore solely originate from them. Orexin-A caused a clear concentration-dependent decrease in cAMP production in forskolin-stimulated CHO-hCB₁ cells (Paper II). The orexin-A-induced decrease in cAMP formation was inhibited by the OX₁ antagonist SB334867, the CB₁ antagonist/inverse agonist AM-251, and THL, which inhibits 2-AG production (Figure 10). This implies that the orexin-A-induced decrease in cAMP in CHO-hCB₁ cells is mediated by the OX₁ receptor-induced release of 2-AG from CHO-hOX₁ cells, which then acts on CB₁ receptors in neighboring CHO-hCB₁ cells (Figure 10). This fits the scheme of retrograde synaptic transmission, where the postsynaptic cell releases 2-AG, which then diffuses to the

presynaptic cell and acts on CB₁ receptors (reviewed in Kano *et al.*, 2009). The mechanism by which 2-AG exits the cells is debated, but there is some evidence for a transporter (Chicca *et al.*, 2012). If there is a transporter, its expression or absence from different cell types might also partly explain why 2-AG release is not seen in all cell types, such as PC-12-hOX₁, and why it is released with different potencies.

Both OX₁ and CB₁ receptors are also coupled to ERK activation in CHO cells, as seen in both the phosphorylation of ERK and downstream ERK activity by activation of the transcription factor Elk-1 (Bouaboula *et al.*, 1995; Ammoun *et al.*, 2003) (Paper III). We utilized a similar cell–cell communication assay (4.14) to explore paracrine 2-AG activity by measuring the Elk-1 activity in CHO-hCB₁ cells upon orexin stimulation of CHO-hOX₁ cells (Paper III). Orexin-A stimulated Elk-1 activity in a THL- and AM-251-sensitive manner (Paper III), similar to the AC assay (Paper II).

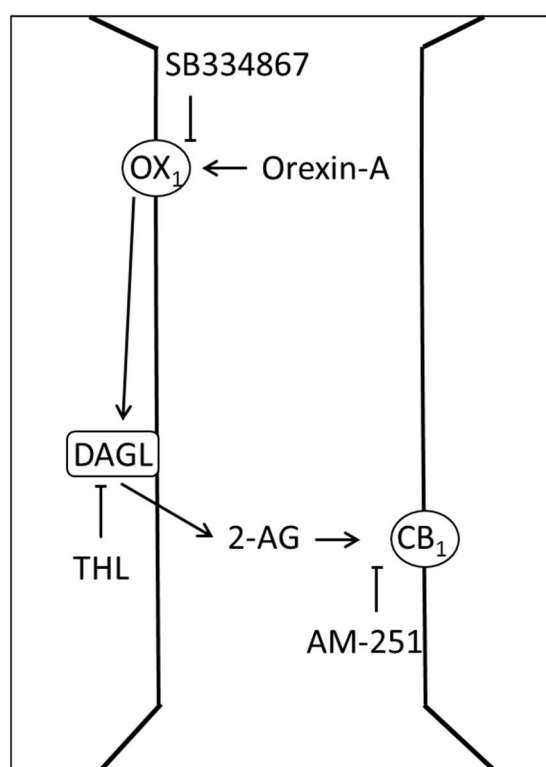


Figure 10. The sites of action of the inhibitors used in the cell–cell communication assay. A cell expressing OX₁ receptors is depicted on the left and a cell with CB₁ receptors on the right. 2-AG produced by OX₁ expressing cell crosses the space between the cells and binds to CB₁ receptor of the other cell. AM-251, CB₁ antagonist/inverse agonist; 2-AG, 2-arachidonoylglycerol; DAGL, diacylglycerol lipase; SB334867, OX₁ antagonist; THL, diacylglycerol lipase inhibitor.

5.3.2 Autocrine signaling

It was previously shown that co-expression of OX₁ and CB₁ receptors strongly potentiates OX₁ receptor signaling to ERK in CHO cells, and less prominently also in

HEK-293 cells (2.6)(Hilairret *et al.*, 2003; Ellis *et al.*, 2006). While the rather convincing results presented in these studies do not directly imply that these receptors are capable of forming heteromeric complexes, the production of 2-AG in these systems was not taken into account. Therefore, we aimed to investigate whether the potentiation of ERK signaling could be explained by the autocrine action of released 2-AG (Paper III).

We started by studying the potentiation of ERK phosphorylation in CHO-hCB₁ cells transduced to also express OX₁ receptors. When the cells with both receptors were activated with orexin-A, a strong ERK response was seen. The response was antagonized (EC₅₀ shifted more than 100-fold) by the CB₁ antagonist SR141716. This is in line with the observations of Hilairret *et al.* (2003). Inhibition of the production of 2-AG with THL, however, produced similar attenuation of the potentiation as SR141716, indicating that the potentiation requires DAGL activity, and thus is probably mediated by 2-AG acting on CB₁ receptors. As both OX₁ and CB₁ receptors are known to activate ERK (2.3.3, 2.5.1, Paper III), it appears logical that when both of these receptors are activated, there is more signaling to ERK, and therefore potentiated ERK phosphorylation, especially as the receptors are likely to utilize different signaling pathways due to their different G-protein coupling (2.2.2, 2.5.1). Our results imply that the released 2-AG can explain the potentiation of ERK signaling, and no receptor heteromerization is necessary.

The activity of ERK itself was studied utilizing an Elk-1-driven reporter assay (4.13). The results of these experiments supported the idea that the potentiation of ERK was due to the autocrine action of released 2-AG. In these experiments, we utilized AM-251 as a CB₁ antagonist, instead of SR141716, as an additional control. THL and AM-251 attenuated the potentiation of Elk-1 signaling in cells expressing both OX₁- and CB₁-receptors to a level similar with the control cells expressing only OX₁. The potentiation of Elk-1 activity was not as strong as that of ERK phosphorylation; the EC₅₀ values were shifted less than 10-fold when the receptors were co-expressed, compared to cells expressing only OX₁. Elk-1 activity was, however, measured after 5 hours of cumulative stimulation, while for ERK phosphorylation the stimulation time was only 10 min and the response is not cumulative. During the 5 hour stimulation, some of the phosphorylated ERK could well have undergone dephosphorylation before activating Elk-1, whereas during 10 min of activation, rapid phosphorylation of ERK was seen, but not the situation after the phosphorylation ceases. In addition, not all phosphorylated ERK targets Elk-1, but also other transcription factors (reviewed in Chang *et al.*, 2003), and some of the phosphorylated ERK remains in the cytoplasm and does not enter the nucleus at all (reviewed in Mebratu and Tesfaigzi, 2009). The phosphorylated ERK that targets transcription factors other than Elk-1, and also ERK that does not enter the nucleus at all, remain undetected in the luciferase assay.

We aimed to determine whether 2-AG release is utilized to a similar extent by other G_q-coupled receptors than OX₁ to signal to CB₁ receptors. Therefore, we transfected the CHO-hCB₁ cells to express human M₁ receptors that have also been reported to utilize endocannabinoids in their signaling (Fukudome *et al.*, 2004). M₁

receptor activation induced similar IP production as activation of OX₁ receptors. Although THL and AM-251 slightly shifted the concentration–response curve of oxotremorine-M (an M₁ agonist) with regard to Elk1 activation and reduced the maximum response, the effect was significantly smaller than for OX₁. OX₁ activation induces strong Ca²⁺ influx, probably stronger than M₁, which could lead to differences in 2-AG release if DAGL is responsive to Ca²⁺ elevation.

The cellular background affected the level of potentiation of Elk-1 signaling. When CHO-hCB₁ cells were transfected to co-express OX₁, the potentiation was slightly stronger than when CHO-hOX₁ cells were transfected to co-express CB₁. There were also differences in the maximal responses to agonists when the receptors were expressed in different backgrounds. In CHO-hCB₁ cells transfected with cDNA encoding OX₁, the maximal responses to orexin-A and the CB₁ agonist HU-210 were similar. In CHO-hOX₁ cells transfected with CB₁, on the other hand, the maximal response to orexin-A was significantly higher than that to HU-210. These differences could be explained by different expression levels and stoichiometry of the receptors, and we therefore decided to determine the expression levels of the receptors in these systems by radioligand binding. Transient expression of CB₁ receptors in CHO-hOX₁ cells or OX₁ receptors in CHO-hCB₁ cells did not affect the expression levels of stably expressed OX₁ and CB₁ receptors in these cells. The expression level of transiently expressed receptors was significantly lower than that of stably expressed receptors, which is not surprising. The results indicate that the interaction between OX₁ and CB₁ receptors is efficient over a wide range of receptor stoichiometries. However, it seems that a certain minimum expression level would be needed for each receptor type. A high enough level of OX₁ receptors would be needed to produce enough 2-AG for any significant stimulation of the CB₁ receptor pathway, and there should be a minimum number of CB₁ receptors to produce effective activation of their signal pathways. On the other hand, at the other ends of expression ranges, the effects may be physiologically saturating for the other components of the cascade (such as DAGL or 2-AG transport). It should be noted, however, that the expression levels of OX₁ receptors cannot be reliably assessed by commercial tools, since receptor quantitation with ¹²⁵I-orexin-A binding suffers from many limitations (Kukkonen, 2013b).

The results of paper III further supported the view that the 2-AG released upon OX₁ activation can activate neighboring cells, but in addition has an autocrine action in cells also expressing CB₁ receptors. Our results challenge the idea that the potentiation of OX₁ signaling would require heteromerization with CB₁ receptors (Hilairret *et al.*, 2003; Ellis *et al.*, 2006). A functional interaction between these two receptors is a more plausible explanation. However, heteromerization of these receptors could provide more efficient signaling from OX₁ receptors to CB₁, as it would bring the receptors closer together, and perhaps make the diffusion distance for released 2-AG as short as possible. We also showed that another GPCR, the M₁ muscarinic receptor, did not produce as strong potentiation of ERK signaling as OX₁. This might be due to differences in the ability of these receptors to activate the production of 2-AG, but this could also reflect their different ability to form

complexes with CB₁. Further experiments are required to assess the possible role of receptor-operated Ca²⁺ influx or other signaling differences among GPCRs in DAGL (or 2-AG transport) activation.

5.4. The role of Ca²⁺ in lipid signaling cascades

As described above (2.3.1), OX₁ receptors increase the intracellular Ca²⁺ concentration by various mechanisms. Receptor-operated Ca²⁺ influx is a central signaling mechanism for OX₁, but the channels responsible for the response and the signaling mechanisms are largely unknown (2.3.1). In 2010, Turunen *et al.* made an interesting finding: MAFP, an AA analog and a general inhibitor of serine hydrolases acting on AA in glycerophospholipids, such as PLA₂, DAGL, and MAGL, was shown to inhibit the receptor-operated Ca²⁺ influx in CHO cells (Turunen *et al.*, 2010). In Paper II, we reassessed this with new pharmacological tools. We observed that PP produced strong inhibition of Ca²⁺ responses, while THL had no effect. DAGL does not appear to be involved in producing Ca²⁺ responses, but the Ca²⁺ influx seems to be somehow mediated by cPLA₂. The action of cPLA₂ releases mediators that are able to induce Ca²⁺ influx, such as LPC and AA or another fatty acid, and the mediators can be further processed by the enzyme cascades. The cPLA₂ product causing the effect in our test system is currently unknown.

Many signaling cascades utilized by OX₁ receptors have been shown to be dependent on extracellular Ca²⁺ (Lund *et al.*, 2000; Ammoun *et al.*, 2006a; Johansson *et al.*, 2007; Turunen *et al.*, 2010). We also observed a similar effect for PLD. At an extracellular Ca²⁺ concentration of 140 nM, the PLD response (Paper I) was abolished, similarly to ERK phosphorylation (Ammoun *et al.*, 2006a). The PLC response is also affected by the extracellular Ca²⁺ concentration, but to a lesser extent (Johansson *et al.*, 2007; Putula and Kukkonen, personal communication). The ³H-AA overflow response to orexin-A is also highly sensitive to extracellular Ca²⁺ (Turunen *et al.*, 2010), and thus both cPLA₂ and DAGL cascades should be abolished upon removal of extracellular Ca²⁺. Even though clearly dependent on extracellular Ca²⁺, PLD, PLC (Lund *et al.*, 2000; Johansson *et al.*, 2007), and ERK (Ammoun *et al.*, 2006a) are only very weakly activated by an ionomycin- or thapsigargin-induced increase in the intracellular Ca²⁺ concentration. On the contrary, an increased intracellular Ca²⁺ concentration by itself is enough to very strongly stimulate cPLA₂ activity (Turunen *et al.*, 2010; Paper II).

These findings can be attacked from several angles. The case for cPLA₂ and DAGL could be easily explained by the fact that they are Ca²⁺-dependent enzymes (Clark *et al.*, 1991; Kramer *et al.*, 1991; Bisogno *et al.*, 2003), and in the cascades leading to ERK, there are potential Ca²⁺-sensitive effectors. PLC is also Ca²⁺-dependent, but it (or at least PLCβ) appears to be, when compared to DAGL or cPLA₂, less affected by a reduction in extracellular Ca²⁺, as also investigated using the M₁ muscarinic receptor (Johansson *et al.*, 2007). However, PLD or its upstream activator nPKC should not be Ca²⁺-sensitive (reviewed in Steinberg, 2004). We have

observed similar discrepancies for other responses such as AC regulation and ERK (Holmqvist *et al.*, 2005; Ammoun *et al.*, 2006a). Thus it can be speculated that these responses to the elevated Ca^{2+} could be more dependent on the effect of receptor-mediated Ca^{2+} influx on orexin receptor itself than on these effectors. Extracellular Ca^{2+} might also influence the binding of orexin-A to OX_1 . Extracellular Ca^{2+} has been shown to affect the binding of ligands to the metabotropic glutamate receptor 1α , by binding to the receptor (Jiang *et al.*, 2014). It has recently been observed that Ca^{2+} is indeed required for orexin-A binding to the OX_1 receptor (Putula and Kukkonen, personal communication). However, this cannot entirely explain the effect of removal of extracellular Ca^{2+} (and especially the effect of blocking of Ca^{2+} influx) on orexin responses (Putula and Kukkonen, personal communication). It can be hypothesized that Ca^{2+} can affect OX_1 receptors in multiple ways. It modulates orexin binding, proximal receptor signaling, and also the downstream effectors. The level of impact on these stages differs between targets. Altogether, the reduction of extracellular Ca^{2+} seems to be a blunt instrument to study these processes.

5.5. Heteromerization of OX_1 , OX_2 , and CB_1 receptors

The idea that heteromerization of OX_1 and CB_1 could make their functional interaction more efficient led us to study their heteromerization. Despite the limitations of some previous studies, discussed above, the evidence for the heteromerization of these receptors is rather compelling. This heteromerization and the trafficking of these complexes has been investigated with for example FRET and biotinylation methods (Hilairret *et al.*, 2003; Ellis *et al.*, 2006; Ward *et al.*, 2011).

We utilized the BRET method to investigate complex formation between Rluc- and GFP^2 -tagged CB_1 , OX_1 and OX_2 receptors in CHO cells. BRET has certain advantages as compared to FRET: while FRET requires excitation with a light source, which can cause high signal background and even photobleaching, BRET is based on the hydrolysis of a substrate for luciferase, and its signal-to-noise ratio is therefore better (reviewed in Salahpour *et al.*, 2012). We started by examining the complex formation between orexin receptors. Both OX_1 and OX_2 receptors formed constitutive homomeric but also heteromeric complexes, as revealed by BRET. The BRET ratios showed saturation when the amount of the GFP^2 construct cDNA was increased in relation to the Rluc construct cDNA. The saturation implies that the formation of the complexes was specific. OX_2 -Rluc- OX_1 GFP^2 showed a strong response and a nicely saturating BRET curve, but when the tags (OX_1 -Rluc- OX_2 GFP^2) were reversed, the signal was weaker, and the combination did not produce as good a saturation curve as the previous one. These differences could be due to the geometry of the complex. It can be speculated that in some types of complexes, the GFP^2 may be more easily reached by the energy released from Rluc than in some others. We then also tested the ability of both OX_1 and OX_2 receptors to form complexes with CB_1 receptors. All receptor combinations gave BRET signals and showed saturation, but we again observed differences in the saturation curves similar to the ones described for the combinations of OX_1 and OX_2 .

Our BRET results are in line with previous studies (Hilairret *et al.*, 2003; Ellis *et al.*, 2006; Ward *et al.*, 2011) reporting heteromeric complexes between CB₁ and OX₁ receptors. In addition, we demonstrated that human OX₂ receptors can also form constitutive homomers and heteromeric complexes with both OX₁ and CB₁. The ability of mouse OX₂ receptor splice variants to form complexes with each other has also recently been shown (Wang *et al.*, 2013a). Our studies, and the previous ones, imply that these receptors are capable of forming complexes, which is typical for many GPCRs. The formation of these receptor complexes has, however, only been demonstrated in recombinant cells, and there is no evidence for it in physiological settings. IUPHAR has three criteria of which at least two have to be fulfilled before dimerization of a certain pair of receptors can be regarded as biologically significant: 1) the dimerization has to be shown in native tissues or primary cells, 2) there has to be proof of dimer-specific signaling, agonist binding, or bivalent ligands for the dimer, and 3) the dimerization has to be validated *in vivo* with, for example, knockout animals or siRNA (Pin *et al.*, 2007; reviewed in Hiller *et al.*, 2013). However, the problem with these criteria is that the effects seen in knockout animals, or when using siRNA techniques recommended for studying multimerization *in vivo*, could be due to lack of the absent receptor and not necessarily the lack of multimerization. Therefore, meeting the third criterion does not necessarily mean that the multimerization is physiologically relevant. Nevertheless, none of these criteria are met in the case of CB₁ or OX₁ dimers/oligomers, because the changes in the signaling reported by Hilairret *et al.* (2003) and Ellis *et al.* (2006) can be explained by more plausible and simple functional interactions. While there are no means for inhibiting the complex formation between these receptors, it is impossible to determine whether the dimerization affects their signaling by, for example, bringing the CB₁ receptors closer to the highest possible concentrations of 2-AG.

Heteromerization as such is interesting and has been investigated by various means and reported for many GPCRs (reviewed in Hiller *et al.*, 2013). The heteromerization of receptors can affect their signaling, trafficking, and pharmacology. For example, bivalent ligands could be used as more efficacious or selective agonists, or as antagonists for the receptors. There is also evidence for disease-specific dimerization of receptors, and the development of bivalent ligands could be especially useful for these types of situations (reviewed in Hiller *et al.*, 2013).

5.5 Comments on interactions between CB₁ and OX₁ receptors

Three out of the four papers of this thesis concern interactions between OX₁ and CB₁ receptors. Other studies have reported evidence of such interactions, but none of them have directly shown production and release of 2-AG upon OX₁ stimulation. Our results reported in Papers II and III demonstrate that OX₁ produces potent 2-AG release, and that this 2-AG can act as a paracrine or autocrine messenger. 2-AG is known to act in retrograde synaptic transmission, and our findings confirm the view that OX₁ activation can produce this retrograde messenger. The study by Haj-

Dahmane *et al.* (2005) provided the first electrophysiological evidence for this in hypothalamic slices, and another study implicated a similar scheme in the ventrolateral PAG (Ho *et al.*, 2011). According to these studies, orexins inhibit the presynaptic activity of both glutamate and GABA neurons via endocannabinoid release. Cristino *et al.* (2013) confirmed that in the lateral hypothalamus, the neurons that innervate orexin neurons and are thus inhibited by orexin-induced endocannabinoid release, can be either glutamatergic or GABAergic. In obese mice, the GABAergic innervation of orexin neurons is dominant (Cristino *et al.*, 2013). There is no doubt that this type of functional interaction between OX₁ and CB₁ receptors exists. There is also evidence that CB₁ receptor mRNA and PPO mRNA are expressed in the same cells (Cota *et al.*, 2003). However, there is no evidence of both OX₁ and CB₁ receptors being expressed in the same cells. Therefore, although heteromerization of these receptors now has been convincingly shown, physiological evidence is lacking. Nevertheless, the concept of heteromerization is interesting, and in Paper IV we examined it with a new method. Even though it seems more plausible that the interaction of these systems is functional, it is not impossible that direct physical receptor interactions could occur.

6. Conclusions

The studies forming this thesis add to our knowledge of OX₁ receptor signaling and especially the roles of lipid mediators. When compared to other GPCRs, especially another strongly G_q-coupled receptor, the human M₁ muscarinic acetylcholine receptor, the OX₁ receptor may show unique signaling features. However, it may also be that in recombinant cells the high receptor expression levels distort the signaling. In our studies, we were able to demonstrate that:

- 1) OX₁ activation of a novel PKC isoform, probably PKC δ , leads to the activation of PLD1 in CHO cells;
- 2) OX₁ activation leads to copious AA release by the action of cPLA₂ in CHO cells. cPLA₂ activity is also required for orexin receptor-operated Ca²⁺ influx;
- 3) OX₁ activation leads to strong release of the endocannabinoid 2-AG from CHO, neuro-2a, and HEK293 cells. The production of 2-AG is evoked by a cascade involving PLC and DAGL activation. The released 2-AG can act in a paracrine or autocrine manner, and is therefore able to mediate retrograde synaptic transmission. In addition, 2-AG may act as a source of free AA;
- 4) Orexin receptor subtypes and CB₁ cannabinoid receptors dimerize in any combination. However, we also demonstrated that the previously reported co-signaling of OX₁ and CB₁ receptors, upon co-expression, essentially requires that 2-AG, elevated by OX₁ receptor signaling, acts on CB₁ receptors. We therefore believe that the heterodimerization of the receptor is of lesser importance.

7. Future perspectives

A novel PKC was previously found to mediate part of the AC stimulation upon OX₁ receptor activation in CHO cells (Holmqvist *et al.*, 2005). For both AC and PLD1, the involved PKC isoform is suggested to be PKC δ , although we did not have access to a very specific inhibitor of this isoform. Recombinant PKC δ is translocated to the plasma membrane in CHO cells upon orexin receptor activation (Holmqvist *et al.*, 2005). This is probably driven by PLC-produced DAG, thus constituting a likely activation route for PKC δ activation. The nPKC-dependent component of the AC stimulation is indeed sensitive to the PLC inhibitor U-73122 (Holmqvist *et al.*, 2005). However, we did not observe any inhibition of PLD1 activation by this inhibitor, although we verified its effect in the PLC assay (Paper I). These findings thus stand in direct contradiction. The tools available constitute one of the problems. U-73122 is not very specific for PLC, and neither is it very stable. We also attempted to examine PKC δ phosphorylation upon OX₁ stimulation, but the antibodies we had at the time did not appear to work, and the project was put on the shelf. Involvement of novel PKC activation in this signaling is nevertheless very interesting – and also the lack of involvement of PKC ϵ – and I would like to determine the upstream mediators of PKC δ activation. Perhaps better antibodies could now be available, or phosphopeptide analysis of PKC δ could be performed. PKC δ inhibitor peptides could perhaps be synthesized in-house, and the role of PKC δ in orexin receptor responses in other tissues could be assessed with them.

The physiological importance of the novel lipid pathways of PA (from PLD), AA (from cPLA₂ and DAGL), and 2-AG (from DAGL) is one major question that arises. There have been previous findings of 2-AG-mediated orexin signaling in the ventrolateral PAG and DR (2.6). However, how general a mechanism for orexin receptor signaling is this? Is the role of 2-AG only to act on CB_{1/2} receptors, or is the production of AA from 2-AG hydrolysis also of importance for orexin signaling? Are orexin receptors particularly prone to activating 2-AG production? Are OX₁ and CB₁ receptors expressed in the same native cells, and is their co-signaling of importance?

We and others (Ellis *et al.*, 2006; Ward *et al.*, 2011) have shown that in recombinant cells (CHO cells in our case), OX₁ and CB₁ receptors are indeed able to form complexes. Complex formation between GPCRs has been shown to affect the trafficking of the receptors (reviewed in Hiller *et al.*, 2013), and from previous studies there is some evidence that the cellular localization of OX₁ and CB₁ complexes is affected by their ligands (Ellis *et al.*, 2006; Ward *et al.*, 2011). It would be interesting to examine how agonists of OX₁ and CB₁ receptors, and especially the 2-AG produced upon OX₁ activation, affect this. The effect of OX₁ activation-induced 2-AG release on the formation of these complexes is also of great interest. Does the 2-AG signaling to CB₁ perhaps enhance the formation of the CB₁–OX₁ heteromer?

PLD and cPLA₂ responses have thus far only been reported in these recombinant CHO cells. Both responses are potently activated, but are they of any significance to orexin physiology? There are rather good pharmacological tools (both

the ones used here and the subtype-nonspecific inhibitor FIPI) to assess these cascades in native cells, so it should be possible to analyze the responses in, for instance, native neurons. The cPLA₂ cascade, based on the inhibitor used, pyrrophenone, is important in regulating the orexin receptor-operated Ca²⁺ influx in CHO cells (Paper II). This influx was reported more than ten years ago, and the channel identity and its activation mechanism have been investigated in many subsequent studies (2.3.1). It would be very interesting to identify which of the many possible products of the cPLA₂ cascade are responsible for the orexin receptor-operated Ca²⁺ influx. This might also give a clue to the channel identity. Orexin receptors are additionally known to activate NSCCs in the brain (2.3.1), and thus studies in CHO cells could also help to identify the signaling mechanisms in native neurons.

8. Acknowledgements

This study was conducted at the Department of Veterinary Biosciences, Faculty of Veterinary medicine, University of Helsinki. It was funded by Research Foundation of the University of Helsinki, Magnus Ehrnrooth Foundation and Academy of Finland. I have also been a member of FinPharma Doctoral Program, Drug Discovery section since 2012.

There are many people who I owe gratitude for helping me out and making this thesis possible. First and most of all I want to thank my supervisor, Professor Jyrki Kukkonen for support and guidance through this whole journey. Without you this thesis simply would not exist. I have learned many practical things and methods while working in Jyrki-lab, but also many other things about science (and life). Working in your lab has been interesting and rewarding but also fun!

I would like to express my gratitude for Professor Mika Scheinin and Professor Raimo Tuominen for reading this thesis and giving helpful and very valuable comments to improve it.

People working in Jyrki-lab have played a huge part in making this thesis come true. First of all I want to thank Jaana for support and friendship during these years. We had great times in the lab but I'm especially thankful for the times outside the lab, such as the memorable conference trips. I also want to thank Pauli for friendship and fun times but also encouragement and advice in many problematic situations. I thank Pirjo for technical assistance and nice coffee breaks. Marjo is acknowledged for arranging lab meetings and helping out with scientific problems. Thank you also for bringing Eppu to work from time to time, it has cheered me up many times.

People who worked in Jyrki-lab for shorter periods have also contributed to this thesis. Sami, thank you for teaching me some useful methods, but also for cheering up the life in the lab. I also want to thank Ulla-Kaisa for helping out with this project and for bringing positive energy to the lab. I also had the pleasure of learning methods in Latvian Biomedical Study Center, in Riga and I would like to thank the people working there. Special thanks to Ilona and Ramona for their hospitality and for sharing their expertise.

In addition to people from our group there are many other people in Viikki who have one way or the other been involved in this process. I would like to thank Ninna for support, understanding and fun lunch breaks, Kirsi L. for many therapeutic conversations and helping me out with problems in the lab, Simo for fun lunch breaks, Esa for company in the lab and for helping with bike-related issues, Kirsi A. for great company but also for valuable advice in many things. I would also like to thank Jenni, Mikael, Maija, Jonna, Johanna, Tiina P-M, Anja, Tarja, Ritva, Tiina S., Ulla and all the people I maybe forgot to mention. It has been great to work and have coffee breaks with you!

I am extremely thankful to Professor Karl Åkerman for helping me out in a very stressful and difficult funding situation. You kindly took me in to your lab and gave me an opportunity to work with a very interesting project. During that time I also had the pleasure of meeting great and brilliant people. I would also like to thank them for good and very educating times. Thank you, Tommy, Linda, Lauri, Laura, Kirsi, Johanna T., Johanna K., Carina and all the others I met in Biomedicum for great company and fun times.

I want to thank my friends for supporting me throughout these years and taking my mind of work. Especially I want to thank Anna for the years that we have

known each other. Your friendship means a lot to me. I also want to thank other friends from years back that I see way too seldom Janiika, Inka, Johanna, Tuomo, Petteri, Nelli and all the others who I used to hang out with in Oulu. I also want to thank Virpi for all the support and “therapy” during the writing process.

People from Esport have had an essential part in this process by cheering me up and helping me deal with stress. I want to thank Leena for the countless times you have listened to my worries but also for many much-needed laughs, Kira for taking my mind off work and for brightening up so many not-so-good days and Hanna and Leo for writing-trousers. I also want to thank Saara P., Annette, Maria V., Paula, Veera, Tanja, Kimmo and many others for friendly and energetic atmosphere and great times at Esport.

I want to thank my family for their encouragement. I thank my aunt Ritva for always having an open door for me and for believing in me. I am also grateful for my grandfather Keijo, Kaarina, Veikko, Marsa, Aarne and Arja for support and hospitality I have always enjoyed when visiting any of you. Vällilä family, Riitta, Jari, Joonas, Iina and the cats, thank you for nice and relaxing times in Porvoo! I'm thankful to my brother Kalle and his wife Marja and their great kids Mico, Jinna and Henni for taking my mind off work.

My deepest gratitude belongs to my parents Pirkko and Ville. You have always encouraged and supported me and that means more to me than I can say. Last but definitely not least I wish to thank Santtu for your endless understanding and patience. These years have not been easy but you have supported me in all situations. I really do appreciate it enormously.

9. References

- Adeghate E., Fernandez-Cabezudo M., Hameed R., El-Hasasna H., El Wasila M., Abbas T., Al-Ramadi B. (2010) Orexin-1 receptor co-localizes with pancreatic hormones in islet cells and modulates the outcome of streptozotocin-induced diabetes mellitus. *PloS one* 5:e8587.
- Adeghate E., Hameed R. (2011) Mechanism of orexin b-stimulated insulin and glucagon release from the pancreas of normal and diabetic rats. *Pancreas* 40:131-136.
- Adie W. J. (1926) A case of true narcolepsy: Onset at the age of 12 years. *Proceedings of the Royal Society of Medicine* 19:2.
- Aguado T., Romero E., Monory K., Palazuelos J., Sendtner M., Marsicano G., Lutz B., Guzman M., Galve-Roperh I. (2007) The cb1 cannabinoid receptor mediates excitotoxicity-induced neural progenitor proliferation and neurogenesis. *J Biol Chem* 282:23892-23898.
- Akbari E., Motamedi F., Davoodi F. G., Noorbakhshnia M., Ghanbarian E. (2011) Orexin-1 receptor mediates long-term potentiation in the dentate gyrus area of freely moving rats. *Behav Brain Res* 216:375-380.
- Akbari E., Motamedi F., Naghdi N., Noorbakhshnia M. (2008) The effect of antagonization of orexin 1 receptors in ca1 and dentate gyrus regions on memory processing in passive avoidance task. *Behav Brain Res* 187:172-177.
- Akbari E., Naghdi N., Motamedi F. (2006) Functional inactivation of orexin 1 receptors in ca1 region impairs acquisition, consolidation and retrieval in morris water maze task. *Behav Brain Res* 173:47-52.
- Akbari E., Naghdi N., Motamedi F. (2007) The selective orexin 1 receptor antagonist sb-334867-a impairs acquisition and consolidation but not retrieval of spatial memory in morris water maze. *Peptides* 28:650-656.
- Akirav I. (2013) Cannabinoids and glucocorticoids modulate emotional memory after stress. *Neurosci Biobehav Rev* 37:2554-2563.
- Al-Barazanji K. A., Wilson S., Baker J., Jessop D. S., Harbuz M. S. (2001) Central orexin-a activates hypothalamic-pituitary-adrenal axis and stimulates hypothalamic corticotropin releasing factor and arginine vasopressin neurones in conscious rats. *J Neuroendocrinol* 13:421-424.
- Alexandre C., Andermann M. L., Scammell T. E. (2013) Control of arousal by the orexin neurons. *Curr Opin Neurobiol* 23:752-759.
- Alger B. E., Teyler T. J. (1976) Long-term and short-term plasticity in the ca1, ca3, and dentate regions of the rat hippocampal slice. *Brain Res* 110:463-480.
- Alvarez C. E., Sutcliffe J. G. (2002) Hypocretin is an early member of the incretin gene family. *Neurosci Lett* 324:169-172.
- Ammoun S., Johansson L., Ekholm M. E., Holmqvist T., Danis A. S., Korhonen L., Sergeeva O. A., Haas H. L., Åkerman K. E., Kukkonen J. P. (2006a) Ox1 orexin receptors activate extracellular signal-regulated kinase (erk) in cho cells via multiple mechanisms: The role of ca²⁺ influx in ox1 receptor signaling. *Mol Endocrinol* 20:80-99.
- Ammoun S., Johansson L., Åkerman K. E. O., Korhonen L., Lindholm D., Kukkonen J. P. (2003) Regulation of map/sap-kinase cascades by orexin receptors. *Neuroscience* 2003 Abstract 161.14.
- Ammoun S., Lindholm D., Wootz H., Åkerman K. E., Kukkonen J. P. (2006b) G-protein-coupled ox1 orexin/hcrtr-1 hypocretin receptors induce caspase-

- dependent and -independent cell death through p38 mitogen-/stress-activated protein kinase. *J Biol Chem* 281:834-842.
- Antoni F. A. (2000) Molecular diversity of cyclic amp signalling. *Front Neuroendocrinol* 21:103-132.
- Arnone M., Maruani J., Chaperon F., Thiebot M. H., Poncelet M., Soubrie P., Le Fur G. (1997) Selective inhibition of sucrose and ethanol intake by sr 141716, an antagonist of central cannabinoid (cb1) receptors. *Psychopharmacology (Berl)* 132:104-106.
- Azhdari-Zarmehri H., Reisi Z., Vaziri A., Haghparast A., Shaigani P. (2013a) Involvement of orexin-2 receptors in the ventral tegmental area and nucleus accumbens in the antinociception induced by the lateral hypothalamus stimulation in rats. *Peptides* 47:94-98.
- Azhdari-Zarmehri H., Semnanian S., Fathollahi Y., Ghaderi Pakdel F. (2013b) Tail flick modification of orexin-a induced changes of electrophysiological parameters in the rostral ventromedial medulla. *Cell journal* 16.
- Azhdari Zarmehri H., Semnanian S., Fathollahi Y., Erami E., Khakpay R., Azizi H., Rohampour K. (2011) Intra-periaqueductal gray matter microinjection of orexin-a decreases formalin-induced nociceptive behaviors in adult male rats. *The journal of pain : official journal of the American Pain Society* 12:280-287.
- Barb C. R., Matteri R. L. (2005) Orexin-b modulates luteinizing hormone and growth hormone secretion from porcine pituitary cells in culture. *Domest Anim Endocrinol* 28:331-337.
- Barreiro M. L., Pineda R., Navarro V. M., Lopez M., Suominen J. S., Pinilla L., Senaris R., Toppari J., Aguilar E., Dieguez C., Tena-Sempere M. (2004) Orexin 1 receptor messenger ribonucleic acid expression and stimulation of testosterone secretion by orexin-a in rat testis. *Endocrinology* 145:2297-2306.
- Bartelt A., Heeren J. (2014) Adipose tissue browning and metabolic health. *Nature reviews Endocrinology* 10:24-36.
- Bartsch T., Levy M. J., Knight Y. E., Goadsby P. J. (2004) Differential modulation of nociceptive dural input to [hypocretin] orexin a and b receptor activation in the posterior hypothalamic area. *Pain* 109:367-378.
- Bengtsson M. W., Makela K., Sjoblom M., Uotila S., Akerman K. E., Herzig K. H., Flemstrom G. (2007) Food-induced expression of orexin receptors in rat duodenal mucosa regulates the bicarbonate secretory response to orexin-a. *American journal of physiology Gastrointestinal and liver physiology* 293:G501-509.
- Bernard R., Lydic R., Baghdoyan H. A. (2002) Hypocretin-1 activates g proteins in arousal-related brainstem nuclei of rat. *Neuroreport* 13:447-450.
- Berridge M. J. (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 1793:933-940.
- Berthoud H. R., Patterson L. M., Sutton G. M., Morrison C., Zheng H. (2005) Orexin inputs to caudal raphe neurons involved in thermal, cardiovascular, and gastrointestinal regulation. *Histochem Cell Biol* 123:147-156.
- Betschart C., Hintermann S., Behnke D., Cotesta S., Fendt M., Gee C. E., Jacobson L. H., Laue G., Ofner S., Chaudhari V., Badiger S., Pandit C., Wagner J., Hoyer D. (2013) Identification of a novel series of orexin receptor antagonists with a distinct effect on sleep architecture for the treatment of insomnia. *J Med Chem* 56:7590-7607.
- Bingham S., Davey P. T., Babbs A. J., Irving E. A., Sammons M. J., Wyles M., Jeffrey P., Cutler L., Riba I., Johns A., Porter R. A., Upton N., Hunter A. J.,

- Parsons A. A. (2001) Orexin-a, an hypothalamic peptide with analgesic properties. *Pain* 92:81-90.
- Bisogno T., Cascio M. G., Saha B., Mahadevan A., Urbani P., Minassi A., Appendino G., Saturnino C., Martin B., Razdan R., Di Marzo V. (2006) Development of the first potent and specific inhibitors of endocannabinoid biosynthesis. *Biochim Biophys Acta* 1761:205-212.
- Bisogno T., Howell F., Williams G., Minassi A., Cascio M. G., Ligresti A., Matias I., Schiano-Moriello A., Paul P., Williams E. J., Gangadharan U., Hobbs C., Di Marzo V., Doherty P. (2003) Cloning of the first sn1-dag lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J Cell Biol* 163:463-468.
- Blanco M., Lopez M., Garcia-Caballero T., Gallego R., Vazquez-Boquete A., Morel G., Senaris R., Casanueva F., Dieguez C., Beiras A. (2001) Cellular localization of orexin receptors in human pituitary. *J Clin Endocrinol Metab* 86:1616-1619.
- Bligh E. G., Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911-917.
- Bliss T. V., Lomo T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* 232:331-356.
- Bootman M. D., Lipp P., Berridge M. J. (2001) The organisation and functions of local Ca^{2+} signals. *J Cell Sci* 114:2213-2222.
- Borgland S. L., Taha S. A., Sarti F., Fields H. L., Bonci A. (2006) Orexin a in the vta is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. *Neuron* 49:589-601.
- Borsook D., Becerra L., Carlezon W. A., Jr., Shaw M., Renshaw P., Elman I., Levine J. (2007) Reward-aversion circuitry in analgesia and pain: Implications for psychiatric disorders. *Eur J Pain* 11:7-20.
- Bouaboula M., Perrachon S., Milligan L., Canat X., Rinaldi-Carmona M., Portier M., Barth F., Calandra B., Pecceu F., Lupker J., Maffrand J. P., Le Fur G., Casellas P. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* 272:22330-22339.
- Bouaboula M., Poinot-Chazel C., Bourrie B., Canat X., Calandra B., Rinaldi-Carmona M., Le Fur G., Casellas P. (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor cb1. *Biochem J* 312 (Pt 2):637-641.
- Bouaboula M., Poinot-Chazel C., Marchand J., Canat X., Bourrie B., Rinaldi-Carmona M., Calandra B., Le Fur G., Casellas P. (1996) Signaling pathway associated with stimulation of cb2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of krox-24 expression. *Eur J Biochem* 237:704-711.
- Boutrel B., Kenny P. J., Specio S. E., Martin-Fardon R., Markou A., Koob G. F., De Lecea L. (2005) Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. *Proc Natl Acad Sci U S A* 102:19168-19173.
- Boutrel B., Steiner N., Halfon O. (2013) The hypocretins and the reward function: What have we learned so far? *Frontiers in behavioral neuroscience* 7:59.
- Broberger C., De Lecea L., Sutcliffe J. G., Hokfelt T. (1998) Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in

- the rodent lateral hypothalamus: Relationship to the neuropeptide γ and agouti gene-related protein systems. *J Comp Neurol* 402:460-474.
- Brown H. A., Gutowski S., Moomaw C. R., Slaughter C., Sternweis P. C. (1993) Adp-ribosylation factor, a small gtp-dependent regulatory protein, stimulates phospholipase d activity. *Cell* 75:1137-1144.
- Brown R. M., Khoo S. Y., Lawrence A. J. (2013) Central orexin (hypocretin) 2 receptor antagonism reduces ethanol self-administration, but not cue-conditioned ethanol-seeking, in ethanol-preferring rats. *Int J Neuropsychopharmacol* 16:2067-2079.
- Burdakov D., Jensen L. T., Alexopoulos H., Williams R. H., Fearon I. M., O'Kelly I., Gerasimenko O., Fugger L., Verkhatsky A. (2006) Tandem-pore k^+ channels mediate inhibition of orexin neurons by glucose. *Neuron* 50:711-722.
- Burdyga G., Lal S., Varro A., Dimaline R., Thompson D. G., Dockray G. J. (2004) Expression of cannabinoid cb1 receptors by vagal afferent neurons is inhibited by cholecystokinin. *J Neurosci* 24:2708-2715.
- Cai X. J., Widdowson P. S., Harrold J., Wilson S., Buckingham R. E., Arch J. R., Tadayyon M., Clapham J. C., Wilding J., Williams G. (1999) Hypothalamic orexin expression: Modulation by blood glucose and feeding. *Diabetes* 48:2132-2137.
- Cannon B., Nedergaard J. (2010) Metabolic consequences of the presence or absence of the thermogenic capacity of brown adipose tissue in mice (and probably in humans). *Int J Obes (Lond)* 34 Suppl 1:S7-16.
- Carlson G., Wang Y., Alger B. E. (2002) Endocannabinoids facilitate the induction of ltp in the hippocampus. *Nature neuroscience* 5:723-724.
- Cavuto P., Mcainch A. J., Hatzinikolas G., Cameron-Smith D., Wittert G. A. (2007) Effects of cannabinoid receptors on skeletal muscle oxidative pathways. *Mol Cell Endocrinol* 267:63-69.
- Chang F., Steelman L. S., Lee J. T., Shelton J. G., Navolanic P. M., Blalock W. L., Franklin R. A., Mccubrey J. A. (2003) Signal transduction mediated by the ras/raf/mek/erk pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia* 17:1263-1293.
- Chemelli R. M., Willie J. T., Sinton C. M., Elmquist J. K., Scammell T., Lee C., Richardson J. A., Williams S. C., Xiong Y., Kisanuki Y., Fitch T. E., Nakazato M., Hammer R. E., Saper C. B., Yanagisawa M. (1999) Narcolepsy in orexin knockout mice: Molecular genetics of sleep regulation. *Cell* 98:437-451.
- Chen C., Xu R. (2003) The in vitro regulation of growth hormone secretion by orexins. *Endocrine* 22:57-66.
- Chen H., Yang L., Chen F., Yan J., Yang N., Wang Y. J., Zhu Z. R., Hu Z. A., Sui J. F., Hu B. (2013) Functional inactivation of orexin 1 receptors in the cerebellum disrupts trace eyeblink conditioning and local theta oscillations in guinea pigs. *Behav Brain Res* 250:114-122.
- Chen X. P., Yang W., Fan Y., Luo J. S., Hong K., Wang Z., Yan J. F., Chen X., Lu J. X., Benovic J. L., Zhou N. M. (2010) Structural determinants in the second intracellular loop of the human cannabinoid cb1 receptor mediate selective coupling to g(s) and g(i). *Br J Pharmacol* 161:1817-1834.
- Chen X. W., Mu Y., Huang H. P., Guo N., Zhang B., Fan S. Y., Xiong J. X., Wang S. R., Xiong W., Huang W., Liu T., Zheng L. H., Zhang C. X., Li L. H., Yu Z. P., Hu Z. A., Zhou Z. (2008) Hypocretin-1 potentiates nmda receptor-

- mediated somatodendritic secretion from locus ceruleus neurons. *J Neurosci* 28:3202-3208.
- Cheng J. K., Chou R. C., Hwang L. L., Chiou L. C. (2003) Antiallodynic effects of intrathecal orexins in a rat model of postoperative pain. *J Pharmacol Exp Ther* 307:1065-1071.
- Chevalleyre V., Castillo P. E. (2003) Heterosynaptic LTD of hippocampal GABAergic synapses: A novel role of endocannabinoids in regulating excitability. *Neuron* 38:461-472.
- Chicca A., Marazzi J., Nicolussi S., Gertsch J. (2012) Evidence for bidirectional endocannabinoid transport across cell membranes. *J Biol Chem* 287:34660-34682.
- Choi D. L., Davis J. F., Magrisso I. J., Fitzgerald M. E., Lipton J. W., Benoit S. C. (2012) Orexin signaling in the paraventricular thalamic nucleus modulates mesolimbic dopamine and hedonic feeding in the rat. *Neuroscience* 210:243-248.
- Civelli O., Reinscheid R. K., Zhang Y., Wang Z., Fredriksson R., Schiöth H. B. (2013) G protein-coupled receptor deorphanizations. *Annu Rev Pharmacol Toxicol* 53:127-146.
- Clark J. D., Lin L. L., Kriz R. W., Ramesha C. S., Sultzman L. A., Lin A. Y., Milona N., Knopf J. L. (1991) A novel arachidonic acid-selective cytosolic PLA₂ contains a Ca²⁺-dependent translocation domain with homology to PKC and GAP. *Cell* 65:1043-1051.
- Console-Bram L., Marcu J., Abood M. E. (2012) Cannabinoid receptors: Nomenclature and pharmacological principles. *Prog Neuropsychopharmacol Biol Psychiatry* 38:4-15.
- Cota D., Marsicano G., Tschöp M., Grubler Y., Flachskamm C., Schubert M., Auer D., Yassouridis A., Thone-Reineke C., Ortman S., Tomassoni F., Cervino C., Nisoli E., Linthorst A. C., Pasquali R., Lutz B., Stalla G. K., Pagotto U. (2003) The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* 112:423-431.
- Cota D., Steiner M. A., Marsicano G., Cervino C., Herman J. P., Grubler Y., Stalla J., Pasquali R., Lutz B., Stalla G. K., Pagotto U. (2007) Requirement of cannabinoid receptor type 1 for the basal modulation of hypothalamic-pituitary-adrenal axis function. *Endocrinology* 148:1574-1581.
- Crespo I., Gomez De Heras R., Rodriguez De Fonseca F., Navarro M. (2008) Pretreatment with subeffective doses of rimonabant attenuates orexigenic actions of orexin A-hypocretin 1. *Neuropharmacology* 54:219-225.
- 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:E2229-2238.
- Date Y., Mondal M. S., Matsukura S., Ueta Y., Yamashita H., Kaiya H., Kangawa K., Nakazato M. (2000) Distribution of orexin/hypocretin in the rat median eminence and pituitary. *Brain Res Mol Brain Res* 76:1-6.
- De La Herran-Arita A. K., Drucker-Colin R. (2012) Models for narcolepsy with cataplexy drug discovery. *Expert opinion on drug discovery* 7:155-164.
- De Lecea L., Kilduff T. S., Peyron C., Gao X., Foye P. E., Danielson P. E., Fukuhara C., Battenberg E. L., Gautvik V. T., Bartlett F. S., 2nd, Frankel W. N., Van Den Pol A. N., Bloom F. E., Gautvik K. M., Sutcliffe J. G. (1998) The

- hypocretins: Hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 95:322-327.
- De Vries T. J., De Vries W., Janssen M. C., Schoffelmeer A. N. (2005) Suppression of conditioned nicotine and sucrose seeking by the cannabinoid-1 receptor antagonist sr141716a. *Behav Brain Res* 161:164-168.
- De Vries T. J., Shaham Y., Homberg J. R., Crombag H., Schuurman K., Dieben J., Vanderschuren L. J., Schoffelmeer A. N. (2001) A cannabinoid mechanism in relapse to cocaine seeking. *Nat Med* 7:1151-1154.
- Dennis E. A., Cao J., Hsu Y. H., Magriotti V., Kokotos G. (2011) Phospholipase a2 enzymes: Physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem Rev* 111:6130-6185.
- Devane W. A., Dysarz F. A., 3rd, Johnson M. R., Melvin L. S., Howlett A. C. (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* 34:605-613.
- Devane W. A., Hanus L., Breuer A., Pertwee R. G., Stevenson L. A., Griffin G., Gibson D., Mandelbaum A., Etinger A., Mechoulam R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258:1946-1949.
- Di Marzo V., Goparaju S. K., Wang L., Liu J., Batkai S., Jarai Z., Fezza F., Miura G. I., Palmiter R. D., Sugiura T., Kunos G. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* 410:822-825.
- Di Marzo V., Matias I. (2005) Endocannabinoid control of food intake and energy balance. *Nature neuroscience* 8:585-589.
- Digby J. E., Chen J., Tang J. Y., Lehnert H., Matthews R. N., Randeve H. S. (2006) Orexin receptor expression in human adipose tissue: Effects of orexin-a and orexin-b. *J Endocrinol* 191:129-136.
- Dipatrizio N. V., Simansky K. J. (2008) Activating parabrachial cannabinoid cb1 receptors selectively stimulates feeding of palatable foods in rats. *J Neurosci* 28:9702-9709.
- Doreulee N., Alania M., Chikovani M., Chkhartishvili B., Skhirtladze C. (2009) Orexin-a induces long-term depression of nmda responses in ca-1 field of hippocampal slices. *Georgian medical news* 65-70.
- Dube M. G., Horvath T. L., Kalra P. S., Kalra S. P. (2000) Evidence of npy y5 receptor involvement in food intake elicited by orexin a in sated rats. *Peptides* 21:1557-1560.
- Dunwiddie T., Lynch G. (1978) Long-term potentiation and depression of synaptic responses in the rat hippocampus: Localization and frequency dependency. *J Physiol* 276:353-367.
- Eggermann E., Serafin M., Bayer L., Machard D., Saint-Mleux B., Jones B. E., Muhlethaler M. (2001) Orexins/hypocretins excite basal forebrain cholinergic neurones. *Neuroscience* 108:177-181.
- Ehrstrom M., Gustafsson T., Finn A., Kirchgessner A., Gryback P., Jacobsson H., Hellstrom P. M., Naslund E. (2005) Inhibitory effect of exogenous orexin a on gastric emptying, plasma leptin, and the distribution of orexin and orexin receptors in the gut and pancreas in man. *J Clin Endocrinol Metab* 90:2370-2377.
- Ekoski E., Aitio O., Tornquist K., Yli-Kauhaluoma J., Tuominen R. K. (2010) Hiv-1 tat-peptide inhibits protein kinase c and protein kinase a through substrate competition. *Eur J Pharm Sci* 40:404-411.

- Ellis J., Pediani J. D., Canals M., Milasta S., Milligan G. (2006) Orexin-1 receptor-cannabinoid cb1 receptor heterodimerization results in both ligand-dependent and -independent coordinated alterations of receptor localization and function. *J Biol Chem* 281:38812-38824.
- Erami E., Azhdari-Zarmehri H., Ghasemi-Dashkhasan E., Esmaeili M. H., Semnanian S. (2012) Intra-paragigantocellularis lateralis injection of orexin-a has an antinociceptive effect on hot plate and formalin tests in rat. *Brain Res* 1478:16-23.
- Espana R. A., Melchior J. R., Roberts D. C., Jones S. R. (2011) Hypocretin 1/orexin a in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration. *Psychopharmacology (Berl)* 214:415-426.
- Espana R. A., Oleson E. B., Locke J. L., Brookshire B. R., Roberts D. C., Jones S. R. (2010) The hypocretin-orexin system regulates cocaine self-administration via actions on the mesolimbic dopamine system. *Eur J Neurosci* 31:336-348.
- Esseltine J. L., Scott J. D. (2013) Akap signaling complexes: Pointing towards the next generation of therapeutic targets? *Trends Pharmacol Sci* 34:648-655.
- Exton J. H. (1996) Regulation of phosphoinositide phospholipases by hormones, neurotransmitters, and other agonists linked to g proteins. *Annu Rev Pharmacol Toxicol* 36:481-509.
- Fahrner M., Derler I., Jardin I., Romanin C. (2013) The stim1/orai signaling machinery. *Channels (Austin)* 7:330-343.
- Flemström G., Sjöblom M., Jedstedt G., Åkerman K. E. (2003) Short fasting dramatically decreases rat duodenal secretory responsiveness to orexin a but not to vip or melatonin. *American journal of physiology Gastrointestinal and liver physiology* 285:G1091-1096.
- Fonseca B. M., Costa M. A., Almada M., Correia-Da-Silva G., Teixeira N. A. (2013) Endogenous cannabinoids revisited: A biochemistry perspective. *Prostaglandins & other lipid mediators* 102-103:13-30.
- Foster D. A. (2009) Phosphatidic acid signaling to mtor: Signals for the survival of human cancer cells. *Biochim Biophys Acta* 1791:949-955.
- Freedland C. S., Poston J. S., Porrino L. J. (2000) Effects of sr141716a, a central cannabinoid receptor antagonist, on food-maintained responding. *Pharmacol Biochem Behav* 67:265-270.
- Frohman M. A., Sung T. C., Morris A. J. (1999) Mammalian phospholipase d structure and regulation. *Biochim Biophys Acta* 1439:175-186.
- Fronczek R., Van Geest S., Frolich M., Overeem S., Roelandse F. W., Lammers G. J., Swaab D. F. (2012) Hypocretin (orexin) loss in alzheimer's disease. *Neurobiol Aging* 33:1642-1650.
- Fujiki N., Yoshida Y., Zhang S., Sakurai T., Yanagisawa M., Nishino S. (2006) Sex difference in body weight gain and leptin signaling in hypocretin/orexin deficient mouse models. *Peptides* 27:2326-2331.
- Fukudome Y., Ohno-Shosaku T., Matsui M., Omori Y., Fukaya M., Tsubokawa H., Taketo M. M., Watanabe M., Manabe T., Kano M. (2004) Two distinct classes of muscarinic action on hippocampal inhibitory synapses: M2-mediated direct suppression and m1/m3-mediated indirect suppression through endocannabinoid signalling. *Eur J Neurosci* 19:2682-2692.
- Funato H., Tsai A. L., Willie J. T., Kisanuki Y., Williams S. C., Sakurai T., Yanagisawa M. (2009) Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metab* 9:64-76.

- Galdino G., Romero T. R., Silva J. F., Aguiar D. C., De Paula A. M., Cruz J. S., Parrella C., Piscitelli F., Duarte I. D., Di Marzo V., Perez A. C. (2014) The endocannabinoid system mediates aerobic exercise-induced antinociception in rats. *Neuropharmacology* 77:313-324.
- Gales C., Van Durm J. J., Schaak S., Pontier S., Percherancier Y., Audet M., Paris H., Bouvier M. (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-g protein complexes. *Nature structural & molecular biology* 13:778-786.
- Gallily R., Breuer A., Mechoulam R. (2000) 2-arachidonylethanolamide, an endogenous cannabinoid, inhibits tumor necrosis factor- α production in murine macrophages, and in mice. *Eur J Pharmacol* 406:R5-7.
- Gao Q., Frohman M. A. (2012) Roles for the lipid-signaling enzyme mitopld in mitochondrial dynamics, pirna biogenesis, and spermatogenesis. *BMB reports* 45:7-13.
- Gardner E. L. (2005) Endocannabinoid signaling system and brain reward: Emphasis on dopamine. *Pharmacol Biochem Behav* 81:263-284.
- Georgescu D., Zachariou V., Barrot M., Mieda M., Willie J. T., Eisch A. J., Yanagisawa M., Nestler E. J., Dileone R. J. (2003) Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. *J Neurosci* 23:3106-3111.
- Ghayur T., Hugunin M., Talanian R. V., Ratnofsky S., Quinlan C., Emoto Y., Pandey P., Datta R., Huang Y., Kharbanda S., Allen H., Kamen R., Wong W., Kufe D. (1996) Proteolytic activation of protein kinase c delta by an ice/ced 3-like protease induces characteristics of apoptosis. *J Exp Med* 184:2399-2404.
- Ghomashchi F., Stewart A., Hefner Y., Ramanadham S., Turk J., Leslie C. C., Gelb M. H. (2001) A pyrrolidine-based specific inhibitor of cytosolic phospholipase a(2) α blocks arachidonic acid release in a variety of mammalian cells. *Biochim Biophys Acta* 1513:160-166.
- Ghosh M., Tucker D. E., Burchett S. A., Leslie C. C. (2006) Properties of the group iv phospholipase a2 family. *Prog Lipid Res* 45:487-510.
- Glass M., Felder C. C. (1997) Concurrent stimulation of cannabinoid cb1 and dopamine d2 receptors augments camp accumulation in striatal neurons: Evidence for a gs linkage to the cb1 receptor. *J Neurosci* 17:5327-5333.
- Gonzalez S., Schmid P. C., Fernandez-Ruiz J., Krebsbach R., Schmid H. H., Ramos J. A. (2003) Region-dependent changes in endocannabinoid transmission in the brain of morphine-dependent rats. *Addict Biol* 8:159-166.
- Gotter A. L., Webber A. L., Coleman P. J., Renger J. J., Winrow C. J. (2012) International union of basic and clinical pharmacology. Lxxxvi. Orexin receptor function, nomenclature and pharmacology. *Pharmacol Rev* 64:389-420.
- Guo Y., Feng P. (2012) Ox2r activation induces pkc-mediated erk and creb phosphorylation. *Exp Cell Res* 318:2004-2013.
- Göncz E., Strowski M. Z., Grotzinger C., Nowak K. W., Kaczmarek P., Sassek M., Mergler S., El-Zayat B. F., Theodoropoulou M., Stalla G. K., Wiedenmann B., Plockinger U. (2008) Orexin-a inhibits glucagon secretion and gene expression through a foxo1-dependent pathway. *Endocrinology* 149:1618-1626.
- Hagan J. J., Leslie R. A., Patel S., Evans M. L., Wattam T. A., Holmes S., Benham C. D., Taylor S. G., Routledge C., Hemmati P., Munton R. P., Ashmeade T. E., Shah A. S., Hatcher J. P., Hatcher P. D., Jones D. N., Smith M. I., Piper D. C.,

- Hunter A. J., Porter R. A., Upton N. (1999) Orexin a activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci U S A* 96:10911-10916.
- Haj-Dahmane S., Shen R. Y. (2005) The wake-promoting peptide orexin-b inhibits glutamatergic transmission to dorsal raphe nucleus serotonin neurons through retrograde endocannabinoid signaling. *J Neurosci* 25:896-905.
- Halls M. L., Cooper D. M. (2011) Regulation by ca^{2+} -signaling pathways of adenylyl cyclases. *Cold Spring Harbor perspectives in biology* 3:a004143.
- Hamm H. E. (1998) The many faces of g protein signaling. *J Biol Chem* 273:669-672.
- Hara J., Beuckmann C. T., Nambu T., Willie J. T., Chemelli R. M., Sinton C. M., Sugiyama F., Yagami K., Goto K., Yanagisawa M., Sakurai T. (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 30:345-354.
- Hara J., Yanagisawa M., Sakurai T. (2005) Difference in obesity phenotype between orexin-knockout mice and orexin neuron-deficient mice with same genetic background and environmental conditions. *Neurosci Lett* 380:239-242.
- Harris G. C., Wimmer M., Aston-Jones G. (2005) A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* 437:556-559.
- Henage L. G., Exton J. H., Brown H. A. (2006) Kinetic analysis of a mammalian phospholipase d: Allosteric modulation by monomeric gtpases, protein kinase c, and polyphosphoinositides. *J Biol Chem* 281:3408-3417.
- Henry D. J., Chavkin C. (1995) Activation of inwardly rectifying potassium channels (girk1) by co-expressed rat brain cannabinoid receptors in xenopus oocytes. *Neurosci Lett* 186:91-94.
- Herkenham M., Lynn A. B., Little M. D., Johnson M. R., Melvin L. S., De Costa B. R., Rice K. C. (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci U S A* 87:1932-1936.
- Hewavitharana T., Wedegaertner P. B. (2012) Non-canonical signaling and localizations of heterotrimeric g proteins. *Cell Signal* 24:25-34.
- Higgs S., Williams C. M., Kirkham T. C. (2003) Cannabinoid influences on palatability: Microstructural analysis of sucrose drinking after delta(9)-tetrahydrocannabinol, anandamide, 2-arachidonoyl glycerol and sr141716. *Psychopharmacology (Berl)* 165:370-377.
- Hilaret S., Bouaboula M., Carriere D., Le Fur G., Casellas P. (2003) Hypersensitization of the orexin 1 receptor by the cb1 receptor: Evidence for cross-talk blocked by the specific cb1 antagonist, sr141716. *J Biol Chem* 278:23731-23737.
- Hill K. J., Webber A. C., Hill S. J. (2003) A role of protein kinase c mu in signalling from the human adenosine a1 receptor to the nucleus. *Br J Pharmacol* 139:721-732.
- Hiller C., Kuhhorn J., Gmeiner P. (2013) Class a g-protein-coupled receptor (gpcr) dimers and bivalent ligands. *J Med Chem* 56:6542-6559.
- Ho Y. C., Lee H. J., Tung L. W., Liao Y. Y., Fu S. Y., Teng S. F., Liao H. T., Mackie K., Chiou L. C. (2011) Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. *J Neurosci* 31:14600-14610.
- Holland P. R., Akerman S., Goadsby P. J. (2005) Orexin 1 receptor activation attenuates neurogenic dural vasodilation in an animal model of trigeminovascular nociception. *J Pharmacol Exp Ther* 315:1380-1385.

- Hollander J. A., Lu Q., Cameron M. D., Kamenecka T. M., Kenny P. J. (2008) Insular hypocretin transmission regulates nicotine reward. *Proc Natl Acad Sci U S A* 105:19480-19485.
- Hollander J. A., Pham D., Fowler C. D., Kenny P. J. (2012) Hypocretin-1 receptors regulate the reinforcing and reward-enhancing effects of cocaine: Pharmacological and behavioral genetics evidence. *Frontiers in behavioral neuroscience* 6:47.
- Holmqvist T., Johansson L., Östman M., Ammoun S., Åkerman K. E., Kukkonen J. P. (2005) Ox1 orexin receptors couple to adenylyl cyclase regulation via multiple mechanisms. *J Biol Chem* 280:6570-6579.
- Howlett A. C. (1984) Inhibition of neuroblastoma adenylyl cyclase by cannabinoid and nantadol compounds. *Life Sci* 35:1803-1810.
- Howlett A. C., Qualy J. M., Khachatrian L. L. (1986) Involvement of gi in the inhibition of adenylyl cyclase by cannabimimetic drugs. *Mol Pharmacol* 29:307-313.
- Hu J., El-Fakahany E. E. (1993) Role of intercellular and intracellular communication by nitric oxide in coupling of muscarinic receptors to activation of guanylate cyclase in neuronal cells. *J Neurochem* 61:578-585.
- Huang H., Acuna-Goycolea C., Li Y., Cheng H. M., Obrietan K., Van Den Pol A. N. (2007) Cannabinoids excite hypothalamic melanin-concentrating hormone but inhibit hypocretin/orexin neurons: Implications for cannabinoid actions on food intake and cognitive arousal. *J Neurosci* 27:4870-4881.
- Hungs M., Fan J., Lin L., Lin X., Maki R. A., Mignot E. (2001) Identification and functional analysis of mutations in the hypocretin (orexin) genes of narcoleptic canines. *Genome Res* 11:531-539.
- Hutcheson D. M., Quarta D., Halbout B., Rigal A., Valerio E., Heidbreder C. (2011) Orexin-1 receptor antagonist sb-334867 reduces the acquisition and expression of cocaine-conditioned reinforcement and the expression of amphetamine-conditioned reward. *Behav Pharmacol* 22:173-181.
- Ida T., Nakahara K., Kuroiwa T., Fukui K., Nakazato M., Murakami T., Murakami N. (2000a) Both corticotropin releasing factor and neuropeptide y are involved in the effect of orexin (hypocretin) on the food intake in rats. *Neurosci Lett* 293:119-122.
- Ida T., Nakahara K., Murakami T., Hanada R., Nakazato M., Murakami N. (2000b) Possible involvement of orexin in the stress reaction in rats. *Biochem Biophys Res Commun* 270:318-323.
- Ito N., Yabe T., Gamo Y., Nagai T., Oikawa T., Yamada H., Hanawa T. (2008) I.C.V. Administration of orexin-a induces an antidepressive-like effect through hippocampal cell proliferation. *Neuroscience* 157:720-732.
- Jacob W., Marsch R., Marsicano G., Lutz B., Wotjak C. T. (2012) Cannabinoid cb1 receptor deficiency increases contextual fear memory under highly aversive conditions and long-term potentiation in vivo. *Neurobiol Learn Mem* 98:47-55.
- Jaeger L. B., Farr S. A., Banks W. A., Morley J. E. (2002) Effects of orexin-a on memory processing. *Peptides* 23:1683-1688.
- Jain M. R., Horvath T. L., Kalra P. S., Kalra S. P. (2000) Evidence that npy y1 receptors are involved in stimulation of feeding by orexins (hypocretins) in sated rats. *Regul Pept* 87:19-24.

- Jang J. H., Lee C. S., Hwang D., Ryu S. H. (2012) Understanding of the roles of phospholipase d and phosphatidic acid through their binding partners. *Prog Lipid Res* 51:71-81.
- Jaszberenyi M., Bujdoso E., Pataki I., Telegdy G. (2000) Effects of orexins on the hypothalamic-pituitary-adrenal system. *J Neuroendocrinol* 12:1174-1178.
- Jbilo O., Ravinet-Trillou C., Arnone M., Buisson I., Bribes E., Peleraux A., Penarier G., Soubrie P., Le Fur G., Galiegue S., Casellas P. (2005) The cb1 receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. *FASEB J* 19:1567-1569.
- Jenkins G. H., Fiset P. L., Anderson R. A. (1994) Type i phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem* 269:11547-11554.
- Jenkins G. M., Frohman M. A. (2005) Phospholipase d: A lipid centric review. *Cell Mol Life Sci* 62:2305-2316.
- Jiang J. Y., Nagaraju M., Meyer R. C., Zhang L., Hamelberg D., Hall R. A., Brown E. M., Conn P. J., Yang J. J. (2014) Extracellular calcium modulates actions of orthosteric and allosteric ligands on metabotropic glutamate receptor 1alpha. *J Biol Chem* 289:1649-1661.
- Johansson L., Ekholm M. E., Kukkonen J. P. (2007) Regulation of ox(1) orexin/hypocretin receptor-coupling to phospholipase c by ca(2+) influx. *Br J Pharmacol* 150:97-104.
- Johansson L., Ekholm M. E., Kukkonen J. P. (2008) Multiple phospholipase activation by ox(1) orexin/hypocretin receptors. *Cell Mol Life Sci* 65:1948-1956.
- Jones D. N., Gartlon J., Parker F., Taylor S. G., Routledge C., Hemmati P., Munton R. P., Ashmeade T. E., Hatcher J. P., Johns A., Porter R. A., Hagan J. J., Hunter A. J., Upton N. (2001) Effects of centrally administered orexin-b and orexin-a: A role for orexin-1 receptors in orexin-b-induced hyperactivity. *Psychopharmacology (Berl)* 153:210-218.
- Jöhren O., Bruggemann N., Dendorfer A., Dominiak P. (2003) Gonadal steroids differentially regulate the messenger ribonucleic acid expression of pituitary orexin type 1 receptors and adrenal orexin type 2 receptors. *Endocrinology* 144:1219-1225.
- Jöhren O., Neidert S. J., Kummer M., Dendorfer A., Dominiak P. (2001) Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* 142:3324-3331.
- Kajimura S., Saito M. (2014) A new era in brown adipose tissue biology: Molecular control of brown fat development and energy homeostasis. *Annu Rev Physiol* 76:225-249.
- Kajiyama S., Kawamoto M., Shiraishi S., Gaus S., Matsunaga A., Suyama H., Yuge O. (2005) Spinal orexin-1 receptors mediate anti-hyperalgesic effects of intrathecally-administered orexins in diabetic neuropathic pain model rats. *Brain Res* 1044:76-86.
- Kamano H., Burk B., Noben-Trauth K., Klempnauer K. H. (1995) Differential splicing of the mouse b-myb gene. *Oncogene* 11:2575-2582.
- Kaminski N. E., Abood M. E., Kessler F. K., Martin B. R., Schatz A. R. (1992) Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol Pharmacol* 42:736-742.

- Kaminski T., Smolinska N., Nitkiewicz A., Przala J. (2010) Expression of orexin receptors 1 (ox1r) and 2 (ox2r) in the porcine hypothalamus during the oestrous cycle. *J Physiol Pharmacol* 61:363-371.
- Kano M., Ohno-Shosaku T., Hashimotodani Y., Uchigashima M., Watanabe M. (2009) Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev* 89:309-380.
- Karnani M. M., Apergis-Schoute J., Adamantidis A., Jensen L. T., De Lecea L., Fugger L., Burdakov D. (2011) Activation of central orexin/hypocretin neurons by dietary amino acids. *Neuron* 72:616-629.
- Karteris E., Chen J., Randeve H. S. (2004) Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. *J Clin Endocrinol Metab* 89:1957-1962.
- Karteris E., Machado R. J., Chen J., Zervou S., Hillhouse E. W., Randeve H. S. (2005) Food deprivation differentially modulates orexin receptor expression and signalling in the rat hypothalamus and adrenal cortex. *Am J Physiol Endocrinol Metab* 288:E1089-1100.
- Karteris E., Randeve H. S., Grammatopoulos D. K., Jaffe R. B., Hillhouse E. W. (2001) Expression and coupling characteristics of the crh and orexin type 2 receptors in human fetal adrenals. *J Clin Endocrinol Metab* 86:4512-4519.
- Kaslin J., Nystedt J. M., Ostergard M., Peitsaro N., Panula P. (2004) The orexin/hypocretin system in zebrafish is connected to the aminergic and cholinergic systems. *J Neurosci* 24:2678-2689.
- Kauer J. A., Malenka R. C. (2007) Synaptic plasticity and addiction. *Nat Rev Neurosci* 8:844-858.
- Kawada Y., Ueno S., Asayama K., Tsutsui M., Utsunomiya K., Toyohira Y., Morisada N., Tanaka K., Shirahata A., Yanagihara N. (2003) Stimulation of catecholamine synthesis by orexin-a in bovine adrenal medullary cells through orexin receptor 1. *Biochem Pharmacol* 66:141-147.
- Kay K., Parise E. M., Lilly N., Williams D. L. (2014) Hindbrain orexin 1 receptors influence palatable food intake, operant responding for food, and food-conditioned place preference in rats. *Psychopharmacology (Berl)* 231:419-427.
- Kelley A. E., Baldo B. A., Pratt W. E. (2005) A proposed hypothalamic-thalamic-striatal axis for the integration of energy balance, arousal, and food reward. *J Comp Neurol* 493:72-85.
- Kessler B. A., Stanley E. M., Frederick-Duus D., Fadel J. (2011) Age-related loss of orexin/hypocretin neurons. *Neuroscience* 178:82-88.
- Kirchgessner A. L. (2002) Orexins in the brain-gut axis. *Endocr Rev* 23:1-15.
- Kirchgessner A. L., Liu M. (1999) Orexin synthesis and response in the gut. *Neuron* 24:941-951.
- Kirkwood A., Dudek S. M., Gold J. T., Aizenman C. D., Bear M. F. (1993) Common forms of synaptic plasticity in the hippocampus and neocortex in vitro. *Science* 260:1518-1521.
- Kis G. K., Ocsko T., Galfi M., Radacs M., Molnar Z., Rakosi K., Molnar A. H., Laszlo F., Varga C., Laszlo F. A. (2011) The effects of orexins on monoaminergic-induced changes in vasopressin level in rat neurohypophyseal cell cultures. *Neuropeptides* 45:385-389.
- Kiwaki K., Kotz C. M., Wang C., Lanningham-Foster L., Levine J. A. (2004) Orexin a (hypocretin 1) injected into hypothalamic paraventricular nucleus and

- spontaneous physical activity in rats. *Am J Physiol Endocrinol Metab* 286:E551-559.
- Kobilka B. K. (2007) G protein coupled receptor structure and activation. *Biochim Biophys Acta* 1768:794-807.
- Kohlmeier K. A., Watanabe S., Tyler C. J., Burlet S., Leonard C. S. (2008) Dual orexin actions on dorsal raphe and laterodorsal tegmentum neurons: Noisy cation current activation and selective enhancement of ca^{2+} transients mediated by l-type calcium channels. *J Neurophysiol* 100:2265-2281.
- Kok S. W., Roelfsema F., Overeem S., Lammers G. J., Strijers R. L., Frolich M., Meinders A. E., Pijl H. (2002) Dynamics of the pituitary-adrenal ensemble in hypocretin-deficient narcoleptic humans: Blunted basal adrenocorticotropin release and evidence for normal time-keeping by the master pacemaker. *J Clin Endocrinol Metab* 87:5085-5091.
- Kortenjann M T. O., Shaw Pe. (1994) Inhibition of v-raf-dependent c-fos expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase erk2. *Mol Cell Biol* 14:4815-4824.
- Kotz C. M., Teske J. A., Levine J. A., Wang C. (2002) Feeding and activity induced by orexin a in the lateral hypothalamus in rats. *Regul Pept* 104:27-32.
- Kramer R. M., Roberts E. F., Manetta J., Putnam J. E. (1991) The ca^{2+} -sensitive cytosolic phospholipase a2 is a 100-kda protein in human monoblast u937 cells. *J Biol Chem* 266:5268-5272.
- Kukkonen J. P. (2011) A menage a trois made in heaven: G-protein-coupled receptors, lipids and trp channels. *Cell Calcium* 50:9-26.
- Kukkonen J. P. (2013a) Lipid signaling cascades of orexin/hypocretin receptors. *Biochimie*.
- Kukkonen J. P. (2013b) Physiology of the orexinergic/hypocretinergic system: A revisit in 2012. *American journal of physiology Cell physiology* 304:C2-32.
- Kukkonen J. P., Leonard C. S. (2013) Orexin/hypocretin receptor signalling cascades. *Br J Pharmacol*.
- Laricchiuta D., Centonze D., Petrosini L. (2013) Effects of endocannabinoid and endovanilloid systems on aversive memory extinction. *Behav Brain Res* 256:101-107.
- Larsson K. P., Peltonen H. M., Bart G., Louhivuori L. M., Penttonen A., Antikainen M., Kukkonen J. P., Åkerman K. E. (2005) Orexin-a-induced ca^{2+} entry: Evidence for involvement of trpc channels and protein kinase c regulation. *J Biol Chem* 280:1771-1781.
- Lauckner J. E., Hille B., Mackie K. (2005) The cannabinoid agonist win55,212-2 increases intracellular calcium via cb1 receptor coupling to gq/11 g proteins. *Proc Natl Acad Sci U S A* 102:19144-19149.
- Lawrence A. J., Cowen M. S., Yang H. J., Chen F., Oldfield B. (2006) The orexin system regulates alcohol-seeking in rats. *Br J Pharmacol* 148:752-759.
- Leonard C. S., Kukkonen J. P. (2013) Orexin/hypocretin receptor signalling: A functional perspective. *Br J Pharmacol*.
- Li B., Chen F., Ye J., Chen X., Yan J., Li Y., Xiong Y., Zhou Z., Xia J., Hu Z. (2010) The modulation of orexin a on hcn currents of pyramidal neurons in mouse prelimbic cortex. *Cereb Cortex* 20:1756-1767.
- Lichtman A. H., Cook S. A., Martin B. R. (1996) Investigation of brain sites mediating cannabinoid-induced antinociception in rats: Evidence supporting periaqueductal gray involvement. *J Pharmacol Exp Ther* 276:585-593.

- Liguori G., Assisi L., Squillaciotti C., Paino S., Mirabella N., Vittoria A. (2012) Presence, distribution and steroidogenic effect of the peptides orexin a and receptor 1 for orexins in the testis of the south american camelid alpaca (*vicugna pacos*). *Gen Comp Endocrinol* 179:137-142.
- Lin H. H. (2013) G-protein-coupled receptors and their (bio) chemical significance win 2012 nobel prize in chemistry. *Biomedical journal* 36:118-124.
- Lin L., Faraco J., Li R., Kadotani H., Rogers W., Lin X., Qiu X., De Jong P. J., Nishino S., Mignot E. (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98:365-376.
- Liscovitch M., Chalifa V., Pertile P., Chen C. S., Cantley L. C. (1994) Novel function of phosphatidylinositol 4,5-bisphosphate as a cofactor for brain membrane phospholipase d. *J Biol Chem* 269:21403-21406.
- Lopez De Jesus M., Stope M. B., Oude Weernink P. A., Mahlke Y., Borgermann C., Ananaba V. N., Rimmbach C., Rosskopf D., Michel M. C., Jakobs K. H., Schmidt M. (2006) Cyclic amp-dependent and epac-mediated activation of r-ras by g protein-coupled receptors leads to phospholipase d stimulation. *J Biol Chem* 281:21837-21847.
- Lopez M., Senaris R., Gallego R., Garcia-Caballero T., Lago F., Seoane L., Casanueva F., Dieguez C. (1999) Orexin receptors are expressed in the adrenal medulla of the rat. *Endocrinology* 140:5991-5994.
- Lopez M., Seoane L., Garcia M. C., Lago F., Casanueva F. F., Senaris R., Dieguez C. (2000) Leptin regulation of prepro-orexin and orexin receptor mrna levels in the hypothalamus. *Biochem Biophys Res Commun* 269:41-45.
- Louhivuori L. M., Jansson L., Nordstrom T., Bart G., Nasman J., Akerman K. E. (2010) Selective interference with trpc3/6 channels disrupts ox1 receptor signalling via ncx and reveals a distinct calcium influx pathway. *Cell Calcium* 48:114-123.
- Lu X. Y., Bagnol D., Burke S., Akil H., Watson S. J. (2000) Differential distribution and regulation of ox1 and ox2 orexin/hypocretin receptor messenger rna in the brain upon fasting. *Horm Behav* 37:335-344.
- Lubkin M., Stricker-Krongrad A. (1998) Independent feeding and metabolic actions of orexins in mice. *Biochem Biophys Res Commun* 253:241-245.
- Lund P. E., Shariatmadari R., Uustare A., Dethoux M., Parmentier M., Kukkonen J. P., Åkerman K. E. O. (2000) The orexin ox1 receptor activates a novel ca²⁺ influx pathway necessary for coupling to phospholipase c. *J Biol Chem* 275:30806-30812.
- Mackie K., Lai Y., Westenbroek R., Mitchell R. (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit q-type calcium currents in att20 cells transfected with rat brain cannabinoid receptor. *J Neurosci* 15:6552-6561.
- Madden C. J., Tupone D., Morrison S. F. (2012) Orexin modulates brown adipose tissue thermogenesis. *Biomolecular concepts* 3:381-386.
- Magalhaes A. C., Dunn H., Ferguson S. S. (2012) Regulation of gpcr activity, trafficking and localization by gpcr-interacting proteins. *Br J Pharmacol* 165:1717-1736.
- Malendowicz L. K., Hochol A., Ziolkowska A., Nowak M., Gottardo L., Nussdorfer G. G. (2001) Prolonged orexin administration stimulates steroid-hormone secretion, acting directly on the rat adrenal gland. *International journal of molecular medicine* 7:401-404.

- Malendowicz L. K., Tortorella C., Nussdorfer G. G. (1999) Orexins stimulate corticosterone secretion of rat adrenocortical cells, through the activation of the adenylate cyclase-dependent signaling cascade. *J Steroid Biochem Mol Biol* 70:185-188.
- Martin W. J., Coffin P. O., Attias E., Balinsky M., Tsou K., Walker J. M. (1999) Anatomical basis for cannabinoid-induced antinociception as revealed by intracerebral microinjections. *Brain Res* 822:237-242.
- Martin W. J., Patrick S. L., Coffin P. O., Tsou K., Walker J. M. (1995) An examination of the central sites of action of cannabinoid-induced antinociception in the rat. *Life Sci* 56:2103-2109.
- Martynska L., Wolinska-Witort E., Chmielowska M., Bik W., Baranowska B. (2011) Evaluation of orexin a activity on lh and fsh release from primary culture pituitary cells in immature and mature female rats. *Neuro endocrinology letters* 32:769-773.
- Mathes C. M., Ferrara M., Rowland N. E. (2008) Cannabinoid-1 receptor antagonists reduce caloric intake by decreasing palatable diet selection in a novel dessert protocol in female rats. *American journal of physiology Regulatory, integrative and comparative physiology* 295:R67-75.
- Matsuda L. A., Lolait S. J., Brownstein M. J., Young A. C., Bonner T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346:561-564.
- Mayr B., Montminy M. (2001) Transcriptional regulation by the phosphorylation-dependent factor creb. *Nature reviews Molecular cell biology* 2:599-609.
- Mazzocchi G., Malendowicz L. K., Gottardo L., Aragona F., Nussdorfer G. G. (2001) Orexin a stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. *J Clin Endocrinol Metab* 86:778-782.
- Mebratu Y., Tesfaigzi Y. (2009) How erk1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle* 8:1168-1175.
- Mechoulam R., Spatz M., Shohami E. (2002) Endocannabinoids and neuroprotection. *Sci STKE* 2002:re5.
- Mendoza M. C., Er E. E., Blenis J. (2011) The ras-erk and pi3k-mtor pathways: Cross-talk and compensation. *Trends Biochem Sci* 36:320-328.
- Meves H. (2008) Arachidonic acid and ion channels: An update. *Br J Pharmacol* 155:4-16.
- Mignen O., Shuttleworth T. J. (2000) I(arc), a novel arachidonate-regulated, noncapacitative ca(2+) entry channel. *J Biol Chem* 275:9114-9119.
- Mignot E., Lammers G. J., Ripley B., Okun M., Nevsimalova S., Overeem S., Vankova J., Black J., Harsh J., Bassetti C., Schrader H., Nishino S. (2002) The role of cerebrospinal fluid hypocretin measurement in the diagnosis of narcolepsy and other hypersomnias. *Arch Neurol* 59:1553-1562.
- Milasta S., Evans N. A., Ormiston L., Wilson S., Lefkowitz R. J., Milligan G. (2005) The sustainability of interactions between the orexin-1 receptor and beta-arrestin-2 is defined by a single c-terminal cluster of hydroxy amino acids and modulates the kinetics of erk mapk regulation. *Biochem J* 387:573-584.
- Milligan G., Kostenis E. (2006) Heterotrimeric g-proteins: A short history. *Br J Pharmacol* 147 Suppl 1:S46-55.
- Mitchell R., McCulloch D., Lutz E., Johnson M., Mackenzie C., Fennell M., Fink G., Zhou W., Sealson S. C. (1998) Rhodopsin-family receptors associate with small g proteins to activate phospholipase d. *Nature* 392:411-414.

- Miyasaka K., Masuda M., Kanai S., Sato N., Kurosawa M., Funakoshi A. (2002) Central orexin-a stimulates pancreatic exocrine secretion via the vagus. *Pancreas* 25:400-404.
- Molina-Holgado F., Molina-Holgado E., Guaza C. (1998) The endogenous cannabinoid anandamide potentiates interleukin-6 production by astrocytes infected with theiler's murine encephalomyelitis virus by a receptor-mediated pathway. *FEBS Lett* 433:139-142.
- Monda M., Viggiano A., Fuccio F., De Luca V. (2004) Injection of orexin a into the diagonal band of broca induces sympathetic and hyperthermic reactions. *Brain Res* 1018:265-271.
- Monda M., Viggiano A., Mondola P., De Luca V. (2001) Inhibition of prostaglandin synthesis reduces hyperthermic reactions induced by hypocretin-1/orexin a. *Brain Res* 909:68-74.
- Monteleone P., Piscitelli F., Scognamiglio P., Monteleone A. M., Canestrelli B., Di Marzo V., Maj M. (2012) Hedonic eating is associated with increased peripheral levels of ghrelin and the endocannabinoid 2-arachidonoyl-glycerol in healthy humans: A pilot study. *J Clin Endocrinol Metab* 97:E917-924.
- Moreno G., Perello M., Gaillard R. C., Spinedi E. (2005) Orexin a stimulates hypothalamic-pituitary-adrenal (hpa) axis function, but not food intake, in the absence of full hypothalamic npy-ergic activity. *Endocrine* 26:99-106.
- Morris A. J., Frohman M. A., Engebrecht J. (1997) Measurement of phospholipase d activity. *Anal Biochem* 252:1-9.
- Mu J., Zhuang S. Y., Kirby M. T., Hampson R. E., Deadwyler S. A. (1999) Cannabinoid receptors differentially modulate potassium a and d currents in hippocampal neurons in culture. *J Pharmacol Exp Ther* 291:893-902.
- Munro S., Thomas K. L., Abu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365:61-65.
- Nagayama T., Sinor A. D., Simon R. P., Chen J., Graham S. H., Jin K., Greenberg D. A. (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci* 19:2987-2995.
- Naidu P. S., Kinsey S. G., Guo T. L., Cravatt B. F., Lichtman A. H. (2010) Regulation of inflammatory pain by inhibition of fatty acid amide hydrolase. *J Pharmacol Exp Ther* 334:182-190.
- Nakabayashi M., Suzuki T., Takahashi K., Totsune K., Muramatsu Y., Kaneko C., Date F., Takeyama J., Darnel A. D., Moriya T., Sasano H. (2003) Orexin-a expression in human peripheral tissues. *Mol Cell Endocrinol* 205:43-50.
- Nakajima Y., Nakajima S. (2010) Measurement of orexin (hypocretin) and substance p effects on constitutively active inward rectifier k(+) channels in brain neurons. *Methods Enzymol* 484:613-630.
- Nambu T., Sakurai T., Mizukami K., Hosoya Y., Yanagisawa M., Goto K. (1999) Distribution of orexin neurons in the adult rat brain. *Brain Res* 827:243-260.
- Nanmoku T., Isobe K., Sakurai T., Yamanaka A., Takekoshi K., Kawakami Y., Goto K., Nakai T. (2002) Effects of orexin on cultured porcine adrenal medullary and cortex cells. *Regul Pept* 104:125-130.
- Narita M., Nagumo Y., Hashimoto S., Khotib J., Miyatake M., Sakurai T., Yanagisawa M., Nakamachi T., Shioda S., Suzuki T. (2006) Direct involvement of orexinergic systems in the activation of the mesolimbic dopamine pathway and related behaviors induced by morphine. *J Neurosci* 26:398-405.

- Nishino S., Ripley B., Overeem S., Nevsimalova S., Lammers G. J., Vankova J., Okun M., Rogers W., Brooks S., Mignot E. (2001) Low cerebrospinal fluid hypocretin (orexin) and altered energy homeostasis in human narcolepsy. *Ann Neurol* 50:381-388.
- Nowak K. W., Strowski M. Z., Switonska M. M., Kaczmarek P., Singh V., Fabis M., Mackowiak P., Nowak M., Malendowicz L. K. (2005) Evidence that orexins a and b stimulate insulin secretion from rat pancreatic islets via both receptor subtypes. *International journal of molecular medicine* 15:969-972.
- O'keefe L., Simcocks A. C., Hryciw D. H., Mathai M. L., Mcainch A. J. (2014) The cannabinoid receptor 1 and its role in influencing peripheral metabolism. *Diabetes Obes Metab* 16:294-304.
- Ocsko T., Galfi M., Radacs M., Molnar Z., Kis G. K., Rakosi K., Molnar A. H., Laszlo F., Laszlo F. A., Varga C. (2012) Effects of orexin-monoaminergic interactions on oxytocin secretion in rat neurohypophyseal cell cultures. *Regul Pept* 175:43-48.
- Ohno-Shosaku T., Maejima T., Kano M. (2001) Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. *Neuron* 29:729-738.
- Osisami M., Ali W., Frohman M. A. (2012) A role for phospholipase d3 in myotube formation. *PloS one* 7:e33341.
- Ouedraogo R., Naslund E., Kirchgessner A. L. (2003) Glucose regulates the release of orexin-a from the endocrine pancreas. *Diabetes* 52:111-117.
- Overeem S., Kok S. W., Lammers G. J., Vein A. A., Frolich M., Meinders A. E., Roelfsema F., Pijl H. (2003) Somatotropic axis in hypocretin-deficient narcoleptic humans: Altered circadian distribution of gh-secretory events. *Am J Physiol Endocrinol Metab* 284:E641-647.
- Pagotto U., Marsicano G., Cota D., Lutz B., Pasquali R. (2006) The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev* 27:73-100.
- Pan X., Ikeda S. R., Lewis D. L. (1996) Rat brain cannabinoid receptor modulates n-type ca²⁺ channels in a neuronal expression system. *Mol Pharmacol* 49:707-714.
- Panikashvili D., Mechoulam R., Beni S. M., Alexandrovich A., Shohami E. (2005) Cbl cannabinoid receptors are involved in neuroprotection via nf-kappa b inhibition. *J Cereb Blood Flow Metab* 25:477-484.
- Pasumarthi R. K., Reznikov L. R., Fadel J. (2006) Activation of orexin neurons by acute nicotine. *Eur J Pharmacol* 535:172-176.
- Patel S., Roelke C. T., Rademacher D. J., Cullinan W. E., Hillard C. J. (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* 145:5431-5438.
- Peltonen H. M., Åkerman K. E., Bart G. (2010) A role for pkd1 and pkd3 activation in modulation of calcium oscillations induced by orexin receptor 1 stimulation. *Biochim Biophys Acta* 1803:1206-1212.
- Petersen A. M., Pedersen B. K. (2005) The anti-inflammatory effect of exercise. *J Appl Physiol* (1985) 98:1154-1162.
- Peyron C., Faraco J., Rogers W., Ripley B., Overeem S., Charnay Y., Nevsimalova S., Aldrich M., Reynolds D., Albin R., Li R., Hungs M., Pedrazzoli M., Padigaru M., Kucherlapati M., Fan J., Maki R., Lammers G. J., Bouras C., Kucherlapati R., Nishino S., Mignot E. (2000) A mutation in a case of early

- onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 6:991-997.
- Peyron C., Tighe D. K., Van Den Pol A. N., De Lecea L., Heller H. C., Sutcliffe J. G., Kilduff T. S. (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18:9996-10015.
- Pillolla G., Melis M., Perra S., Muntoni A. L., Gessa G. L., Pistis M. (2007) Medial forebrain bundle stimulation evokes endocannabinoid-mediated modulation of ventral tegmental area dopamine neuron firing in vivo. *Psychopharmacology (Berl)* 191:843-853.
- Pin J. P., Neubig R., Bouvier M., Devi L., Filizola M., Javitch J. A., Lohse M. J., Milligan G., Palczewski K., Parmentier M., Spedding M. (2007) International union of basic and clinical pharmacology. Lxvii. Recommendations for the recognition and nomenclature of g protein-coupled receptor heteromultimers. *Pharmacol Rev* 59:5-13.
- Plaza-Zabala A., Flores A., Martin-Garcia E., Saravia R., Maldonado R., Berrendero F. (2013) A role for hypocretin/orexin receptor-1 in cue-induced reinstatement of nicotine-seeking behavior. *Neuropsychopharmacology* 38:1724-1736.
- Plaza-Zabala A., Martin-Garcia E., De Lecea L., Maldonado R., Berrendero F. (2010) Hypocretins regulate the anxiogenic-like effects of nicotine and induce reinstatement of nicotine-seeking behavior. *J Neurosci* 30:2300-2310.
- Porkka-Heiskanen T., Alanko L., Kalinchuk A., Heiskanen S., Stenberg D. (2004) The effect of age on prepro-orexin gene expression and contents of orexin a and b in the rat brain. *Neurobiol Aging* 25:231-238.
- Prus A. J., James J. R., Rosecrans J. A. (2009) Conditioned place preference. In: *Methods of behavior analysis in neuroscience*(Buccafusco, J. J., ed) Boca Raton (FL).
- Quarta D., Valerio E., Hutcheson D. M., Hedou G., Heidbreder C. (2010) The orexin-1 receptor antagonist sb-334867 reduces amphetamine-evoked dopamine outflow in the shell of the nucleus accumbens and decreases the expression of amphetamine sensitization. *Neurochem Int* 56:11-15.
- Rainero I., Rubino E., Gallone S., Fenoglio P., Picci L. R., Giobbe L., Ostacoli L., Pinessi L. (2011) Evidence for an association between migraine and the hypocretin receptor 1 gene. *The journal of headache and pain* 12:193-199.
- Ramanjaneya M., Conner A. C., Chen J., Kumar P., Brown J. E., Jöhren O., Lehnert H., Stanfield P. R., Randeve H. S. (2009) Orexin-stimulated map kinase cascades are activated through multiple g-protein signalling pathways in human h295r adrenocortical cells: Diverse roles for orexins a and b. *J Endocrinol* 202:249-261.
- Ramanjaneya M., Conner A. C., Chen J., Stanfield P. R., Randeve H. S. (2008) Orexins stimulate steroidogenic acute regulatory protein expression through multiple signaling pathways in human adrenal h295r cells. *Endocrinology* 149:4106-4115.
- Randeve H. S., Karteris E., Grammatopoulos D., Hillhouse E. W. (2001) Expression of orexin-a and functional orexin type 2 receptors in the human adult adrenals: Implications for adrenal function and energy homeostasis. *J Clin Endocrinol Metab* 86:4808-4813.
- Ravinet Trillou C., Delgorge C., Menet C., Arnone M., Soubrie P. (2004) Cb1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int J Obes Relat Metab Disord* 28:640-648.

- Rebois R. V., Warner D. R., Basi N. S. (1997) Does subunit dissociation necessarily accompany the activation of all heterotrimeric g proteins? *Cell Signal* 9:141-151.
- Reibaud M., Obinu M. C., Ledent C., Parmentier M., Bohme G. A., Imperato A. (1999) Enhancement of memory in cannabinoid cb1 receptor knock-out mice. *Eur J Pharmacol* 379:R1-2.
- Rotter A., Bayerlein K., Hansbauer M., Weiland J., Sperling W., Kornhuber J., Biermann T. (2012) Orexin a expression and promoter methylation in patients with cannabis dependence in comparison to nicotine-dependent cigarette smokers and nonsmokers. *Neuropsychobiology* 66:126-133.
- Rouet-Benzineb P., Rouyer-Fessard C., Jarry A., Avondo V., Pouzet C., Yanagisawa M., Laboisie C., Laburthe M., Voisin T. (2004) Orexins acting at native ox(1) receptor in colon cancer and neuroblastoma cells or at recombinant ox(1) receptor suppress cell growth by inducing apoptosis. *J Biol Chem* 279:45875-45886.
- Rumenapp U., Geiszt M., Wahn F., Schmidt M., Jakobs K. H. (1995) Evidence for adp-ribosylation-factor-mediated activation of phospholipase d by m3 muscarinic acetylcholine receptor. *Eur J Biochem* 234:240-244.
- Russell S. H., Small C. J., Dakin C. L., Abbott C. R., Morgan D. G., Ghatei M. A., Bloom S. R. (2001) The central effects of orexin-a in the hypothalamic-pituitary-adrenal axis in vivo and in vitro in male rats. *J Neuroendocrinol* 13:561-566.
- Sadeghi S., Reisi Z., Azhdari-Zarmehri H., Haghparast A. (2013) Involvement of orexin-1 receptors in the ventral tegmental area and the nucleus accumbens in antinociception induced by lateral hypothalamus stimulation in rats. *Pharmacol Biochem Behav* 105:193-198.
- Sakurai T. (1999) Orexins and orexin receptors: Implication in feeding behavior. *Regul Pept* 85:25-30.
- Sakurai T. (2007) The neural circuit of orexin (hypocretin): Maintaining sleep and wakefulness. *Nat Rev Neurosci* 8:171-181.
- Sakurai T. (2013) Orexin deficiency and narcolepsy. *Curr Opin Neurobiol* 23:760-766.
- Sakurai T., Amemiya A., Ishii M., Matsuzaki I., Chemelli R. M., Tanaka H., Williams S. C., Richardson J. A., Kozlowski G. P., Wilson S., Arch J. R., Buckingham R. E., Haynes A. C., Carr S. A., Annan R. S., McNulty D. E., Liu W. S., Terrett J. A., Elshourbagy N. A., Bergsma D. J., Yanagisawa M. (1998) Orexins and orexin receptors: A family of hypothalamic neuropeptides and g protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-585.
- Sakurai T., Nagata R., Yamanaka A., Kawamura H., Tsujino N., Muraki Y., Kageyama H., Kunita S., Takahashi S., Goto K., Koyama Y., Shioda S., Yanagisawa M. (2005) Input of orexin/hypocretin neurons revealed by a genetically encoded tracer in mice. *Neuron* 46:297-308.
- Salahpour A., Espinoza S., Masri B., Lam V., Barak L. S., Gainetdinov R. R. (2012) Bret biosensors to study gpcr biology, pharmacology, and signal transduction. *Front Endocrinol (Lausanne)* 3:105.
- Samson W. K., Gosnell B., Chang J. K., Resch Z. T., Murphy T. C. (1999) Cardiovascular regulatory actions of the hypocretins in brain. *Brain Res* 831:248-253.
- Sanchez M. G., Ruiz-Llorente L., Sanchez A. M., Diaz-Laviada I. (2003) Activation of phosphoinositide 3-kinase/pkb pathway by cb(1) and cb(2) cannabinoid

- receptors expressed in prostate pc-3 cells. Involvement in raf-1 stimulation and ngf induction. *Cell Signal* 15:851-859.
- Sarchielli P., Rainero I., Coppola F., Rossi C., Mancini M., Pinessi L., Calabresi P. (2008) Involvement of corticotrophin-releasing factor and orexin-a in chronic migraine and medication-overuse headache: Findings from cerebrospinal fluid. *Cephalalgia* 28:714-722.
- Satoh Y., Uchida M., Fujita A., Nishio H., Takeuchi T., Hata F. (2001) Possible role of orexin a in nonadrenergic, noncholinergic inhibitory response of muscle of the mouse small intestine. *Eur J Pharmacol* 428:337-342.
- Savinainen J. R., Saario S. M., Niemi R., Jarvinen T., Laitinen J. T. (2003) An optimized approach to study endocannabinoid signaling: Evidence against constitutive activity of rat brain adenosine a1 and cannabinoid cb1 receptors. *Br J Pharmacol* 140:1451-1459.
- Schioth H. B., Fredriksson R. (2005) The G-protein coupled receptor classification system in comparative perspective. *Gen Comp Endocrinol* 142:94-101.
- Schmidt M., Dekker F. J., Maarsingh H. (2013) Exchange protein directly activated by camp (epac): A multidomain camp mediator in the regulation of diverse biological functions. *Pharmacol Rev* 65:670-709.
- Schmidt M., Rumenapp U., Bienek C., Keller J., Von Eichel-Streiber C., Jakobs K. H. (1996) Inhibition of receptor signaling to phospholipase d by clostridium difficile toxin b. Role of rho proteins. *J Biol Chem* 271:2422-2426.
- Schweitzer P. (2000) Cannabinoids decrease the k(+) m-current in hippocampal cal neurons. *J Neurosci* 20:51-58.
- Scott S. A., Selvy P. E., Buck J. R., Cho H. P., Criswell T. L., Thomas A. L., Armstrong M. D., Arteaga C. L., Lindsley C. W., Brown H. A. (2009) Design of isoform-selective phospholipase d inhibitors that modulate cancer cell invasiveness. *Nat Chem Biol* 5:108-117.
- Sears R. M., Fink A. E., Wigstrand M. B., Farb C. R., De Lecea L., Ledoux J. E. (2013) Orexin/hypocretin system modulates amygdala-dependent threat learning through the locus coeruleus. *Proc Natl Acad Sci U S A* 110:20260-20265.
- Selbach O., Bohla C., Barbara A., Doreulee N., Eriksson K. S., Sergeeva O. A., Haas H. L. (2010) Orexins/hypocretins control bistability of hippocampal long-term synaptic plasticity through co-activation of multiple kinases. *Acta Physiol (Oxf)* 198:277-285.
- Selbach O., Doreulee N., Bohla C., Eriksson K. S., Sergeeva O. A., Poelchen W., Brown R. E., Haas H. L. (2004) Orexins/hypocretins cause sharp wave- and theta-related synaptic plasticity in the hippocampus via glutamatergic, gabaergic, noradrenergic, and cholinergic signaling. *Neuroscience* 127:519-528.
- Sellayah D., Bharaj P., Sikder D. (2011) Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab* 14:478-490.
- Seno K., Okuno T., Nishi K., Murakami Y., Watanabe F., Matsuura T., Wada M., Fujii Y., Yamada M., Ogawa T., Okada T., Hashizume H., Kii M., Hara S., Hagishita S., Nakamoto S., Yamada K., Chikazawa Y., Ueno M., Teshirogi I., Ono T., Ohtani M. (2000) Pyrrolidine inhibitors of human cytosolic phospholipase a(2). *J Med Chem* 43:1041-1044.

- Sharf R., Guarnieri D. J., Taylor J. R., Dileone R. J. (2010) Orexin mediates morphine place preference, but not morphine-induced hyperactivity or sensitization. *Brain Res* 1317:24-32.
- Sharf R., Sarhan M., Dileone R. J. (2008) Orexin mediates the expression of precipitated morphine withdrawal and concurrent activation of the nucleus accumbens shell. *Biol Psychiatry* 64:175-183.
- Sharkey K. A., Darmani N. A., Parker L. A. (2014) Regulation of nausea and vomiting by cannabinoids and the endocannabinoid system. *Eur J Pharmacol* 722:134-146.
- Shen J., Tanida M., Yao J. F., Nijima A., Nagai K. (2008) Biphasic effects of orexin-a on autonomic nerve activity and lipolysis. *Neurosci Lett* 444:166-171.
- Shen M., Piser T. M., Seybold V. S., Thayer S. A. (1996) Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J Neurosci* 16:4322-4334.
- Shen Y., Zhao Y., Zheng D., Chang X., Ju S., Guo L. (2013) Effects of orexin a on glut4 expression and lipid content via mapk signaling in 3t3-l1 adipocytes. *J Steroid Biochem Mol Biol* 138:376-383.
- Sheng W. S., Hu S., Min X., Cabral G. A., Lokensgard J. R., Peterson P. K. (2005) Synthetic cannabinoid win55,212-2 inhibits generation of inflammatory mediators by il-1beta-stimulated human astrocytes. *Glia* 49:211-219.
- Shinohara Y., Inui T., Yamamoto T., Shimura T. (2009) Cannabinoid in the nucleus accumbens enhances the intake of palatable solution. *Neuroreport* 20:1382-1385.
- Shirai Y., Kashiwagi K., Yagi K., Sakai N., Saito N. (1998) Distinct effects of fatty acids on translocation of gamma- and epsilon-subspecies of protein kinase c. *J Cell Biol* 143:511-521.
- Shoblock J. R., Welty N., Aluisio L., Fraser I., Motley S. T., Morton K., Palmer J., Bonaventure P., Carruthers N. I., Lovenberg T. W., Boggs J., Galici R. (2011) Selective blockade of the orexin-2 receptor attenuates ethanol self-administration, place preference, and reinstatement. *Psychopharmacology (Berl)* 215:191-203.
- Shuttleworth T. J. (2009) Arachidonic acid, arc channels, and orai proteins. *Cell Calcium* 45:602-610.
- Sinor A. D., Irvin S. M., Greenberg D. A. (2000) Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. *Neurosci Lett* 278:157-160.
- Skrzypski M., Kaczmarek P., Le T. T., Wojciechowicz T., Pruszynska-Oszmalek E., Szczepankiewicz D., Sassek M., Arafat A., Wiedenmann B., Nowak K. W., Strowski M. Z. (2012) Effects of orexin a on proliferation, survival, apoptosis and differentiation of 3t3-l1 preadipocytes into mature adipocytes. *FEBS Lett* 586:4157-4164.
- Skrzypski M., T T. L., Kaczmarek P., Pruszynska-Oszmalek E., Pietrzak P., Szczepankiewicz D., Kolodziejski P. A., Sassek M., Arafat A., Wiedenmann B., Nowak K. W., Strowski M. Z. (2011) Orexin a stimulates glucose uptake, lipid accumulation and adiponectin secretion from 3t3-l1 adipocytes and isolated primary rat adipocytes. *Diabetologia* 54:1841-1852.
- Smart D., Jerman J. C., Brough S. J., Rushton S. L., Murdock P. R., Jewitt F., Elshourbagy N. A., Ellis C. E., Middlemiss D. N., Brown F. (1999) Characterization of recombinant human orexin receptor pharmacology in a chinese hamster ovary cell-line using fliper. *Br J Pharmacol* 128:1-3.

- Smith R. J., Aston-Jones G. (2012) Orexin / hypocretin 1 receptor antagonist reduces heroin self-administration and cue-induced heroin seeking. *Eur J Neurosci* 35:798-804.
- Soltoff S. P. (2007) Rottlerin: An inappropriate and ineffective inhibitor of pkcdelta. *Trends Pharmacol Sci* 28:453-458.
- Song D., Bandsma R. H., Xiao C., Xi L., Shao W., Jin T., Lewis G. F. (2011) Acute cannabinoid receptor type 1 (cb1r) modulation influences insulin sensitivity by an effect outside the central nervous system in mice. *Diabetologia* 54:1181-1189.
- Soya S., Shoji H., Hasegawa E., Hondo M., Miyakawa T., Yanagisawa M., Mieda M., Sakurai T. (2013) Orexin receptor-1 in the locus coeruleus plays an important role in cue-dependent fear memory consolidation. *J Neurosci* 33:14549-14557.
- Stanley B. G., Leibowitz S. F. (1984) Neuropeptide y: Stimulation of feeding and drinking by injection into the paraventricular nucleus. *Life Sci* 35:2635-2642.
- Starowicz K., Makuch W., Osikowicz M., Piscitelli F., Petrosino S., Di Marzo V., Przewlocka B. (2012) Spinal anandamide produces analgesia in neuropathic rats: Possible cb(1)- and trpv1-mediated mechanisms. *Neuropharmacology* 62:1746-1755.
- Steinberg S. F. (2004) Distinctive activation mechanisms and functions for protein kinase cdelta. *Biochem J* 384:449-459.
- Sugiura T., Kondo S., Sukagawa A., Nakane S., Shinoda A., Itoh K., Yamashita A., Waku K. (1995) 2-arachidonoylglycerol: A possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215:89-97.
- Sutcliffe J. G., De Lecea L. (1999) Novel neurotransmitters for sleep and energy homeostasis. *Results Probl Cell Differ* 26:239-255.
- Suyama H., Kawamoto M., Shiraishi S., Gaus S., Kajiyama S., Yuge O. (2004) Analgesic effect of intrathecal administration of orexin on neuropathic pain in rats. *In Vivo* 18:119-123.
- Takahashi N., Okumura T., Yamada H., Kohgo Y. (1999) Stimulation of gastric acid secretion by centrally administered orexin-a in conscious rats. *Biochem Biophys Res Commun* 254:623-627.
- Tang J., Chen J., Ramanjaneya M., Punn A., Conner A. C., Randevara H. S. (2008) The signalling profile of recombinant human orexin-2 receptor. *Cell Signal* 20:1651-1661.
- Taslimi Z., Haghparast A., Hassanpour-Ezatti M., Safari M. S. (2011) Chemical stimulation of the lateral hypothalamus induces conditioned place preference in rats: Involvement of ox1 and cb1 receptors in the ventral tegmental area. *Behav Brain Res* 217:41-46.
- Taylor C. W., Broad L. M. (1998) Pharmacological analysis of intracellular ca²⁺ signalling: Problems and pitfalls. *Trends Pharmacol Sci* 19:370-375.
- Telegdy G., Adamik A. (2002) The action of orexin a on passive avoidance learning. Involvement of transmitters. *Regul Pept* 104:105-110.
- Terao A., Apte-Deshpande A., Morairty S., Freund Y. R., Kilduff T. S. (2002) Age-related decline in hypocretin (orexin) receptor 2 messenger rna levels in the mouse brain. *Neurosci Lett* 332:190-194.
- Thannickal T. C., Moore R. Y., Nienhuis R., Ramanathan L., Gulyani S., Aldrich M., Cornford M., Siegel J. M. (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 27:469-474.

- Thornton T. M., Rincon M. (2009) Non-classical p38 map kinase functions: Cell cycle checkpoints and survival. *International journal of biological sciences* 5:44-51.
- Trivedi P., Yu H., Macneil D. J., Van Der Ploeg L. H., Guan X. M. (1998) Distribution of orexin receptor mRNA in the rat brain. *FEBS Lett* 438:71-75.
- Tsujino N., Sakurai T. (2013) Role of orexin in modulating arousal, feeding, and motivation. *Frontiers in behavioral neuroscience* 7:28.
- Tupone D., Madden C. J., Cano G., Morrison S. F. (2011) An orexinergic projection from perifornical hypothalamus to raphe pallidus increases rat brown adipose tissue thermogenesis. *J Neurosci* 31:15944-15955.
- Turunen P. M., Ekholm M. E., Somerharju P., Kukkonen J. P. (2010) Arachidonic acid release mediated by ox(1) orexin receptors. *Br J Pharmacol* 159:212 - 221.
- Turunen P. M., Jääntti M. H., Kukkonen J. P. (2012) Ox1 orexin receptor signaling via arachidonic acid and endocannabinoid release. *Manuscript*.
- Twitchell W., Brown S., Mackie K. (1997) Cannabinoids inhibit n- and p/q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol* 78:43-50.
- Uramura K., Funahashi H., Muroya S., Shioda S., Takigawa M., Yada T. (2001) Orexin-a activates phospholipase c- and protein kinase c-mediated ca²⁺ signaling in dopamine neurons of the ventral tegmental area. *Neuroreport* 12:1885-1889.
- Wagner I. V., Perwitz N., Drenckhan M., Lehnert H., Klein J. (2011) Cannabinoid type 1 receptor mediates depot-specific effects on differentiation, inflammation and oxidative metabolism in inguinal and epididymal white adipocytes. *Nutr Diabetes* 1:e16.
- Walker J. M., Huang S. M., Strangman N. M., Tsou K., Sanudo-Pena M. C. (1999) Pain modulation by release of the endogenous cannabinoid anandamide. *Proc Natl Acad Sci U S A* 96:12198-12203.
- Van Den Pol A. N. (1999) Hypothalamic hypocretin (orexin): Robust innervation of the spinal cord. *J Neurosci* 19:3171-3182.
- Van Den Pol A. N., Ghosh P. K., Liu R. J., Li Y., Aghajanian G. K., Gao X. B. (2002) Hypocretin (orexin) enhances neuron activity and cell synchrony in developing mouse gfp-expressing locus coeruleus. *J Physiol* 541:169-185.
- Wang C., Pan Y., Zhang R., Bai B., Chen J., Randevara H. S. (2013a) Heterodimerization of mouse orexin type 2 receptor variants and the effects on signal transduction. *Biochim Biophys Acta* 1843:652-663.
- Wang J., Osaka T., Inoue S. (2001) Energy expenditure by intracerebroventricular administration of orexin to anesthetized rats. *Neurosci Lett* 315:49-52.
- Wang J., Osaka T., Inoue S. (2003) Orexin-a-sensitive site for energy expenditure localized in the arcuate nucleus of the hypothalamus. *Brain Res* 971:128-134.
- Wang J., Ueda N. (2009) Biology of endocannabinoid synthesis system. *Prostaglandins & other lipid mediators* 89:112-119.
- Wang W., Pan Y., Li Q., Wang L. (2013b) Orexin: A potential role in the process of obstructive sleep apnea. *Peptides* 42:48-54.
- Ward R. J., Padiani J. D., Milligan G. (2011) Heteromultimerization of cannabinoid cb(1) receptor and orexin ox(1) receptor generates a unique complex in which both protomers are regulated by orexin a. *J Biol Chem* 286:37414-37428.
- Varvel S. A., Lichtman A. H. (2002) Evaluation of cb1 receptor knockout mice in the morris water maze. *J Pharmacol Exp Ther* 301:915-924.

- Watson S. L., Watson C. J., Baghdoyan H. A., Lydic R. (2010) Thermal nociception is decreased by hypocretin-1 and an adenosine a1 receptor agonist microinjected into the pontine reticular formation of sprague dawley rat. *The journal of pain : official journal of the American Pain Society* 11:535-544.
- Wenzel J., Grabinski N., Knopp C. A., Dendorfer A., Ramanjaneya M., Randeve H. S., Ehrhart-Bornstein M., Dominiak P., Jöhren O. (2009) Hypocretin/orexin increases the expression of steroidogenic enzymes in human adrenocortical nci h295r cells. *American journal of physiology Regulatory, integrative and comparative physiology* 297:R1601-1609.
- Verty A. N., Mcgregor I. S., Mallet P. E. (2004) Consumption of high carbohydrate, high fat, and normal chow is equally suppressed by a cannabinoid receptor antagonist in non-deprived rats. *Neurosci Lett* 354:217-220.
- Williams C. M., Kirkham T. C. (1999) Anandamide induces overeating: Mediation by central cannabinoid (cb1) receptors. *Psychopharmacology (Berl)* 143:315-317.
- Williams R. H., Alexopoulos H., Jensen L. T., Fugger L., Burdakov D. (2008) Adaptive sugar sensors in hypothalamic feeding circuits. *Proc Natl Acad Sci U S A* 105:11975-11980.
- Willie J. T., Chemelli R. M., Sinton C. M., Yanagisawa M. (2001) To eat or to sleep? Orexin in the regulation of feeding and wakefulness. *Annu Rev Neurosci* 24:429-458.
- Wilson R. I., Nicoll R. A. (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 410:588-592.
- Voisin T., El Firar A., Rouyer-Fessard C., Gratio V., Laburthe M. (2008) A hallmark of immunoreceptor, the tyrosine-based inhibitory motif itim, is present in the g protein-coupled receptor ox1r for orexins and drives apoptosis: A novel mechanism. *FASEB J* 22:1993-2002.
- Wu W. N., Wu P. F., Zhou J., Guan X. L., Zhang Z., Yang Y. J., Long L. H., Xie N., Chen J. G., Wang F. (2013) Orexin-a activates hypothalamic amp-activated protein kinase signaling through a ca(2)(+)-dependent mechanism involving voltage-gated l-type calcium channel. *Mol Pharmacol* 84:876-887.
- Wu X., Gao J., Yan J., Owyang C., Li Y. (2004) Hypothalamus-brain stem circuitry responsible for vagal efferent signaling to the pancreas evoked by hypoglycemia in rat. *J Neurophysiol* 91:1734-1747.
- Xia J. X., Fan S. Y., Yan J., Chen F., Li Y., Yu Z. P., Hu Z. A. (2009) Orexin a-induced extracellular calcium influx in prefrontal cortex neurons involves l-type calcium channels. *Journal of physiology and biochemistry* 65:125-136.
- Xu T. R., Yang Y., Ward R., Gao L., Liu Y. (2013) Orexin receptors: Multi-functional therapeutic targets for sleeping disorders, eating disorders, drug addiction, cancers and other physiological disorders. *Cell Signal* 25:2413-2423.
- Yamada H., Takahashi N., Tanno S., Nagamine M., Takakusaki K., Okumura T. (2005) A selective orexin-1 receptor antagonist, sb334867, blocks 2-dg-induced gastric acid secretion in rats. *Neurosci Lett* 376:137-142.
- Yamada N., Katsuura G., Tatsuno I., Asaki T., Kawahara S., Ebihara K., Saito Y., Nakao K. (2008) Orexin decreases mrna expressions of nmda and ampa receptor subunits in rat primary neuron cultures. *Peptides* 29:1582-1587.
- Yamamoto T., Nozaki-Taguchi N., Chiba T. (2002) Analgesic effect of intrathecally administered orexin-a in the rat formalin test and in the rat hot plate test. *Br J Pharmacol* 137:170-176.

- Yamamoto T., Saito O., Shono K., Aoe T., Chiba T. (2003) Anti-mechanical allodynic effect of intrathecal and intracerebroventricular injection of orexin-a in the rat neuropathic pain model. *Neurosci Lett* 347:183-186.
- Yamanaka A., Kunii K., Nambu T., Tsujino N., Sakai A., Matsuzaki I., Miwa Y., Goto K., Sakurai T. (2000) Orexin-induced food intake involves neuropeptide y pathway. *Brain Res* 859:404-409.
- Yang B., Ferguson A. V. (2002) Orexin-a depolarizes dissociated rat area postrema neurons through activation of a nonselective cationic conductance. *J Neurosci* 22:6303-6308.
- Yang B., Ferguson A. V. (2003) Orexin-a depolarizes nucleus tractus solitarius neurons through effects on nonselective cationic and k⁺ conductances. *J Neurophysiol* 89:2167-2175.
- Yang B., Samson W. K., Ferguson A. V. (2003) Excitatory effects of orexin-a on nucleus tractus solitarius neurons are mediated by phospholipase c and protein kinase c. *J Neurosci* 23:6215-6222.
- Yang L., Zou B., Xiong X., Pascual C., Xie J., Malik A., Sakurai T., Xie X. S. (2013a) Hypocretin/orexin neurons contribute to hippocampus-dependent social memory and synaptic plasticity in mice. *J Neurosci* 33:5275-5284.
- Yang Y. R., Follo M. Y., Cocco L., Suh P. G. (2013b) The physiological roles of primary phospholipase c. *Advances in biological regulation* 53:232-241.
- Yasuda T., Masaki T., Kakuma T., Hara M., Nawata T., Katsuragi I., Yoshimatsu H. (2005) Dual regulatory effects of orexins on sympathetic nerve activity innervating brown adipose tissue in rats. *Endocrinology* 146:2744-2748.
- Yeoh J. W., James M. H., Jobling P., Bains J. S., Graham B. A., Dayas C. V. (2012) Cocaine potentiates excitatory drive in the perifornical/lateral hypothalamus. *J Physiol* 590:3677-3689.
- Yokobori E., Kojima K., Azuma M., Kang K. S., Maejima S., Uchiyama M., Matsuda K. (2011) Stimulatory effect of intracerebroventricular administration of orexin a on food intake in the zebrafish, danio rerio. *Peptides* 32:1357-1362.
- Yoshida K., McCormack S., Espana R. A., Crocker A., Scammell T. E. (2006) Afferents to the orexin neurons of the rat brain. *J Comp Neurol* 494:845-861.
- Yoshimichi G., Yoshimatsu H., Masaki T., Sakata T. (2001) Orexin-a regulates body temperature in coordination with arousal status. *Exp Biol Med (Maywood)* 226:468-476.
- Zhang J., Li B., Yu L., He Y. C., Li H. Z., Zhu J. N., Wang J. J. (2011) A role for orexin in central vestibular motor control. *Neuron* 69:793-804.
- Zhang S., Blache D., Vercoe P. E., Adam C. L., Blackberry M. A., Findlay P. A., Eidne K. A., Martin G. B. (2005) Expression of orexin receptors in the brain and peripheral tissues of the male sheep. *Regul Pept* 124:81-87.
- Zhao M., Xia L., Chen G. Q. (2012) Protein kinase c delta in apoptosis: A brief overview. *Arch Immunol Ther Exp (Warsz)* 60:361-372.
- Ziolkowska A., Spinazzi R., Albertin G., Nowak M., Malendowicz L. K., Tortorella C., Nussdorfer G. G. (2005) Orexins stimulate glucocorticoid secretion from cultured rat and human adrenocortical cells, exclusively acting via the ox1 receptor. *J Steroid Biochem Mol Biol* 96:423-429.
- Zogopoulos P., Vasileiou I., Patsouris E., Theocharis S. E. (2013) The role of endocannabinoids in pain modulation. *Fundam Clin Pharmacol* 27:64-80.
- Zwirska-Korczała K., Adamczyk-Sowa M., Sowa P., Pilc K., Suchanek R., Pierzchala K., Namysłowski G., Misiolek M., Sadowski K., Kato I., Kuwahara A., Zabielski R. (2007) Role of leptin, ghrelin, angiotensin ii and orexins in 3t3 l1

preadipocyte cells proliferation and oxidative metabolism. J Physiol Pharmacol 58 Suppl 1:53-64.