Phospholipase Cascades in OX₁ Orexin Receptor Signaling

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Academic dissertation

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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 \(Ca^{2+}\) concentrations via multiple mechanisms, including activation of phospholipase C and increased \(Ca^{2+}\) 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 Cδ. 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  $\alpha$-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  $\gamma$-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 arachidonyl fluorophosphonate
MAGL  monoacylglycerol lipase
MAPK/KK mitogen-activated protein kinase kinase kinase
MCH   melanin-concentrating hormone
mTOR mammalian target of rapamycin
NMDA N-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 pyrophenone
PPAR peroxisome proliferator-activated receptor
PPO prepro-orexin
REM rapid eye movement
RGS regulator of G-protein signaling
Rluc Renilla 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 (Schioth 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 \( \alpha \)-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: \( \alpha \), \( \beta \), and \( \gamma \). 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 \( \alpha \)-subunit of the G-protein, and when GTP is bound, the \( \beta \gamma \)-complex dissociates from the \( \alpha \)-subunit. The GTP-bound \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-subunit returns to its inactive, GDP-bound state and reunites with the \( \beta \gamma \)-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 Gα- and Gβγ-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: Gs, Gi/o, Gq, and G12/13. This division is according to their α-subunits (Gs, Gi/o, Gq, and G12/13), and the typical members of these subfamilies have certain classical responses upon receptor activation: Gαs activates adenylyl cyclase (AC), whereas Gαi inhibits it. Gαq, on the other hand, activates phospholipase Cβ (PLCβ), and Gα12/13 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.](image)

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

<table>
<thead>
<tr>
<th>Heterotrimeric G-protein families</th>
<th>G-protein</th>
<th>Main effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gαq/11</td>
<td>PLC ↑</td>
<td></td>
</tr>
<tr>
<td>Gαi</td>
<td>AC ↑</td>
<td></td>
</tr>
<tr>
<td>Gαi/o</td>
<td>AC ↓</td>
<td></td>
</tr>
<tr>
<td>Gα12/13</td>
<td>PLC and PLD ↑</td>
<td></td>
</tr>
</tbody>
</table>

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 Ca$^{2+}$ signaling

Classically, coupling to $G_q$ proteins results in the activation of phospholipase Cβ (PLCβ), which hydrolyzes phosphatidylinositols, especially phosphatidylinositol-4,5-bisphosphate (PIP$_2$). Hydrolyzation of PIP$_2$ produces inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG), both of which are important second messengers (reviewed in Berridge, 2009; Yang et al., 2013b). IP$_3$ activates IP$_3$ receptors in the endoplasmic reticulum (ER), which leads to the release of Ca$^{2+}$ into the cytosol, and hence an increase in the cytoplasmic Ca$^{2+}$ concentration (Exton, 1996). Depletion of ER Ca$^{2+}$ stores is detected by stromal interaction molecule 1 (STIM1), which then leads to the opening of Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channels, now known to consist of Orai1 subunits (reviewed in Berridge, 2009; Fahrner et al., 2013).

Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ as a signaling molecule is reflected in the wide range of channels and pumps that regulate the intracellular Ca$^{2+}$ concentration. The temporal and spatial dynamics of the increase in Ca$^{2+}$ determine the target regulation by Ca$^{2+}$ (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 $G_{sa}$ subunit activates the effector, AC. Alternatively, if the GPCR couples to $G_i$, the $G_{ia}$ subunit inhibits AC activity (reviewed in Antoni, 2000). However, other factors regulate the activity of ACs. The intracellular Ca$^{2+}$ concentration can modulate AC activity: this can occur via the Ca$^{2+}$–calmodulin complex, calcineurin, Ca$^{2+}$-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 Gᵣ proteins (Sakurai et al., 1998), but later it became clear that they actually also couple to Gₓ and Gᵢₒ 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/lysosomal 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 G₉
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 Na\(^+\)/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 G\(_{\alpha_s}\), direct inhibition via G\(_{\alpha_i}\), and indirect and conditional activation via 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., 2007).
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 PLC$\beta$. There are six subtypes of phospholipase C (PLC): $\beta$, $\gamma$, $\delta$, $\epsilon$, $\zeta$, and $\eta$, 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 Orai1 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 PKCδ) (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 Cζ. 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
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 Herrana-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; Hollander 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 \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors ( AMPARs, another family of ionotropic glutamate receptors) in the postsynaptic 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 an increase in the number of AMPARs on the postsynaptic membrane. The \(\text{OX}_1\) 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 (Erani 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-fastened 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 adrenocorticotropic 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 corticol 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
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 cameldid, 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 (PPARγ) 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 PPARγ 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, Δ⁹-tetrahydrocannabinol (Δ⁹-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 Δ⁹-THC exerts its effects in animals.

In 1984, Howlett *et al.* (1984) reported that Δ⁸-THC and Δ⁹-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 Gᵢ 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 CB₁ and CB₂, respectively. CB₁ is mainly expressed in the CNS, while CB₂ 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, CB₁ 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 CB₁ (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 Gᵢ proteins was implied for cannabinoid receptors even before the receptors had been identified (Howlett *et al.*, 1986). Both CB₁ and CB₂ receptors couple to Gᵢ, and have been shown to inhibit AC (Howlett, 1984; Kaminski *et al.*, 1992). Coupling to other G-proteins, such as Gₛ and Gᵢ, has also been suggested for CB₁ (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. CB₁ and CB₂ activate ERK1 and ERK2, PKC and PI3K (Bouaboula *et al.*, 1995; Bouaboula *et al.*, 1996; Sanchez *et al.*, 2003). CB₁ 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 Ca²⁺ 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-1β (IL-1β)-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. CB<sub>1</sub> 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 (TNFα) 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-κB in mice, thereby inhibiting the release of pro-inflammatory cytokines (Panikashvili et al., 2005). This effect was attenuated by the CB<sub>1</sub> antagonist SR141716, and 2-AG did not produce these effects in CB<sub>1</sub>-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 CB<sub>1</sub> 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. CB<sub>1</sub> 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 CB$_1$ 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 (Hilairet 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 Gq 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 (Hilairet 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 CB$_1$ and OX$_1$ 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, CB$_1$ receptors were internalized when exposed to the agonist WIN55,212-2. When expressed together with OX$_1$, CB$_1$ receptors were also internalized when the cells were exposed to orexin-A, and the maximally effective orexin-A concentration caused equal CB$_1$ receptor internalization as WIN55,212-2. Orexin-A was ten times more potent in causing the internalization of OX$_1$-CB$_1$ heteromers than of OX$_1$ homomers, and this was suggested to be due to higher affinity of orexin-A to OX$_1$–CB$_1$ complexes than to OX$_1$–OX$_1$ complexes (Ward et al., 2011).

Although the reports described above have presented strong evidence of the formation of heteromeric complexes between OX$_1$ and CB$_1$ 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 OX$_1$ stimulation and their effect on signaling in cells expressing both OX$_1$ and CB$_1$ 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 OX$_1$ or CB$_1$ receptors were used. For CHO-hOX$_1$, 400 mg/ml geneticin, and for CHO-hCB$_1$, 0.25 mg/ml phleomycin was also included in the culture medium. Neuro-2a-hOX$_1$, PC12-hOX$_1$, 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-hOX$_1$ cells was also supplemented with 5% horse serum. All cells were cultured on plastic cell culture dishes (56 cm$^2$ bottom area) in a humidified air-ventilated incubator with 5% CO$_2$ 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 OX$_1$ and CB$_1$ 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 hOX$_1$ or hCB$_1$ 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 μl of methanol (scraping volume plus washing volume). Next, 500 μl of chloroform was added to the samples and they were vortex-mixed and incubated for 15 min at room temperature (RT). After this, 400 μl 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 μl) 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:isoctane: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, humidified 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 ^3^H-overflow from cells labeled with ^3^H-arachidonic acid and ^3^H-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 ^3^H-AA or ^3^H-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 ^3^H-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 ^3^H-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 ^3^H-ATP+^3^H-ADP from ^3^H-cAMP fractions with sequential Dowex/alumina
chromatography. The amount of radioactivity in the fractions was determined by liquid scintillation counting (4.7).

4.11 Ca\(^{2+}\) measurements (II)

For Ca\(^{2+}\) 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 Ca\(^{2+}\) indicator fura-2 acetoxyethyl ester (4 \(\mu\)M) 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. Ca\(^{2+}\) 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-hCB\(_1\) cells were grown on 6-well plates and transduced with recombinant baculovirus vectors with hOX\(_1\)-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 \(\mu\)l of lysis buffer [50 mM Heps, 150 mM NaCl (pH 7.5) + 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 1.5 mM MgCl\(_2\), 1 mM EDTA, 10 mM Na\(^{1-}\)-pyrophosphate, 1 mM Na\(^{1-}\)-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 \(\mu\)g 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 Gi 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 $^3$H-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 (BRET$^2$) assay (IV)

Dimerization of OX$_1$, OX$_2$ and CB$_1$ receptors was studied using the bioluminescence resonance energy transfer (BRET$^2$) method. In this method, cells are transfected with fusion constructs of Renilla luciferase (Rluc) or green fluorescent protein 2 (GFP$^2$) and the receptors of interest. When a receptor tagged with Rluc comes in close proximity to a GFP$^2$-tagged receptor, and a substrate for Rluc is present, the light energy of Rluc may excite the nearby GFP$^2$, producing a signal. These signals can be measured, and the specific GFP$^2$ 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 GFP$^2$ 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 BRET$^2$ 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 ± 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. $^3$H-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 Ca$^{2+}$-imaging experiments (II), where the $\chi^2$ 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 EC$_{50}$ 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 μM). 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 the activation of 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 PKCδ 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 PKCδ, but not PKCε. 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 μM).

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., 2002).
2010), but we did not observe this effect. An activator of cPKC (KAC1-1) did not produce any PLD stimulation, while activators of PKCe (KAE1-1) and PKCδ (KAD1-1) activated PLD. Furthermore, the activity produced by KAE1-1 could be abolished by an inhibitor of PKCe (KIE1-1), which had no effect, however, on PLD activation induced by orexin-A or TPA. Thus, PKCδ emerged as the more interesting of the nPKC isoforms. Unfortunately, a specific peptide inhibitor of PKCδ was not commercially available. Consequently, we used rottlerin as a PKCδ inhibitor, although we were aware that it is not a very selective or good inhibitor (Soltsoff, 2007). Rottlerin produced a clear concentration-dependent inhibition of PLD activation, supporting the notion that PLD was activated by PKCδ.

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 μM 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/PKCδ 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 PKCδ 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, PKCδ 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 PKCδ, 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 Gₛ-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 PKCδ, 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 potently 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 potently activates PLD1 in CHO cells. This solely relies on an nPKC, probably PKCδ (Figure 6). The activation mechanism of this PKC is unclear, but it (or PLD) does not require AC, PLC, PLA₂, Rho family GTPase, Gβγ, or PKD activity. The physiological significance of OX₁ signaling to PLD has not yet been investigated.
Figure 6. Investigated pathways for OX₁ induced phospholipase D (PLD) activation. Crosses on top of the arrows indicate that the pathway is not involved in OX₁ induced activation of PLD. AC, adenylyl cyclase; DAG, diacylglycerol; PKCδ, protein kinase Cδ; 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-hOX₁ 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 PLA₂ 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 ¹⁴C-AA-labeled cells revealed two prominent ¹⁴C-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. PLA₂ 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 cPLA₂α/ζ, 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 cPLA₂α 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 "\(^3\)H-overflow" method \((4.8)\) (Turunen et al., 2010), and observed that the release of the \(^3\)H-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 \(^3\)H-overflow experiments, it appears that \(c\)PLA\(_2\) (\(p\)EC\(_{50}\) ≈ 9.3 from \(^3\)H-overflow, and \(p\)EC\(_{50}\) ≈ 9.8 from TLC separation) is activated more potently than DAGL (\(p\)EC\(_{50}\) ≈ 8.4 from \(^3\)H-overflow, and \(p\)EC\(_{50}\) ≈ 8.3 from TLC separation).

![Figure 7](image.png)

**Figure 7.** Pathways producing arachidonic acid (AA) upon activation of OX\(_1\) orexin receptors by orexin-A in recombinant CHO-hOX\(_1\) cells. 2-AG, 2-arachidonoylglycerol; cPLA\(_2\), Ca\(^{2+}\)-dependent/cytosolic phospholipase A\(_2\); 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 \(^3\)H-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 $^3$H-oleic acid instead of $^3$H-AA, the $^3$H-overflow induced by OX$_1$ activation was much smaller and less potent. PP had no effect on this $^3$H-overflow, while THL inhibited it completely. The enzyme responsible for the effect in $^3$H-OA-labeled cells therefore appears to be DAGL, while cPLA$_2$ is not involved. This would suggest that the cPLA$_2$ species involved in AA release is cPLA$_2\alpha$ and not cPLA$_2\zeta$, as the $\alpha$ 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 cPLA$_2$, 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-hOX$_1$, PC12-hOX$_1$, 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 cPLA$_2$ 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.
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](image)

**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 (Hilairet 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-hCB$_1$ cells transduced to also express OX$_1$ receptors. When the cells with both receptors were activated with orexin-A, a strong ERK response was seen. The response was antagonized (EC$_{50}$ shifted more than 100-fold) by the CB$_1$ antagonist SR141716. This is in line with the observations of Hilairet 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 CB$_1$ receptors. As both OX$_1$ and CB$_1$ 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 CB$_1$ antagonist, instead of SR141716, as an additional control. THL and AM-251 attenuated the potentiation of Elk-1 signaling in cells expressing both OX$_1$- and CB$_1$-receptors to a level similar with the control cells expressing only OX$_1$. The potentiation of Elk-1 activity was not as strong as that of ERK phosphorylation; the EC$_{50}$ values were shifted less than 10-fold when the receptors were co-expressed, compared to cells expressing only OX$_1$. 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 OX$_1$ to signal to CB$_1$ receptors. Therefore, we transfected the CHO-hCB$_1$ cells to express human M$_1$ receptors that have also been reported to utilize endocannabinoids in their signaling (Fukudome et al., 2004). M$_1$
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 (Hilairet 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 Ca\(^{2+}\) influx or other signaling differences among GPCRs in DAGL (or 2-AG transport) activation.

5.4. The role of Ca\(^{2+}\) in lipid signaling cascades

As described above (2.3.1), OX\(_1\) receptors increase the intracellular Ca\(^{2+}\) concentration by various mechanisms. Receptor-operated Ca\(^{2+}\) influx is a central signaling mechanism for OX\(_1\), 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 PLA\(_2\), DAGL, and MAGL, was shown to inhibit the receptor-operated Ca\(^{2+}\) 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 Ca\(^{2+}\) responses, while THL had no effect. DAGL does not appear to be involved in producing Ca\(^{2+}\) responses, but the Ca\(^{2+}\) influx seems to be somehow mediated by cPLA\(_2\). The action of cPLA\(_2\) releases mediators that are able to induce Ca\(^{2+}\) influx, such as LPC and AA or another fatty acid, and the mediators can be further processed by the enzyme cascades. The cPLA\(_2\) product causing the effect in our test system is currently unknown.

Many signaling cascades utilized by OX\(_1\) receptors have been shown to be dependent on extracellular Ca\(^{2+}\) (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 Ca\(^{2+}\) 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 Ca\(^{2+}\) concentration, but to a lesser extent (Johansson et al., 2007; Putula and Kukkonen, personal communication). The \(^{3}\)H-AA overflow response to orexin-A is also highly sensitive to extracellular Ca\(^{2+}\) (Turunen et al., 2010), and thus both cPLA\(_2\) and DAGL cascades should be abolished upon removal of extracellular Ca\(^{2+}\). Even though clearly dependent on extracellular Ca\(^{2+}\), 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 Ca\(^{2+}\) concentration. On the contrary, an increased intracellular Ca\(^{2+}\) concentration by itself is enough to very strongly stimulate cPLA\(_2\) activity (Turunen et al., 2010; Paper II).

These findings can be attacked from several angles. The case for cPLA\(_2\) and DAGL could be easily explained by the fact that they are Ca\(^{2+}\)-dependent enzymes (Clark et al., 1991; Kramer et al., 1991; Bisogno et al., 2003), and in the cascades leading to ERK, there are potential Ca\(^{2+}\)-sensitive effectors. PLC is also Ca\(^{2+}\)-dependent, but it (or at least PLC\(\beta\)) appears to be, when compared to DAGL or cPLA\(_2\), less affected by a reduction in extracellular Ca\(^{2+}\), as also investigated using the M\(_1\) muscarinic receptor (Johansson et al., 2007). However, PLD or its upstream activator nPKC should not be Ca\(^{2+}\)-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 1\(\alpha\) 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 (Hilairet 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 (Hilairet 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 Hilairet 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 PKCδ, 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 Ca^{2+} 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 PKCδ, although we did not have access to a very specific inhibitor of this isoform. Recombinant PKCδ 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 PKCδ 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 PKCδ 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 PKCδ activation in this signaling is nevertheless very interesting – and also the lack of involvement of PKCe – and I would like to determine the upstream mediators of PKCδ activation. Perhaps better antibodies could now be available, or phosphopeptide analysis of PKCδ could be performed. PKCδ inhibitor peptides could perhaps be synthesized in-house, and the role of PKCδ 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 CB1 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-nonselective 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 Ca^{2+} 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 Ca^{2+} 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.
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