Studies on OX_1 orexin receptor coupling to arachidonic acid and endocannabinoid signaling

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II **Pauli M. Turunen,** Maria H. Jäntti, and Jyrki P. Kukkonen. (2012) OX_1 Orexin/Hypocretin Receptor Signaling via Arachidonic Acid and Endocannabinoid Release. *Mol. Pharmacol.* August, 82(2):156-167.

III Hanna M. Peltonen, Johanna M. Magga, Genevieve Bart, **Pauli M. Turunen**, Miia S. H. Antikainen, Jyrki P. Kukkonen, Karl E. Akerman. (2009) Involvement of TRPC3 channels in calcium oscillations mediated by OX_1 orexin receptors. *Biochem Biophys Res Commun.* 385(3), 408–412.

IV **Pauli M. Turunen**, Jaana Putula, Jyrki P. Kukkonen. (2010) Filtration assay for arachidonic acid release. *Anal Biochem.* 407(2), 233–236.

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ABSTRACT

Orexin A and orexin B are hypothalamic neuropeptides secreted by a relatively small group of neurons located within the hypothalamus. Orexins bind and activate two G-protein-coupled receptors termed OX_1 orexin receptor and OX_2 orexin receptor. They regulate several physiological processes including arousal and the sleep/wake cycle, endocrine functions, and feeding and energy metabolism.

Previous studies have established calcium as the central second messenger of the OX_1 receptor. Stimulation of OX_1 leads to increased intracellular Ca^{2+} release via the classical phospholipase C/inositol-1,4,5-trisphosphate pathway, and subsequently also store-operated Ca^{2+} entry. In addition, OX_1 activation triggers receptor-operated Ca^{2+} entry by a mechanism not completely understood. Other intracellular signals originating from the OX_1 receptor include the activation of many signaling enzymes such as protein kinase C, extracellular signal-regulated kinase 1/2 (ERK1/2) and phospholipase D.

The role of phospholipase A_2 (PLA_2) in OX_1 signal transduction was investigated in this thesis. Recombinant Chinise Hamster Ovary-K1 and Human Embryonic Kidney 293 cells were used for the studies. Orexin A binding induced robust release of arachidonic acid (AA). The enzyme species responsible for AA release was suggested to be cytocolic $PLA_2\alpha$. AA release was highly dependent on extracellular Ca^{2+} and partly on the activation of ERK. The involvement of other PLA_2 enzymes was ruled out by pharmacological analysis. $cPLA_2$ activity was also shown to regulate receptor-operated Ca^{2+} entry, but the exact nature of the relaying signal remained unspecified. Thin layer chromatography analysis revealed that orexin-A stimulation also induced the production of 2-arachidonyl glycerol (2-AG), an endocannabinoid, via the phospholipase C-diacylglycerol lipase pathway. OX_1-induced 2-AG production occurred at a biologically relevant magnitude as judged by an artificial cell-cell communication assay developed, establishing the postulated link between orexinergic and endocannabinoid systems.

One part of this research aimed at devising faster ways to assess AA release. As a result, a filtration-based method suitable for agonist screening was developed. A filtration method for AA release enables increased sample throughput with lower noise.

In this study, two new signaling components, AA and 2-AG, originating from OX_1 receptor signaling were identified and the enzymatic species responsible were shown to be $cPLA_2$ and diacylglycerol lipase. These findings are important for mapping of the cellular and molecular functions of orexins and their receptors, especially in the central nervous system.

ABBREVIATIONS

$[Ca^{2+}]_i$	Cytoplasmic Ca^{2+} concentration
2-AG	2-arachidonyl glycerol
aa	Amino acid
AA	Arachidonic acid
AC	Adenylyl cyclase
ARC	Arachidonic acid regulated channel
BSA	Bovine serum albumin
CB_1	CB_1 cannabinoid receptor
CB_2	CB_2 cannabinoid receptor
CCK	Cholecystokinin
CCP	Clathrin-coated pit
CNS	Central nervous system
DAG	Diacylglycerol
DAGK	Diacylglycerol kinase
DAGL	Diacylglycerol lipase
DMV	Dorsal motor nucleus of vagus
DRN	Dorsal raphe nucleus
eCB	Endocannabinoid
ECS	Endocannabinoid system
ENS	Enteric nervous system
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
GEF	Guanine nucleotide exchange factor
GIT	Gastrointestinal tract
GPCR	G-protein-coupled receptor
GP-NAE	Glycerophospho *N*-acetylethanolamine
GRK	GPCR kinase
HBM	Hepes-buffered medium
IP_3	Inositol-1,4,5-trisphosphate
IP_3R	Inositol-1,4,5-trisphosphate Receptor
LC	Locus coeruleus
LHA	Lateral hypothalamic area
LPA	Lysophosphatic acid
MAFP	Methoxyarachidonyl fluorophosponate
MAG	Monoacylglycerol
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NAAA	NAE-hydrolyzing acid amidase
NAT	*N*-acyltransferase
NPY	Neuropeptide Y
OX_1	OX_1 orexin receptor
OX_2	OX_2 orexin receptor
OxA	Orexin-A
OxB	Orexin-B
PA	Phosphatidic acid
PEAse	Palmitoyl ethanolamidase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol(s)
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphaditylinositol-4,5-bisphosphate
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PKA	Protein kinse A
PKC	Protein kinase C
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PPO	Prepro-orexin
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RGS	Regulator of G-protein signaling
ROC	Receptor-operated channel
STIM	Stromal interaction molecule
SOC	Store-operated channels
TGN	Trans-Golgi network
THC	Δ^9-Tetrahydrocannabinol
THL	Tetrahydrolipstatin
TMN	Tuberomammilary nucleus
UTR	Untranslated region
VLPO	Ventrolateral preoptic nucleus
VOC	Voltage-operated channels

1. REVIEW OF THE LITERATURE

1.1. Introduction

The formation of the first functional cell has been one of the most crucial steps in the evolution of life in the shape we recognize it. It took millions of years until the first cells learned to communicate with each other. Communication, or signaling as we like to call it, is of utmost importance for the development of multicellular and hence more complex life forms.

Signaling is about relaying information forward and receiving it in order to create adequate responses. It is needed in the very first step of life when a sperm is swimming toward the ovum and changing direction along the concentration gradients of chemoattractants secreted. Signaling is needed in the fertilization process, and a large amount of signaling is needed during development. Signaling controls gene expression, energy metabolism, and finally cell death.

In the basic scenario, a cell secretes a signaling component or expresses one on its plasma membrane. The target cell then detects the signal with receiver molecule(s) termed receptors. Depending on the chemical nature of the signal, the receptor is either located on the plasma membrane or lies inside the cell in the cytoplasm or nucleus. Water-soluble signal molecules are usually unable to penetrate the hydrophobic plasma membrane, and bind to receptors located on the plasma membrane. The plasma membrane is a selective semi-permeable barrier between the interior of the cell and the outside solution. Lipid-soluble molecules can easily pass through the plasma membrane and bind to their receptors in the cytoplasm or nucleus. In many cases, ligand binding activates signal transduction cascades that alter the behavior of the cell. Activated pathways downstream from the receptor often utilize small molecules termed second messengers. Ca^{2+} ions are common second messengers, but second messengers can also be organic molecules such as cAMP and lipid derivatives. The advantage of second messengers is their ability to rapidly amplify the signal inside the cell.

Occasionally, due to a mutation or several mutations, signaling systems become harmful or even deadly to the organism. The etiological explanation behind numerous diseases, including cancer, is the malfunction of signal transduction or regulation. Better comprehension of the molecular logic of signaling benefits not only basic life science but also medicine. With a deeper understanding of disease-causing signaling pathways, new doors are opened for drugs and therapeutic approaches that might ultimately lead to the conquering of such diseases.

1.2. Calcium signaling

The calcium ion is the most ubiquitous and universal molecule involved in signaling (Clapham, 2007). It is utilized by organisms from bacteria to humans (Berridge *et al.*, 2000; Clapham, 2007; Norris *et al.*, 1996). Ca^{2+} is needed in fertilization and programmed cell death, cell division, secretion, neuronal signaling, and muscle contraction, among other processes. It is intriguing that opposing phenomena such as proliferation and apoptosis or vasodilatation and vasoconstriction are both regulated by an increase in intracellular Ca^{2+} (Parkash *et al.*, 2010), and yet cells are able to distinguish between the signal sources and adjust their functions accordingly. The presence of Ca^{2+} in the course of evolution has generated structural motifs and proteins that specifically bind Ca^{2+} and couple the binding to conformational variations. The most common motif is the so-called EF hand, found in several proteins, including the ubiquitous Ca^{2+} sensor calmodulin (CaM) (Lewit-Bentley *et al.*, 2000). CaM is a dumbbell-shaped small protein that undergoes a dramatic change in conformation upon Ca^{2+} binding (Klee *et al.*, 1980).

This shape-shifting (and Ca^{2+}-sensor) ability is usually coupled to binding to target proteins and regulation of their activity (Klee *et al.*, 1980). Many proteins have a Ca^{2+}-binding domain called the C2 domain embedded into their structure (Hurley, 2006). The positive charge of Ca^{2+} allows C2 proteins to bind anionic lipids located in the inner leaflet of the plasma membrane (Hurley, 2006). Important signaling proteins containing the C2 domain include protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), phospholipase A_2 (PLA_2), and synaptotagmin (Clapham, 2007; Hurley, 2006).

The fundamental concept of Ca^{2+} signaling is outlined with a few principles that are sometimes referred to as the Ca^{2+} signaling network or "toolkit" (Berridge *et al.*, 2000). It consists of: a) the extracellular stimulus

(hormone, neurotransmitter etc.) that activates the intracellular Ca^{2+}-mobilizing signals, the "on-mechanisms" that rapidly leads to an increase in the intracellular concentration of calcium, $[Ca^{2+}]_i$, b) the cellular processes regulated by Ca^{2+}, and finally c) the "off-mechanisms" that remove the Ca^{2+} from the cytoplasm and return the cell to the resting state. $[Ca^{2+}]_i$ in resting cells is held very low (around 100 nM) by stringent regulation of Ca^{2+} localization, energy-requiring pumps and transporters that transfer Ca^{2+} across cellular membranes, and Ca^{2+}-sequestering organelles or molecules (Clapham, 2007). A low cytoplasmic concentration prevents unwanted precipitation of calcium phosphates and establishes the framework for varying Ca^{2+} signal amplitude, timing, and localization (Clapham, 2007).

Extracellular fluid (ECF) contains Ca^{2+} at concentrations several orders of magnitude higher (around 1 mM) than the cytoplasmic concentration, probably reflecting the composition of ancient seas where life developed eons ago. ECF-derived Ca^{2+} is commonly utilized in signal transduction cascades (often termed Ca^{2+} influx). In addition to ECF, Ca^{2+} can be mobilized from intracellular stores (Ca^{2+} release). Mitochondria and the endoplasmic reticulum (ER) are the most important cellular Ca^{2+} storage organelles. They participate in signaling and buffering of free cytoplasmic Ca^{2+} (Clapham, 2007). Cytoplasmic spikes in $[Ca^{2+}]_i$ concentrations are often referred to as Ca^{2+} transients, which can reach a concentration of 1 μM or higher and relay considerable amounts of information within the cell. Even more information can be transmitted by repetitive Ca^{2+} transients termed oscillations. Single Ca^{2+} transients usually activate fast cellular events such as muscle contraction or exocytosis. Oscillations, in turn, regulate more complicated processes such as cell division (Clapham, 2007).

1.2.1 Ca^{2+} influx

Ca^{2+} influx mainly occurs via channels classified as ionotropic receptors (ligand gated ion channels), voltage-operated Ca^{2+} channels (VOCs), receptor-operated channels (ROCs) and store-operated channels (SOCs) according to the opening mechanisms of the channels (Clapham, 2007). Ionotropic receptors are opened upon extracellular ligand binding (Tsien *et al.*, 1990). Excitatory ionotropic receptors include receptors for the neurotransmitters glutamate (AMPA, kainate, NMDA-receptors), acetylcholine (nAChRs), glycine (exicatory glycine N1-3 receptors), serotonin ($5\text{-}HT_3$ receptors) and ATP (P2X receptors). Some of these non-selective cation channels are also permeable for Ca^{2+} (Hayashi *et al.*, 1996; Rathouz *et al.*, 1994).

VOCs function as transducers converting changes in membrane potential into intracellular Ca^{2+} transients (Catterall, 2011). The ten known mammalian members are divided according to their α-subunit into three classes of Ca_v1, Ca_v2 and Ca_v3 (Catterall, 2011; Ertel *et al.*, 2000). VOCs are also classified according to their electrophysiological (e.g. activation voltage, activation/inactivation velocity, conductance) and pharmacological properties (e.g. sensitivity to inhibitors and toxins) into L- (Ca_v1), N- ($Ca_v2.1$), P/Q- ($Ca_v2.2$), R- ($Ca_v2.3$), and T-type (Ca_v3) channels (Catterall, 2011). VOCs are Ca^{2+}-specific channels and mostly expressed in excitable cells, e.g. neurons, and muscle and endocrine cells. In general, VOCs regulate contraction, secretion, and gene expression (Catterall, 2011). In muscle cells (cardiac, smooth and skeletal muscle), VOCs couple to ryanodine receptors either via direct molecular contact or indirectly via Ca^{2+} elevation (Catterall, 2011). The functions of VOCs in excitation-contraction/secretion or synaptic transmission-coupling are tightly regulated by G-protein-mediated signaling pathways by G-protein subunits or phosphorylation (Catterall, 2011).

Receptor-operated channels (ROCs) refer to Ca^{2+} influx pathways that are not voltage-controlled or activated by emptying of ER Ca^{2+} stores (Tsien *et al.*, 1990), but rather are activated upon separate receptor binding and subsequent intracellular signaling. Several intracellular second messengers have been implicated in ROC regulation, including inositol-1,4,5-trisphosphate (IP_3) (Kiselyov *et al.*, 1997; Mozhayeva *et al.*, 1990), diacylglycerol (DAG) (Hofmann *et al.*, 1999), cyclic nucleotides (Finn *et al.*, 1996), arachidonic acid (AA) (Mignen *et al.*, 2000), and G-proteins (Barritt, 1999; Berven *et al.*, 1994; Singer-Lahat *et al.*, 1997). The activation of channels in receptor signaling may also relate to other processes than direct messenger-action, e.g. translocation to the plasma membrane or effects on membrane curvature (Kukkonen, 2011). Until recently, ROCs were only described based on their physiological properties without knowledge of their molecular nature (Barritt, 1999), but the identification of the transient receptor potential (TRP) family of ion channels has dramatically changed the field. The "original" TRP channel was described in *Drosophila* TRP mutants

(Cosens *et al.*, 1969; Hardie *et al.*, 1992; Minke, 1982). In a normal *Drosophila* eye, a light stimulus produces a photoreceptor potential composed of both a transient and sustained phase, but the mutation abolishes the sustained phase (Minke *et al.*, 2002). The loss of the sustained response was finally ascribed by Montell *et al.* to be an inactivation of a Ca^{2+}-permeable channel, coined TRP (Hardie *et al.*, 1992; Montell *et al.*, 1985; Montell *et al.*, 1989). This led to a "cascade" of homology cloning of mammalian orthologs, of which we currently know 28 in humans divided into the subfamilies TRPC, TRPM, TRPV, TRPA, TRPP, and TRPML; however, not all of the TRP homologs may function as channels (Nilius *et al.*, 2007; Venkatachalam *et al.*, 2007). TRPs are Ca^{2+}-permeable nonspecific cation channels that share a common structural theme of six membrane-traversing segments (S1-6) and cytoplasmic N- and C-termini. The pore is formed by S5 and S6. The functional channel is suggested to be a complex of four TRP monomers in either homo- or heteromeric assembly (Minke *et al.*, 2002; Montell, 2011).
TRP channels are regulated by multiple signals. Most of the mammalian TRP channels require PLC activity for functioning. PLC-generated DAG directly activates TRPC2, -3, -6, and -7. On the other hand, DAG inhibits, indirectly via PKC activation, TRPC3, -4, -5, and -6. PLC, phosphaditylinositol-3-kinase (PI3K), and phospholipase D (PLD) can also regulate TRPs by affecting phosphaditylinositol-4,5-bisphosphate (PIP_2) levels in the plasma membrane. Various lipids and and lipid derivatives are implicated in TRP regulation, including AA and other polyunsaturated fatty acids (PUFAs), sphingosine-1-phosphate, lyso-phosphaditylcholine (LPC), platelet-activating factor (PAF), endovanilloids, and eicosanoids. (Kukkonen, 2011; Venkatachalam *et al.*, 2007).

Store-operated Ca^{2+} entry (SOCE) becomes active when ER Ca^{2+} stores are depleted. Depletion is detected by transmembrane ER proteins called STIMs (stromal interaction molecule), of which two mammalian homologues, STIM1 and STIM2, are known (Cahalan, 2009; Zhang *et al.*, 2005). STIM1 was already cloned in 1996 (Parker *et al.*, 1996), but the gene function in SOCE became evident much later (Zhang *et al.*, 2005). STIM1 is the actual Ca^{2+} censor of the ER, while STIM2 regulates basal Ca^{2+} levels (Brandman *et al.*, 2007). A decrease in the Ca^{2+} concentration within the ER leads to dissociation of bound Ca^{2+} from the EF hand of STIM1 (Zhang *et al.*, 2005). Dissociation initiates STIM1 oligomerization and translocation to the junction area between the ER and plasma membranes (Liou *et al.*, 2007). STIM1 then interacts with specific Ca^{2+} channels formed by proteins of the Orai family (Orai1-3) (Feske *et al.*, 2006). The actual molecular mechanism of the interaction is not completely known, nor is it known whether the interaction is direct or indirect (Smyth *et al.*, 2010), but calcium-independent $PLA_2\beta$ ($iPLA_2\beta$) has been suggested to provide the link between STIM1 and Orai1 (Bolotina, 2008). Opening of the Ora1 channels leads to Ca^{2+} influx and replenishment of the ER Ca^{2+} store. In electrophysiology, the current passing through Orai1 is known as I_{CRAC} (Zweifach *et al.*, 1993), described long before the final discovery of Orai1 (Smyth *et al.*, 2010). In addition to Orai1, TRPC subfamily channels have been shown to mediate SOCE as such or in concert with Orai1 via interactions with STIM1 (Cheng *et al.*, 2011), although this has been questioned in other reports (Cheng *et al.*, 2011; DeHaven *et al.*, 2009).

Ca^{2+} entry may also take place via carrier proteins, although this is usually considered much less important than channel-mediated entry. The most well known example of this is the reverse action of the Na^+/Ca^{2+} exchanger (see below).

Ca^{2+} influx is a (cost-) effective signaling method. Highly localized Ca^{2+} hot-spots can be generated in the vicinity of the channels, without any general Ca^{2+} elevation in the cell. The plasma membrane is in many ways an important signaling domain, and many opportunities for Ca^{2+} signals to interact with other signals are generated.

1.2.2. Ca^{2+} release

The research of Michael J. Berridge and coworkers on the secretion mechanism of the blow-fly salivary gland revealed a fundamental signaling pathway for Ca^{2+} release from intracellular stores (reviewed by Berridge, 2009). They demonstrated that the diffusible agent (second messenger) involved was IP_3, derived from the highly phosphorylated inositol-containing phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2), by the action of phospholipase C (PLC) (Berridge, 1983). Finally, the discovery that IP_3 acted on receptor channels on the ER membranes leading to an increase in $[Ca^{2+}]_i$ completed the picture (Furuichi *et al.*, 1989; Spat *et al.*, 1986). IP_3 binds to the cytoplasmic tail of the specific IP_3 receptors (IP_3Rs) located on the ER membranes (Taylor *et al.*, 2002). These receptors are tetrameric and regulated

by both IP_3 and Ca^{2+} in addition to phosphorylation (Bosanac *et al.*, 2004). Three isoforms of IP_3Rs are known with different tissue-dependent expression profiles. IP_3Rs themselves are Ca^{2+} channels that allow Ca^{2+} to pass from the ER into the cytoplasm (Taylor *et al.*, 2002). At lower Ca^{2+} concentrations, Ca^{2+} promotes the opening of the channel (Bootman *et al.*, 1999). In fact, IP_3 cannot open the channel unless Ca^{2+} is bound to the structure (Taylor *et al.*, 2002). At high concentrations, Ca^{2+} closes the channel (Bootman *et al.*, 1999).

PLC activity also produces another second messenger, the lipid remnant of PIP_2, namely DAG (Rhee, 2001). DAG, in contrast to IP_3, remains on the plasma membrane, where it diffuses laterally and can activate multiple important signaling enzymes, including PKC subfamilies c (conventional) and n (novel), protein kinase D (PKD), Ras and DAG kinases (DGK) β and γ (Baier, 2003; Carrasco *et al.*, 2007; Kukkonen, 2011).

The PLC family contains 13 members divided into six subfamilies (β, γ, δ, ε, η, and ζ) (Bunney *et al.*, 2011; Rhee, 2001; Suh *et al.*, 2008). In addition, two or more splice variants are known for most of the PLC family members. The protein repertoire together with different tissue distribution, regulatory mechanisms and physiological functions make PLC signaling very multifaceted (Suh *et al.*, 2008). The best characterized are PLCβs (PLCβ1–4) and PLCγs (PLCγ1and 2). They were originally considered to be activated as a consequence of $G_{q/11}$ activation (Bunney *et al.*, 2011; Rhee, 2001) and by receptor and non-receptor tyrosine kinases, respectively (Bunney *et al.*, 2011; Noh *et al.*, 1995), but more recently, PLCγ has been found to be activated by G-protein-coupled receptors (GPCRs) and PLCβs by Rac GTPases (Bunney *et al.*, 2006; Bunney *et al.*, 2011). Novel-type PLCε are regulated by monomeric G-proteins of Ras and Rho families. PLCδ and -ζ appear to be mainly regulated by an increase in $[Ca^{2+}]_i$ (Kim *et al.*, 1999).

Gα proteins of the $Gα_{q/11}$ family are the most important regulators of PLCβ (Taylor *et al.*, 1991), but the Gβγ complex can activate various PLCβ isoforms (reviewed in Dupre *et al.*, 2009; Smrcka *et al.*, 1993). PLCβs are also subject to phosphorylation by protein kinase A (PKA) and PKC (Rebecchi *et al.*, 2000). An interesting regulatory feature of the PLCβs is their ability to function as GTPase-activating proteins (GAP) for $Gα_{q/11}$ proteins (Berstein *et al.*, 1992; Biddlecome *et al.*, 1996), offering a negative feedback mechanism to control their own activity.

1.2.3. Ca^{2+} removal

For regulated Ca^{2+} signaling it is essential that Ca^{2+} can also be rapidly removed from the cytosol. ATP-driven Ca^{2+} pumps include plasma membrane Ca^{2+} ATPases (PMCAs), extruding Ca^{2+}, and the SR/ER Ca^{2+} ATPases (SERCAs), which underlie the sequestration of Ca^{2+} in the ER/SE (Clapham, 2007). Ca^{2+} can also be transported by the plasma membrane Na^+/Ca^{2+} exchangers (NCXs), which in this 'normal' mode utilize the driving force of Na^+ for Ca^{2+} extrusion (Clapham, 2007). As NCXs are dependent on the electrochemical driving forces for Ca^{2+} and Na^+, they can also work in the so-called reverse mode, leading to Ca^{2+} entry when Na^+ is extruded.

The mitochondrial Ca^{2+} uniporter can be utilized to take in Ca^{2+} driven by the mitochondrial membrane potential (Kirichok *et al.*, 2004). However, mitochondria should not act as Ca^{2+} stores, but Ca^{2+} should be removed upon reduced Ca^{2+} signaling activity. Ca^{2+} uptake depletes the mitochondrial membrane potential, and mitochondria are thus one of the targets of Ca^{2+} toxicity (Szabadkai *et al.*, 2008).

1.3. G-protein-coupled receptors

The concept of GPCRs is well established (Milligan *et al.*, 2006b; Oldham *et al.*, 2008). They represent perhaps the largest and most diverse superfamily of signaling receptors in the mammalian genome. GPCRs (or related molecules) can also be found in yeast, plants and invertebrates, indicating they originated early in evolution (Kroeze *et al.*, 2003). GPCRs are able to respond to various stimuli, including light (i.e. photons), Ca^{2+}, biogenic amines, odorants, nucleotides, lipids, and proteins, among others (Kroeze *et al.*, 2003). The human genome contains at least 800 GPCR sequences (Fredriksson *et al.*, 2003). GPCRs are also important drug targets. It has been estimated that 30% of drugs on the market target GPCRs one way or another (Hopkins *et al.*, 2002).

GPCRs acquired their name from a relatively small cytoplasmic protein complex of three proteins termed heterotrimeric G-proteins. These G-proteins can associate with the cytoplasmic domain of GPCRs and are their 'signaling partners'. A characteristic structural feature of all GPCRs is that they contain seven transmembrane α-helices within a single

polypeptide chain; hence, GPCRs are often called 7TM receptors, heptahelical receptors or, figuratively, serpentine-like receptors. The single polypeptide chain also has small loops outside and inside the cell, preceded and followed by the membrane-spanning α-helices and N-terminal extracellular and C-terminal cytoplasmic domains.

1.3.1. Classification of GPCRs

The classification of GPCRs is complex and several parallel systems still exist. In 1994, Kolakowski introduced the first systematic nomenclature for GPCRs, the A–F classification system (Kolakowski, 1994). In derivatives of Kolakowski's system, classes A, B, and C are more or less written in stone, but on some occasions Frizzled and olfactory receptor types are separated into own classes. Kolakowski classification is based on sequence similarities, disulfide bonds and the presence of key residues. Subsequently, the GRAFS nomenclature was developed by Fredriksson and coworkers. This is based more on presumed phylogenetic criteria and includes *glutamate*, *rhodopsin*, *adhesion*, *frizzled/taste2* and *secretin* families. The major difference compared to A–F classification is the separation of the adhesion and secretin families from class B (Fredriksson *et al.*, 2003). Each classification method has certain limitations and is a compromise at best. The nomenclature used in this thesis follows the GRAFS system.

The rhodopsin family, formerly class A receptors, is the largest group in the GRAFS nomenclature and is named according to the prototypic photon receptor first isolated from bovines in 2000 (Palczewski *et al.*, 2000). This is the largest group, with 670 or so members (Lagerstrom *et al.*, 2008). The rhodopsin family is further divided into four subgroups: α, β, γ and δ. The last of these contains all olfactory receptors, making it the largest single subgroup of the rhodopsin family. The typical structural feature is disulfide bridges connecting extracellular loops between transmembrane helices I and II. Almost every member contains an NSxxNPxxY motif in helix VII and a DRY motif facing the cytoplasm in helix III. DRY is needed both for structural stabilization and G-protein activation. Also typical is a small N-terminal domain compared to other GPCR classes. Rhodopsin family receptors recognize a vast variety of ligands, from tiny photons (with the help of bound 11-*cis*-retinal) to small biogenic amines, lipids, peptides and proteins. Endocannabinoid receptors CB_1 and CB_2 belong to this group, as do orexin receptors OX_1 and OX_2.

Glutamate family receptors were formerly known as class C receptors. They consist of metabotropic glutamate receptors (eight in humans), γ-Aminobutyric acid (GABA) receptors (two in humans), the single calcium-sensing receptor (CASR) and the taste receptor type 1 (TAS1) (Schioth *et al.*, 2005). The typical structural elements of these receptors are two N-terminal lobes that close together surrounding the ligand, referred to as the Venus flytrap according to the common name of the carnivorous plant *Dionaea muscipula* (Schioth *et al.*, 2005).

Adhesion family members (another member of the former class B) have very long N-terminal domains and several modular structures such as EGF repeats, leucine-rich repeats and lectin-like and immunoglobulin domains that are known to mediate protein–protein interactions in adhesion. Indeed, it has been shown that the family of adhesion GPCRs binds to a wide variety of cellular and extracellular matrix targets (Yona *et al.*, 2008). These receptors have been indicated in the immune response, development and cancer (Yona *et al.*, 2008).

Frizzled receptors have only recently been accepted as GPCRs, although they are atypical and not always coupled to heterotrimeric G-proteins (Schulte, 2010). Frizzleds are important during embryonic development controlling cell fate, proliferation and stem cell differentiation, and receive signals from secreted Wnt glycoproteins. In adults, Wnt/Frizzled signaling is usually inactive, but is frequently seen to be functional in cancer cells (Teglund *et al.*, 2010).
The secretin family is the remaining member of the earlier class B (Fredriksson *et al.*, 2003). A hallmark of this receptor family is a large extracellular N-terminal domain of about 60–80 amino acids and numerous cysteins, resulting in stabilizing disulfide bridges and thus sharing some structural features with the adhesion family (Schioth *et al.*, 2005). Ligands of the secretin family of receptors are mainly peptides, including secretin, glucagon, and parathyroid hormone (Fredriksson *et al.*, 2003).

1.3.1.1. Orphan GPCRs

Orphan GPCRs are receptors lacking defined endogenous ligands (Civelli *et al.*, 2006). They are usually discovered by molecular biology approaches based on sequence similarities, but their ligands remain to be elucidated by these techniques (Chung *et al.*, 2008). The "deorphanization" of the receptor of interest is usually done by a technique known as the reverse pharmacology. This is usually carried

out by expressing the corresponding receptor in eukaryotic cells and screening through a chemical library of known and potential ligands for binding or signal transduction abilities (Chung *et al.*, 2008). This has proven to be a very successful method and new high-throughput assays are further speeding up the process. It is, however, not known whether all orphan GPCRs have endogenous ligands or whether the 'hits' are physiologically relevant (Civelli *et al.*, 2006).

1.3.1.2. GPCR oligomerization

In recent years, experimental evidence has accumulated suggesting that GRCPs can exist as dimers or oligomers. Dimerization can occur between identical or structurally related GRCPs, or even between members of different classes (Breitwieser, 2004; Milligan *et al.*, 2006a), and dimerization (or oligomerization) is the natural state of many GPCRs (Lohse, 2010). The protein–protein interactions in complex formation are finally beginning to be understood. Interestingly, both covalent and non-covalent binding have been indicated (Gonzalez-Maeso, 2011), and TM-I and TM-IV appear to be central contact sites. The dynamic scale of the complexes is also variable, ranging from seconds to almost stable complexes. For the muscarinic M_1 receptor, about 30% of the receptors are in complexes at any given time point (Hern *et al.*, 2010). Some GPCRs (such as the β_2-adrenoceptor and $GABA_B$ receptor) form dimers during protein synthesis within the ER and are internalized as dimers (Milligan, 2008). Oligomerization of GPCRs may alter their ligand binding, activation, desensitization, trafficking, and signaling (Gonzalez-Maeso, 2011) in a manner that have both physiological significance and an impact on the future drug discovery strategies (Milligan, 2008). However, for most receptors there is thus far no firm evidence for the effect of dimerization on the properties mentioned above.

1.3.2. Heterotrimeric G-proteins

Despite the enormous diversity within the GPCR family, there are relatively few G-proteins they interact with. These small proteins are cytoplasmic peripheral membrane proteins. In humans, 21 Gα subunits, 6 Gβ subunits and 12 Gγ subunits are known (Downes and Gautam, 1999; review in Oldham *et al.*, 2008). G-proteins are classified according to the Gα subunit and can be further divided into four subclasses: $G\alpha_s$ (Fung *et al.*, 1981), $G\alpha_i$ (Hildebrandt *et al.*, 1983), $G\alpha_{q/11}$ (Strathmann *et al.*, 1990), and $G\alpha_{12/13}$ (Strathmann and Simon, 1991; reviewed in Simon *et al.*, 1991). The typical structure of Gα contains two structural units: a GTPase domain and a helical domain. The GTPase domain is highly conserved and present in every member of the G-protein superfamily. This domain has two important functions: hydrolysis of bound GTP and acting as a binding site for the Gβγ complex and GPCRs. The helical domain has six α-helices forming a bundle that closes the bound nucleotide in a tight pocket like a cover. In order to remain in membranes, each Gα is palmitoylated at its N-terminus, and some also have myristoyl modification (Oldham *et al.*, 2008). Gγ subunits additionally contain lipid linkers. They can be farnesylated or geranylated, ensuring the membrane association of the complex (Dupre *et al.*, 2009).

1.3.2.1. $G\alpha_s$ family

The story of G-proteins already began in 1958 when Sutherland and Rall described enzymatic activity producing the cyclic nucleotide cAMP (Sutherland *et al.*, 1958). It took about two decades until the signal-relaying G-protein was described (Limbird *et al.*, 1980; Pfeuffer, 1979). Typical features of $G\alpha_s$ family proteins include the ability to stimulate adenylyl cyclase (AC) and activation by cholera toxin (Oldham *et al.*, 2008).

1.3.2.2. $G\alpha_i$ family

In some experiments, G-proteins appeared to inhibit rather than activate AC; this led to the discovery of $G\alpha_i$ subunits from cyc^- S49 cells (Bokoch *et al.*, 1984; Codina *et al.*, 1984; Hildebrandt *et al.*, 1983). A little later, another member was discovered with a different structure (Pines *et al.*, 1985). It was named $G\alpha_o$ ("o" for other). The subgroup currently contains $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o1}$, $G\alpha_{o2}$, and $G\alpha_z$. Three additional members of this subgroup are specific to sensory organs: $G\alpha_{gust}$ participates in the bitter and sweet sensation in gustation, and $G\alpha_{t1}$ and $G\alpha_{t2}$ in phototransduction in the retina. All members except $G\alpha_z$ are inactivated by pertussis toxin.

1.3.2.3. $G\alpha_{q/11}$ family

Currently, the $G\alpha_{q/11}$ family consists of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15}/G\alpha_{16}$ Gα proteins (Mizuno *et al.*, 2009; Oldham *et al.*, 2008). GPCRs that connect to $G\alpha_{q/11}$ (or simply G_q) G-proteins are known for their ability to temporarily increase the intracellular Ca^{2+} concentration. Upon receptor activation, $G\alpha_{q/11}$ activates PLCβ (Taylor *et al.*, 1991) and the

subsequently produced IP_3 binds to its receptors in the smooth ER membrane and opens Ca^{2+} channels, leading to an increase in $[Ca^{2+}]_i$ (see section 1.2.2 for more detailed discussion on Ca^{2+} and G_q). Other G_q-activated downstream signaling components include DAG-activated PKC, monomeric G-proteins and MAPK (Mizuno *et al.*, 2009). Recently, $G\alpha_{16}$ has been shown to activate Ras independent of PLCβ (Liu *et al.*, 2011).

1.3.2.4. $G\alpha_{12/13}$ family

The members of this family are structurally distinct compared to other Gα families. $G\alpha_{12/13}$ proteins have versatile functions. They are especially involved in targeting Rho via RhoGEFs (guanine nucleotide exchange factors) containing a $G\alpha_{12/13}$-RGS (regulator of G-protein signaling) homology domain, and regulate a wide variety of biological processes such as embryonic development, cell growth, migration, apoptosis, and neuronal responses (Suzuki *et al.*, 2009). More than 30 $G\alpha_{12/13}$-coupling GPCRs are known, including receptors for angiotensin-2, serotonin, and lysophosphatidic acid (LPA) (Riobo *et al.*, 2005).

1.3.2.5. Gβγ subunits

Originally, the Gβγ complex was thought to be unimportant in signal transduction and to merely act as a docking site for and a negative regulator of Gα (Neer, 1995). Nowadays, Gβγ is also known to be a crucial signal transducer (Clapham *et al.*, 1997; Dupre *et al.*, 2009). Gβγ forms a functional unit that is tightly bound and remains as a heterodimer in physiological conditions; the complex only dissociates under denaturating conditions (Schmidt *et al.*, 1992). In humans there are six Gβ and 12 Gγ proteins, but not all possible Gβγ-pairs have been detected *in vivo*. This might be related to incompatible structural features of the different subunits or simply tissue-specific expression profiles. Nevertheless, the existing pool of combinations is impressive and seemingly adequate to accomplish the tasks needed (Milligan *et al.*, 2006b).

Gβ features a β-propeller secondary structure with 7 alternating tryptophan-aspartate (WD) repeats (Sondek *et al.*, 1996), found in 40 or so distinct proteins forming a unique group (Neer *et al.*, 1994; Smith *et al.*, 1999). Five β-subunits are known; β1–4 are very homologous, while β5 is structurally different. The overall homology of specific Gβγ orthologs in mammals is nearly 100% (Dupre *et al.*, 2009). Gβγ has been shown to directly regulate various signaling components, including PLCβs (Camps *et al.*, 1992), G-protein-coupled inwardly-rectifying potassium channels (GIRK) 1 and 2 (Huang *et al.*, 1995), AC (Tang *et al.*, 1991), MAPK (Crespo *et al.*, 1994), N-type voltage-gated Ca^{2+} channels (Zamponi *et al.*, 1997), and Ras (Mattingly *et al.*, 1996).

1.3.2.6. General concept of the GPCR cycle and regulation of the signaling

According to the classical model of the guanine nucleotide cycle (Kimple *et al.*, 2011), in the resting state the Gαβγ complex is associated with the receptor and GDP bound to the Gα subunit. Binding of a ligand induces a conformational change in the receptor structure that is relayed to the cytoplasmic domain. The change in the receptor structure enhances the exchange of the bound GDP for the abundant GTP in the Gα subunit; the receptor functions as a GEF (Oldham *et al.*, 2008). The nucleotide exchange leads to the dissociation of the trimeric Gαβγ complex from the cytoplasmic part of the GPCRs. Gα is further dissociated from Gβγ and both are able to diffuse (to some degree) along the plasma membrane and activate specific target proteins such as AC, PLC, monomeric G-protein GEFs, and ion channels (Oldham *et al.*, 2008). Regulation of targets ends when the intrinsic GTPase activity of the Gα subunit catalyzes the hydrolysis of GTP to GDP (Coleman *et al.*, 1994). The subsequent conformational change and increased affinity towards GPCR and Gβγ then restores the resting complex.

The rate of GTP hydrolysis can be enhanced by RGS proteins (Kimple *et al.*, 2011; Ross *et al.*, 2000). These proteins function as the GTPase-activating proteins (GAPs), and they all contain a hallmark structural element, a nine-α-helix bundle (Tesmer *et al.*, 1997). Their action has been explained by stabilization of the Gα in a transition state and lowering of the free energy required for GTP hydrolysis (Tesmer *et al.*, 1997). Altogether, 37 RGS-domain-bearing proteins with variable preferences for Gα subunit families have been identified in humans (Kimple *et al.*, 2011).

After agonist-induced activation of GPCR and the corresponding intracellular signaling pathway, signaling must be down-regulated in order to maintain the responsiveness of the cell to new stimuli. The built-in GTPase activity of the Gα subunit and RGS proteins partly affects the attenuation of the signaling cascade, but GPCRs are also controlled by membrane trafficking, leading to the rapid desensitization of GPCR signaling (Jean-Alphonse *et al.*, 2011). Ligand-induced internalization occurs

via clathrin-coated pits (CCP) with the assistance of the arrestin family of adaptor proteins (DeWire *et al.*, 2007). β-Arrestins 1 and 2 are recruited from the cytoplasm by GPCR kinase (GRK)-catalyzed phosphorylation of the GPCR. Phosphorylation creates a docking site for arrestins, and following the binding of β-arrestin to GPCR, uncouples the trimeric Gαβγ complex and transiently inactivates the receptor. β-Arrestins also guide GPCRs to CCP, where the complex is internalized by endocytosis. The internalizated receptor then has two potential fates: it might be dephosphorylated within the endosome and recycled back to the plasma membrane, fully capable of binding the ligand again, or it may be directed to lysosomes for degradation (Jean-Alphonse *et al.*, 2011).

1.4. Orexins and orexin receptors

1.4.1. Overview

1.4.1.1. Discovery of orexins

Orexins are secreted neuropeptides that have been known for only a little more than a decade. They were found simultaneously in 1998 by two independent research groups applying different scientific approaches. Yanagisawa and coworkers isolated a 33 amino acid (aa) peptide and showed that it could activate an orphan receptor, at that time termed HFGAN72 (Sakurai *et al.*, 1998). Another peptide, 28 aa in length, also activated HFGAN72. Subsequently, a second receptor with sequence similarity to HFGAN72 was cloned and it responded to both peptides as well. A 130 aa precursor peptide was shown to encode both 33 aa and 28 aa peptides that are cleaved proteolytically into two final peptides. These peptides were named orexins (from the Greek word for appetite) for their ability to induce food intake in satiated rats following injection into the lateral ventricle (Sakurai *et al.*, 1998). The 33 aa peptide was named orexin-A (OxA) and the 28 aa peptide orexin-B (OxB). Those no-longer-orphan receptors became the OX_1 and OX_2 orexin receptors. The precursor peptide was termed prepro-orexin (PPO) (Sakurai *et al.*, 1998).

At the same time, the research group of Sutcliffe – using directional tag PCR subtraction – showed that a certain hypothalamic mRNA species named clone 35 was expressed in the lateral hypothalamus. Clone 35 encoded a potential secretory peptide (containing a signal sequence) of 130 aa in length that gave rise to two peptides (de Lecea *et al.*, 1999). They named the peptides hypocretins according to their site of discovery (hypothalamus) and sequence resemblance to the hormone secretin. Hypocretin-1 is therefore equivalent to orexin-A and hypocretin-2 to orexin-B. The gene for prepro-orexin is called *hcrt*. The nomenclature varies between publications, but this thesis follows the orexin nomenclature proposed by Sakurai *et al.*

Mammalian (at least canine, human, murine and porcine) prepro-orexin is constructed of 130–131 amino acids. Human prepro-orexin is located on chromosome 17, more precisely in position 17q21 (Sakurai *et al.*, 1999). The gene contains two exons: 5'-UTR (untranslated region) and 7 aa of the signal sequence are located in the first exon and the rest of the signal sequence and both orexin-A and orexin-B sequences in exon 2.

1.4.1.2. Orexin-A and orexin-B

OxA consists of 33 amino acids and two intrachain disulfide bridges, between cysteins 6 and 12 and between 7 and 14, respectively. The N-terminal glutamine of OxA is post-translationally modified to a pyroglutamyl residue, while the C-terminal end is amidated (Sakurai *et al.*, 1998). OxA amino acid sequences are identical in humans, mice, rats, dogs, and pigs (Kukkonen *et al.*, 2002). OxB is a 28 amino acid linear peptide that shares an identical seven amino acid segment with secretin. The C-terminal part of OxA and OxB is relatively similar, including the post-translational amidation; the overall sequence homology between OxA and OxB is 46% (Figure 1.) (Sakurai *et al.*, 1998).

The three-dimensional structures of orexin-A and -B have been resolved. They are both constructed of two α-helices at an angle of approximately 70 degrees to each other (Kim *et al.*, 2004; Lee *et al.*, 1999), and the overall 3D structures are quite similar, explaining their ability to bind the same receptors (Kim *et al.*, 2004). OxA is able to penetrate the blood–brain barrier, whereas OxB is not. This is probably caused by the higher lipophilicity of OxA and the shorter plasma half-life of OxB (Kastin *et al.*, 1999). The physiological significance of this is not known.

1.4.1.3. Orexin receptors

Orexin receptors belong to the GPCR superfamily and more specifically to the rhodopsin family in the GRAFS classification. OX_1 contains 425 amino acids and the sequence homology between humans and rats is 91–98% (Sakurai *et al.*, 1998). OX_2R is slightly

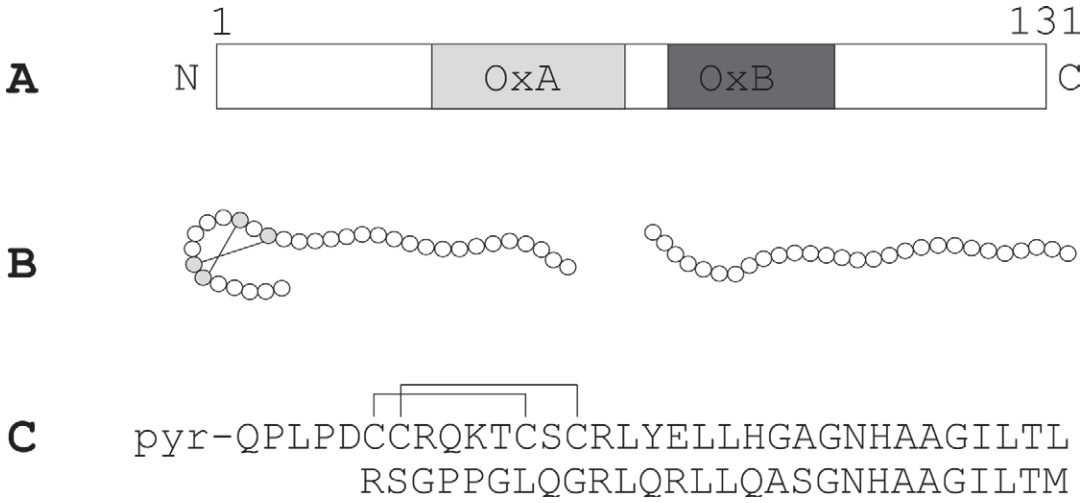

Figure 1. A) Schematic structure of the prepro-orexin. B) Orexin A contains two disulphide bridges in contrast to linear orexin B. C) The C-terminal aa sequences of both peptides are very homologous.

larger, with 444 aa. The aa sequence similarity between OX_1 and OX_2 is about 64% (Sakurai *et al.*, 1998). Both receptors are likely to couple to G-proteins of the families G_i, G_s, and G_q (Holmqvist *et al.*, 2005; Randeva *et al.*, 2001) (Figure 2.). Studies on the expression systems have suggested that OX_2R has a rather equal affinity for OxA and OxB, while OX_1R shows a ten-fold higher affinity/potency for OxA (Sakurai *et al.*, 1998), although this is greatly dependent on the expression system (Putula *et al.*, 2011).

1.4.1.4. Expression and tissue distribution of orexins and orexin receptors

Orexins are produced by orexinergic (OX) neurons, a relatively small population of neuronal cells located in the hypothalamus, especially in the lateral hypothalamic area (LHA), posterior hypothalamus (PH), and perifornical area (PF) (de Lecea *et al.*, 1998; Peyron *et al.*, 1998; Sakurai *et al.*, 1998). It has been estimated that in human brains there are approximately 70 000 orexinergic neurons (Peyron *et al.*, 1998). In rat brains the number is roughly 3000 (Nambu *et al.*, 1999). Despite the small number of OX neurons, they send projections to many brain areas (Figure 3.), suggesting that orexins modulate multiple systems (Kirchgessner, 2002). The most notable innervation targets include some other areas of the hypothalamus, the olfactory bulb, cerebral cortex, thalamus, brainstem, and spinal cord (Date *et al.*, 1999; Mondal *et al.*, 1999; Peyron *et al.*, 1998; Sutcliffe *et al.*, 2000; van den Pol, 1999; Willie *et al.*, 2001). Within the hypothalamus, OX neurons project to the arcuate nucleus and form synapses to cells containing neuropeptide Y (NPY) (Date *et al.*, 1999; Horvath *et al.*, 1999a; Peyron *et al.*, 1998). The close proximity of NPY neurons and OX neurons has also been shown to occur in the hypothalamic paraventricular nucleus (Broberger *et al.*, 1998; Horvath *et al.*, 1999a). This is very interesting, since NPY is a known orexinergic peptide (Kalra *et al.*, 1991), so it is possible that orexin, in part, acts through NPY when stimulating feeding.

The expression of orexin receptors is convergent with that of OX neurons. However, there is a notable difference in expression patterns between OX_1R and OX_2R (Marcus *et al.*, 2001). OX_1R mRNA is found in the ventromedial hypothalamic nucleus (VMH) and anterior part of the hypothalamus near the suprachiasmatic nucleus (Trivedi *et al.*, 1998). OX_1R is also expressed in the hippocampal CA1 and CA2 areas, in the raphe nuclei, and the locus coeruleus (Marcus *et al.*, 2001). Hypothalamic expression of OX_2R is mostly found in the tuberomammilary nucleus, LHA, PVN, and arcuate nucleus, and outside hypothalamus, especially in the amygdala, bed nucleus of the stria terminalis, and nucleus accumbens (Marcus *et al.*, 2001; Trivedi *et al.*, 1998).

Orexins and orexin receptors are also expressed in peripheral tissues, although the origin of orexin in peripheral tissue is not completely understood (Heinonen *et al.*, 2008). Orexins are suggested to be

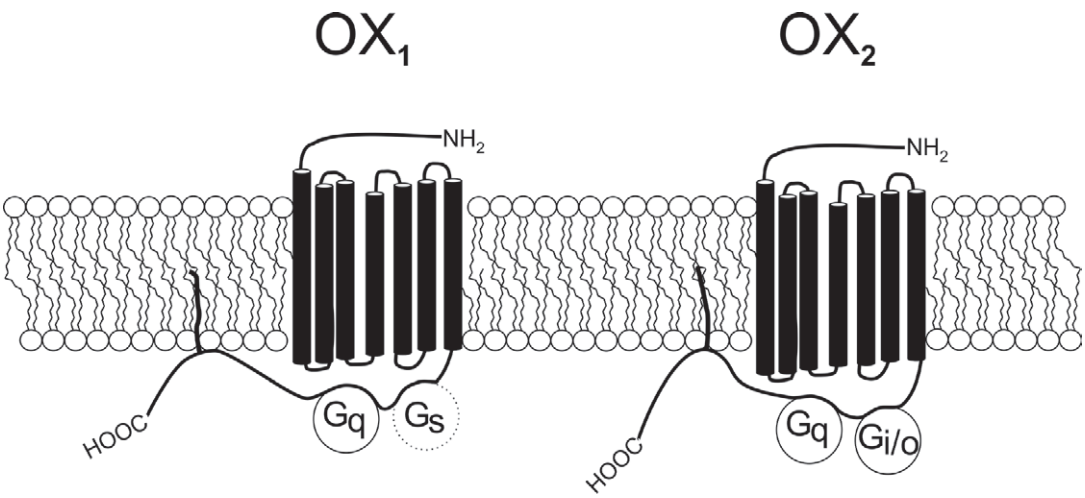

Figure 2. Schematic structure of OX_1 and OX_2 orexin receptors on the plasma membrane and the G-proteins they couple.

found in vagal afferent neurons, the gastrointestinal tract and surrounding enteric nervous system (ENS), pancreas, adrenal gland, kidney, testis and ovary, lung, heart, thyroid gland, and both brown and white adipose tissue (Heinonen *et al.*, 2008; Kirchgessner, 2002).

1.4.2. Orexin receptor signaling to the cell interior

1.4.2.1. Increase in intracellular calcium levels

One of the first measured responses to orexin receptor challenge ever recorded was an increase in $[Ca^{2+}]_i$ (Sakurai *et al.*, 1998). Subsequently, the Ca^{2+} response has been described in several cell lines, including CHO-K1 (Lund *et al.*, 2000), PC12 (Holmqvist *et al.*, 2002), Neuro-2A (Holmqvist *et al.*, 2002), and HEK-293 (Magga *et al.*, 2006), as well as in cultured neurons from the hypothalamus and dorsal root ganglia (Eriksson *et al.*, 2001; van den Pol *et al.*, 1998). In recombinant CHO-hOX_1 cells, the source of Ca^{2+} seems to differ according to the OxA concentration. At lower concentration Ca^{2+} entry is independent of PLC activity, indicating a receptor-operated Ca^{2+} entry pathway (Ekholm *et al.*, 2007; Kukkonen *et al.*, 2001; Larsson *et al.*, 2005; Lund *et al.*, 2000). This pathway is able to promote the activation of PLC-dependent IP_3 production, Ca^{2+} release and further entry via SOC (Kukkonen *et al.*, 2001; Larsson *et al.*, 2005; Lund *et al.*, 2000). Higher concentrations of orexins activate the PLC response directly without any ROC activity (Kukkonen *et al.*, 2001; Lund *et al.*, 2000). The identity of the ROC is not fully verified, but likely candidates are TRPC channels (Nasman *et al.*, 2006; Peltonen *et al.*, 2009).

1.4.2.2. Activation of adenylyl cyclase

The family of ACs consists of nine membrane-bound isoforms and one soluble isoform. ACs are divided into four main groups: group 1 (AC1, AC3, and AC8), group 2 (AC2, AC4, and AC7), group 3 (AC5 and AC6), and group 4 (AC9). The soluble isoform forms a group of its own (Patel *et al.*, 2001). All membrane-bound AC isoforms are regulated by G_s. Group 1 enzymes are Ca^{2+}-sensitive. Group 2 and 3 enzymes respond to PKC and Gβγ. All isoforms except AC9 are activated by alkaloid forskolin. Activation of ACs leads to the production of cAMP and activation of protein kinase A (PKA), cAMP-regulated ion channels, and EPAC (GEF for Rap1) (Patel *et al.*, 2001). Stimulation of both orexin receptors has been shown to activate AC and cAMP production in recombinant cell lines (Holmqvist *et al.*, 2005; Tang *et al.*, 2008) and native cells (Gorojankina *et al.*, 2007; Malendowicz *et al.*, 1999). Coupling of OX_1 to AC in the CHO-hOX_1 expression system appears to be a complex process. In addition to direct G_s activation, a novel PKC isoform, PKCδ, is involved in AC activation, while Ca^{2+} has a permissive role in the activation (Holmqvist *et al.*, 2005). OX_1 is also able to inhibit AC via G_{i-} coupling (Holmqvist *et al.*, 2005). Conversely, there have been studies in which no AC regulation has been seen (Larsson *et al.*, 2003; Magga *et al.*, 2006; van den Pol *et al.*, 1998). This discrepancy might

reflect either methodological issues or differences in cell line/tissue-specific isoform expression and activation profiles, or the subcellular localization of various AC isoforms (Ostrom *et al.*, 2012). In addition, the heart-specific isoform AC6 seems to participate in signaling without cAMP production (Gao *et al.*, 2011); therefore, cAMP measurement may not always reflect AC activation.

1.4.2.3. Activation of protein kinases by OX_1

The kinase cascades initiated by OxA binding to OX_1 are complex and the underlying signaling pathways affect each other. The activation of PLCβ leads to the production of DAG and concomitant activation of PKC. Holmqvist *et al.* (2005) identified the PKCδ isoform to be the target of OX_1, and similar results were seen in other studies (Jäntti *et al.*, 2011). The MAP kinase family consists of four members, ERK1/2, JNK, ERK5 and p38. The common feature of these kinases is their activation by cascade-like phosphorylation. OX_1 challenge leads to ERK1/2 and p38 kinase phosphorylation (Ammoun *et al.*, 2006a; Ammoun *et al.*, 2006b). Downstream effectors contributing to ERK1/2 activation include at least Ca^{2+} influx, PLC/PKC, Ras, Src, and PI3K (Ammoun *et al.*, 2006a). Similar results have been recorded with the OX_2 expression system (Tang *et al.*, 2008). MAP kinases regulate several cellular processes, including proliferation and apoptosis. Interestingly, OxA-induced apoptosis is regulated by p38 MAPK acting independently of Ca^{2+} influx, p53, and caspases (Ammoun *et al.*, 2006b).

1.4.2.4. Activation of phospholipases by OX_1

Phospholipases are enzymes that hydrolyze glycerophospholipids (Murakami et al., 2011). Their nomenclature originates from enzymatic specificity. PLA_1 releases fatty acid bound to the sn1 position in the glycerol backbone, while PLA_2 is more specific towards the sn2 position. An enzyme having both of the aforementioned activities may be termed PLB. PLC cleaves phospholipids releasing DAG and the head group along with the sn3 phosphate, and finally PLD releases phosphatidic acid (PA) and the head group without the sn3 phosphate (Figure 4.). Phospholipases might also be selective to phospholipid species or fatty acids bound to the glycerol backbone (Murakami *et al.*, 2011).

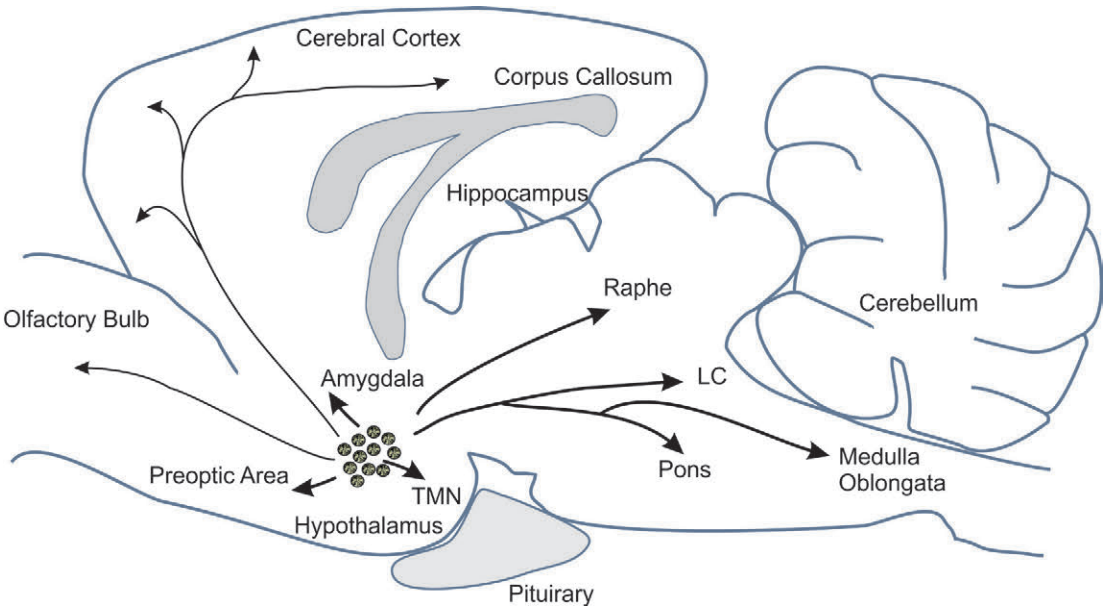

Figure 3. Summary of the organization of the orexin neuronal system in sagittal section of rat brain. The OX neurons (indicated as rasterized spheres) are located solely in the hypothalamus, but they send axonal projections to many brain areas (black arrows). The strongest projections go to the brain stem. (LC, locus coeruleus; TMN, tuberomammilary nucleus.) Picture is adapted from *Ohno K and Sakurai T, 2008*.

1.4.2.4.1. Phospholipase A_2

1.4.2.4.1.1. Overview

Our knowledge of phospholipase A_2 (PLA_2) has increased tremendously within the last two decades. PLA_2s are a relatively large and versatile family of lipid-hydrolyzing enzymes sharing common specificity towards the sn2 position of the glycerol backbone in phospholipids, a position often occupied by arachidonic acid or other polyunsaturated fatty acids. The released arachidonic acid can then act as a precursor for eicosanoid or endocannabinoid biosynthesis, while the remaining 2-lysophospholipid has signaling functions itself (Murakami *et al.*, 2011). PLA_2s have several important functions in many pathological processes, which has raised huge interest among pharmaceutical companies in developing specific inhibitors against different members of this superfamily (Burke *et al.*, 2009).

The first PLA_2s were discovered from various animal venoms (snakes, bee), the first already in 1890. The nomenclature is somewhat obscure and owes its origin to the classification of old and new world snake lipases (group I and group II), and group III came along with the discovery of the unique PLA_2 of bee venom. The discovery of intracellular PLA_2 made a systematic nomenclature essential (Schaloske *et al.*, 2006). To date, at least 30 different PLA_2 are known in mammals. Based on their structure, catalytic mechanism and localization, PLA_2s are categorized into six classes, most of which contain several isoforms (Murakami *et al.*, 2011).

1.4.2.4.1.2. Secretory PLA_2 family

PLA_2s derived from animal venoms are currently known as group I or secretory PLA_2 ($sPLA_2$). This group contains ten members, which all are low molecular weight proteins around 13–15 kDa (Murakami *et al.*, 2011), with the exception of the mammalian enzyme with a size about 55 kDa (Valentin *et al.*, 2000). The $sPLA_2$ family is evolutionarily old and also present in plants (reviewed in Lee *et al.*, 2005) and lower animals, including insects (Ryu *et al.*, 2003), mollusks (McIntosh *et al.*, 1995), and reptiles (Kini, 2003). They have a requirement for Ca^{2+} and they all utilize a similar catalytic strategy (His/Asp-dyad and nucleophilic attack) of bond cleavage from the sn2 position (Scott *et al.*, 1990). Besides being central components in animal-derived venoms, sPLA2s participate in many physiological processes. Interestingly, a receptor for group IB $sPLA_2$ has been discovered (reviewed by Hanasaki *et al.*, 2002). PLA_2-R is a type 1 glycosylated membrane protein. Its large N-terminal portion contains a fibronectin-like type II domain and various carbohydrate recognition domains (CDRs). Structurally, it is related to C-type lectins, but it has no close homologs in the mammalian genome (Higashino *et al.*, 1994).

1.4.2.4.1.3. Cytoplasmic PLA_2s or GIVAs

Cytoplasmic PLA_2s or $cPLA_2$s (group IV A or GIVA) have six members, including the prototypic $cPLA_2\alpha$. Other members include $PLA_2\beta$, $PLA_2\gamma$, $PLA_2\delta$, $PLA_2\varepsilon$, and $PLA_2\zeta$ (these are occasionally referred to as IVA–F, respectively). From an evolutionary perspective, $cPLA_2$s are relatively new and appeared alongside vertebrate evolution and the development of eicosanoid signaling (Murakami *et al.*, 2011). Cytoplasmic PLA_2s belong to the serine hydrolase family featuring a catalytic Ser/Asp-dyad at the bottom of a funnel-like structure in the catalytic domain, as indicated in the crystal structure (Dessen *et al.*, 1999). Typical for the serine hydrolase family are multiple enzymatic activities, including PLA_1, PLA_2, lysophospholipase and acyltransferase activities (Murakami *et al.*, 2011). All members (except cPLA2γ) of the $cPLA_2$ family have a C2 domain in their N-terminus that directs the enzyme to the plasma membrane in a Ca^{2+}-dependent manner. The only member showing specificity towards arachidonic acid is $cPLA_2\alpha$.

1.4.2.4.1.3.1. Regulation of $cPLA_2\alpha$ activation

The activation mechanism of $cPLA_2\alpha$ is quite well known. Increasing $[Ca^{2+}]_i$ leads to $cPLA_2$ translocation

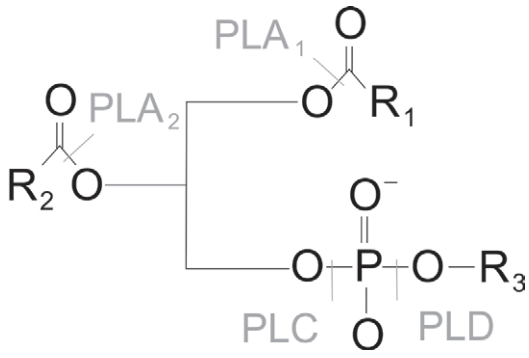

Figure 4. The cleavage sites of phospholipid by common phospholipases. The enzymes are indicated in grey color.

to membranes within seconds. Interestingly, the target membranes are the intracellular (perinuclear) membranes (ER and Golgi) rather than the plasma membrane. The explanation lies in the C2 domain structure of $cPLA_2$ and different lipid composition of the organelle membranes. The C2 domain of $cPLA_2$ binds to PC, unlike the C2 domain in PKC, which prefers phospaditylserine. This has been verified in a clever study in which the C2 domains of $cPLA_2\alpha$ and PKCα were switched (Stahelin *et al.*, 2003). Translocation profiles changed accordingly, and the chimeric $cPLA_2\alpha$ with C2-PKCα shifted to the plasma membrane, while PKCα with C2-$PLA_2\alpha$ moved to the perinuclear membrane. Translocation to the perinuclear membrane brings $cPLA_2$ close to COX enzymes; indeed, prostaglandin metabolism in $cPLA_2\alpha$ containing C2-PKCα is diminished (Murakami *et al.*, 2003). Interestingly, both chimeric $cPLA_2\alpha$ and the wild-type enzyme showed a similar AA-releasing potential (Murakami *et al.*, 2003). The enzyme is further activated by phosphorylation of Ser505 by MAPK (Lin *et al.*, 1993). This induces a change in the conformation of the protein, revealing hydrophobic residues that promote the interactions of the catalytic domain with the membrane, even when $[Ca^{2+}]_i$ has declined. MAPK also phosphorylates Ser727 in the C-terminal part of the enzyme. This phosphorylation masks the binding site for the regulatory protein complex of p11 and annexin A2 that binds to unphosphorylated Ser727 and prevents membrane interactions and enzyme translocation (Hefner *et al.*, 2000; Tian *et al.*, 2008). $cPLA_2\alpha$ has a putative lysine-rich binding site for PIP_2; this lipid is an important activator of the enzyme (Tucker *et al.*, 2009).

1.4.2.4.1.3.2. $cPLA_2$s in health and disease

The generation of a cPLA2α knockout mouse model (*Pla2g4a*$^{-/-}$) (Uozumi *et al.*, 1997) has increased our knowledge of the biological roles of $cPLA_2\alpha$. *Pla2g4a*$^{-/-}$ mice show reduced eicosanoid signaling and impairments in immunity (Murakami *et al.*, 2011). Interestingly, *Pla2g4a*$^{-/-}$ mice seem less prone to many pathological conditions caused by immune system malfunctions. *Pla2g4a*$^{-/-}$ mice show generally reduced symptoms in airway anaphylactic responses (Uozumi *et al.*, 1997), beomycin-induced pulmonary fibrosis (Nagase *et al.*, 2002), the ARDS (adult respiratory distress syndrome) model (Nagase *et al.*, 2000), rheumatoid arthritis (Hegen *et al.*, 2003), and EAE (experimental autoimmune encephalitis), an animal model of multiple sclerosis (Marusic *et al.*, 2005), indicating an active role for $cPLA_2\alpha$ in all these pathophysiological processes. In addition, involvement of cPLA2α has been indicated in lung and prostate cancers (Patel *et al.*, 2008; Weiser-Evans *et al.*, 2009), the onset of labor, fertility, and ovulation (Uozumi *et al.*, 1997). Recently, a new potential function of $cPLA_2$ in Golgi trafficking has been proposed (San Pietro *et al.*, 2009). Vesicle-directed proteins transfer from the rough ER (rER) to the Golgi apparatus for further maturation and vesicle packing, and this involves intracisternal connections. Wedge-shaped phospholipids created by $cPLA_2$ activity help to form the positive curvature needed in tubule formation. Indeed, various methods to inhibit $cPLA_2$ (siRNA, pharmacological inhibitors, dominant-negative constructs) block intracisternal trafficking in the Golgi apparatus, but do not inhibit ER-to-Golgi, Golgi-to-ER or trans-golgi network (TGN)-to-plasma membrane transport (San Pietro *et al.*, 2009).

1.4.2.4.1.3.3. Other members of the GIVA family

Much less is known about the remaining members of the GIVA family. $cPLA_2\beta$ features an additional JimC-domain, making it larger than other family members. It possesses an approximately 1500 times higher lysophospholipase activity than PLA_2 activity. It is constitutively located on mitochondrial membranes. Ubiquitously expressed $cPLA_2\gamma$ is an unusual GIVA family member, since it lacks the characteristic C2-domain (Underwood *et al.*, 1998). Membrane remodeling and homeostasis are believed to be the main cellular functions of $cPLA_2\gamma$ due to prominent lysophospholipase and transacetylase activities (Yamashita *et al.*, 2009). $cPLA_2\delta$ shows both PLA_1 and PLA_2 activities and it translocates to perinuclear membranes, but with much slower kinetics than c $PLA_2\alpha$ (Ohto *et al.*, 2005). $cPLA_2\varepsilon$ and $cPLA_2\zeta$ are found in the thyroid and ε also in muscle and the heart (Ohto *et al.*, 2005). $cPLA_2\varepsilon$ has weak enzymatic activity, but $cPLA_2\zeta$ has been shown to release AA or OA and to show some selectivity for PE over PC (Ghosh *et al.*, 2007).

Ca^{2+}-independent intracellular PLA_2s are known as $iPLA_2$s, group VI or patatin-like phospholipase domain-containing lipases (PNPLAs). They are the third subgroup and the second largest with nine members (PNPLA1–9). Their physiological functions vary from membrane homeostasis and energy metabolism to signaling (reviewed by Murakami *et al.*, 2011). The best characterized member of this family is GVIA $PLA_2\beta$ (Balboa *et al.*, 1997). Several splice variants of $iPLA_2\beta$ are known, but only two are active *in vivo*. The structural hallmark of $iPLA_2\beta$ is the eight ankyrin

repeats in the N-terminus (Larsson *et al.*, 1998), indicating potential oligomerization. A putative binding site for calmodulin is located near the C-terminus (Jenkins *et al.*, 2001). iPLA$_2$β shares a similar catalytic mechanism with cPLA$_2$α, but does not display any specificity towards arachidonic acid in the sn2 position or for the phospoholipid head group (Balsinde *et al.*, 2005). This versatile enzyme also posses transacetylase and lysophospholipase activities (Winstead *et al.*, 2000). iPLA$_2$β involvement is indicated in the regulation of monocyte migration (Mishra *et al.*, 2008), apoptosis (Atsumi *et al.*, 2000), ovarian cancer (Li *et al.*, 2010), and cardiac ischemia (McHowat *et al.*, 1998).

The remaining three subgroups are platelet-activating factor acetylhydrolase (PAF-AH), also known as lipoprotein-associated PLA$_2$, with four members (Tellis *et al.*, 2009), lysosomal PLA$_2$s (Kim *et al.*, 2004) with two members, and the recently discovered adipose-specific PLA$_2$ (Duncan *et al.*, 2008). They have been shown to regulate various physiological processes, including neuronal migration during development (Manya *et al.*, 1998), surfactant metabolism (Shayman *et al.*, 2011), and adipocyte lipolysis (Duncan *et al.*, 2008).

For a long time, inhibitors of PLA$_2$s have been substrate analogues such as MAFP (methoxy arachidonylfluorophosphonate) or ETYA (5,8,11,14-eicosatetraynoic acid). The use of such inhibitors is problematic because of the nonspecific inhibition of almost all arachidonic acid-metabolizing enzymes. The recent development of more specific and even subgroup-selective inhibitors has markedly changed PLA$_2$ research. One of the first commercially available cPLA$_2$-specific inhibitors was pyrrophenone (Ono *et al.*, 2002), which was also used in this thesis (Paper II).

1.4.2.4.2. Activation of other phospholipases by OX$_1$

Activation of the PLC family has already been discussed in section 1.2.2. Briefly, orexins/orexin receptors induce the activation of PLC and production of the second messengers DAG and IP$_3$ from PIP$_2$. This leads to the activation of PKC and an increase in $[Ca^{2+}]_i$.

OX$_1$ are able to activate yet another lipase, phospholipase D (PLD), which hydrolyzes PC to PA and choline. Two mammalian PLDs have been described, PLD1 and PLD2, each having two splice variants that are denoted with the letters a and b (PLD1a, PLD1b, PLD2a and PLD2b) (Colley *et al.*, 1997a; Colley *et al.*, 1997b; Hammond *et al.*, 1997; Steed *et al.*, 1998). Both enzymes are initially activated by GPCRs, and other regulators include phosphoinositides (especially PIP$_2$), Arf and Rho GTPases, and phosphorylation by PKC and MAPKs (Hammond *et al.*, 1997; Jenkins *et al.*, 2005; Muthalif *et al.*, 2000). PLD activation, however, does not necessarily require PLC activation (Balboa *et al.*, 1998; Jäntti *et al.*, 2011). PA, the main product of PLD activation, is an intracellular signaling molecule that can regulate proteins such as phosphaditylinositol-4-phosphate 5-kinase (PIP5K) (Moritz *et al.*, 1992), mTOR (Fang *et al.*, 2001), Raf (Ghosh *et al.*, 2003), and protein phosphatase-1γ (Jones *et al.*, 2002). PLD might partly activate itself: PA activates PIP5K, which in turn produces more PIP$_2$ (an activator of PLD), creating a possible feed-forward loop. PA can be converted to DAG by phosphatidic acid phosphohydrolases (Carman *et al.*, 2006; Sciorra *et al.*, 1999) or to LPA by PLA$_1$, sPLA$_2$, or autotaxin/lyso-PLD (reviewed in Mills *et al.*, 2003). LPA can act as a GPCR ligand and regulate various biological roles, ranging from cell proliferation to wound healing and the reversal of differentiation (Mills *et al.*, 2003).

OX$_1$ coupling to PLD in CHO cells was first suggested in 2008 based on indirect evidence (Johansson *et al.*, 2008), and later confirmed in detail by our group (Jäntti *et al.*, 2011). Based on the pharmacological evidence, the activated isoform is PLD1. A novel PKC, probably PKCδ, is required to activate PLD1 in this cascade (Jäntti *et al.*, 2011).

1.4.2.5. Signaling through arachidonic acid and ARC channels

Arachidonic acid (AA) is a 20-carbon, polyunsaturated fatty acid (PUFA). It is not considered as an essential fatty acid for humans as such, but its precursor, linoleic acid, is, meaning that human metabolism cannot produce linoleic acid *de novo* and therefore we must acquire linoleic acid from the diet. There are, however, some mammalian species that lack the enzymatic machinery to convert linoleic acid to AA. AA is best known for its role as a precursor in eicosanoid biosynthesis. AA has subsequently been shown to be important for endocannabinoid biosynthesis (Piomelli, 2003) and to act as such in signal transduction, activating certain isoforms of PKC (i.e. PKCα) (Lopez-Nicolas *et al.*, 2006), PIK3 (Hughes-Fulford *et al.*, 2006), and the heat shock protein response (Jurivich *et al.*, 1994). Activation of OX$_1$ induces robust release of the AA (Papers I–IV)

by $cPLA_2$ activity (Paper II). AA release is dependent on the Ca^{2+} influx and possibly the activation of MAPK (Paper I).

The idea that AA itself might regulate Ca^{2+} currents came from the studies of Shuttleworth and coworkers (Shuttleworth *et al.*, 1998) on the HEK293 cell line and muscarinic receptors. They showed that AA was able to activate Ca^{2+} entry without any apparent PLC activation. The electrophysiological properties of the measured Ca^{2+} conductance also suggested a distinct channel population. Within a decade, the concept of AA-regulated Ca^{2+} conductance channels (ARC channels) had been established. ARC is a Ca^{2+}-selective, low conductance channel acting in a store-independent manner in the plasma membrane. The ARC channel has recently been suggested to consist of three Orai1 and two Orai3 proteins (but not Orai2) in a pentameric assembly (Mignen *et al.*, 2009).

1.4.3. Physiological effect of orexins

1.4.3.1. Regulation of metabolism

The tissue expression profile of orexins and orexin receptors probably reflects their biological role. At the time of their discovery, it was suggested that orexins act as regulators of feeding (Sakurai *et al.*, 1998). This finding was supported by the fact that orexins and orexin receptors are found in many brain areas related to feeding, reward and direct neuronal regulation of the gastrointestinal tract (GIT). They are also present in peripheral tissues related to feeding and metabolic regulation (GIT and ENS, the pancreas, adipose tissue and adrenal and thyroid glands). PPO mRNA levels are upregulated in fasting (Sakurai *et al.*, 1998). Interestingly, similar regulation is also seen in the flatfish, *Verasper moseri* (Amiya *et al.*, 2012), possibly indicating an evolutionarily old mechanism of metabolic regulation. OX neurons are able to respond to various metabolic signals originating from the periphery (Tsuneki *et al.*, 2012). These include glucose, leptin, and grehlin (Moriguchi *et al.*, 1999; Yamanaka *et al.*, 2003). OX neurons also respond to amino acids (aa) that revert the inhibitory effect of glucose on these neurons (Karnani *et al.*, 2011). The stimulation of OX neurons is dependent on nonessential aa. Previously, only essential aa have been shown to set off neuronal activation (Blouet *et al.*, 2009; Hao *et al.*, 2005). In certain physiological conditions, the ability to detect nonessential aa is beneficial. Nonessential aa more readily cross the blood–brain barrier than essential aa (Oldendorf *et al.*, 1976). Altered concentration ratios of essential and non-essential aa in the brain could therefore indicate deficiency of essential aa in the blood. This might relate to an inadequate dietary composition or an overall increase in aa in the blood due to muscle protein catabolism during prolonged starvation. The ability of OX neurons to sense differences in aa levels might function as a feedback mechanism to induce feeding as a response to a deficiency of aa (Karnani *et al.*, 2011). Centrally, but not intraperitoneally administrated antibodies against orexin peptides directly reduced food intake, pointing to the role of endogenous orexins in feeding (Yamada *et al.*, 2000). Recently, Okamura and Takakusaki presented a trigger role for OxA in the regulation of the cephalic phase of feeding, including anticipatory secretion priming the gut to digestion (Okumura *et al.*, 2008). Their theory is a synthesis of several details including localization of OX neurons in the LH and the long-known role of LH in the regulation of feeding, including gastric acid secretion (Tache *et al.*, 1990).

OxA has been shown to directly stimulate gastric acid secretion when administrated centrally, while intraperitoneal injection fails to induce secretion (Takahashi *et al.*, 1999). OxA appears to be a specific brain-derived gastric acid secretion-stimulating substance, because many tested chemicals or peptides are ineffective or inhibitory (Okumura *et al.*, 1994; Okumura *et al.*, 1990; Okumura *et al.*, 1991; Okumura *et al.*, 2000). The stimulatory action of OxA is relayed by the vagal system, since atropine or vagetomy completely blocks the secretion (Takahashi *et al.*, 1999). The dorsal motor nucleus of the vagus (DMV) contains orexin-immunoreactive nerve terminals and also OX_1 receptors, and GIT-projecting neurons of the DMV are directly activated by OxA to produce gastric secretion and motility (Grabauskas *et al.*, 2003; Krowicki *et al.*, 2002). Orexins also affect pancreatic exocrine secretion, an important part of the cephalic phase response, in a vagus-dependent manner (Miyasaka *et al.*, 2002). Hypoglycemia has been shown to stimulate OX neurons (Briski *et al.*, 2001; Cai *et al.*, 2001). Injection of the OX_1 antagonist SB334867 into the DMV has been shown to block insulin-induced hypoglycemia stimulation of pancreatic nerve firing, while injection of OxA increased firing 30-fold (Wu *et al.*, 2004). These findings support the idea of OxA acting as a trigger molecule in the cephalic phase of feeding, which not only activates the secretion but also the motility of the gut. Interestingly, central administration of OxA also induces feeding-related behavior, such as grooming and burrowing (Ida *et al.*, 1999).

Orexins also have a regulatory role in pancreatic endocrine section. Based on immunohistochemical

analysis, Quedraogo and coworkers suggested the presence of OxA and OX_1 in pancreatic α- and β-cells (Ouedraogo *et al.*, 2003). OxA has been shown to inhibit glucagon secretion by down-regulating preproglucagon via Ca^{2+} and the transcription factors CREB and Foxo-1 (Goncz *et al.*, 2008).

Very recently, an interesting and new role for orexins in the regulation of brown adipose tissue (BAT) development has been described (Sellayah *et al.*, 2011; Tupone *et al.*, 2011). It has been shown that orexin signaling is needed to initiate the neonatal BAT developmental program. *PPO*-null mice demonstrated striking differences in BAT morphology, which is the most likely reason why these mice may develop obesity.

1.4.3.2. Regulation of sleep/wakefulness and arousal

Furthermore, orexins/receptors are found in brain areas known to control the sleep/wake cycle and arousal (Adamantidis *et al.*, 2007; Sakurai *et al.*, 2010). The effect of orexins on feeding might be indirect, for instance with increased arousal resulting in more efficient food search/feeding, since prolonged OxA treatment does not increase food intake or body weight in the same way, for example, as NPY. Orexins have been shown to affect both slow-wave and REM (rapid eye movement) sleep. Several studies have suggested that the promotion of wakefulness by orexin occurs via the stimulation of histamine-producing neurons of the hypothalamic tuberomammilary nucleus (TMN) (Bayer *et al.*, 2001; Eriksson *et al.*, 2001), noradrenergic neurons of the locus coeruleus (LC) (Hagan *et al.*, 1999), and cholinergic laterodorsal tegmental neurons (Burlet *et al.*, 2002). Noradrenergic neurons fire rapidly during wakefulness, reduce firing during NREM sleep, and fall into almost total silence during REM sleep. On the contrary, GABA/galaninergic neurons of the hypothalamic ventrolateral preoptic nucleus (VLPO) are active during sleep. These neurons reciprocally inhibit each other and maintain the states of wakefulness and sleep. OX neurons send dense projections to the aforementioned monoaminergic nuclei (Nambu *et al.*, 1999; Peyron *et al.*, 1998), and orexins have been shown to increase the firing of noradrenergic neurons of the LC (Horvath *et al.*, 1999b), dorsal raphe nucleus (Brown *et al.*, 2001), and TMN (Eriksson *et al.*, 2001). In general, orexin neurons are fully active during active wakefulness and remain almost silent during sleep (Sakurai *et al.*, 2010). OX neurons, monoaminergic/cholinergic neurons in the brain stem waking center, and GABAergic neurons in the VLPO are connected to each other in a triangular manner: OX neurons send excitatory projections to monoaminergic neurons, which in turn send inhibitory projections back to the OX neurons (Figure 5.). VLPO sleep-promoting neurons inhibit both OX neurons and monoaminergic neurons (Sakurai, 2007). During arousal, VLPO and monoaminergic neurons inhibit each other, while OX neurons stabilize the waking state by enhancing signaling in the wake center (Sakurai, 2007). Recently, (Yamanaka *et al.*, 2010) described a positive feedback loop of orexin-activated OX neurons. They showed that OX neurons project to themselves and are able to activate themselves both directly and indirectly (via increased excitatory input from orexin-activated glutaminergic neurons) through OX_2. They proposed that this mechanism might function to maintain the activity of OX neurons during wakefulness (Yamanaka *et al.*, 2010).

1.4.3.3. Orexins in narcolepsy

Narcolepsy is a chronic neurological condition or disorder characterized by excessive daytime sleepiness and falling asleep at inappropriate moments and situations (sleep attacks), and also by a reduced onset of REM sleep (Ohno *et al.*, 2008). Night-time sleep is often fragmented and associated with hypnagogic hallucinations prior to falling asleep and sleep paralysis in awakening (Ohno *et al.*, 2008). Narcoleptic patients often suffer from cataplexy, a sudden weakening of the muscle tone. Cataplexy attacks are usually set off by emotional stimuli, and the degree of attacks ranges from minor jaw-dropping to a complete collapse of the postural muscles (Sakurai *et al.*, 2010).

The clue that orexins and orexin receptors are involved in narcolepsy came from animal studies. Dogs have a genetic mutation destroying the functionality of OX_2R (Lin *et al.*, 1999). The phenotype resembles that of human narcoleptic patients. Likewise, *PPO* gene knockout mice show narcoleptic symptoms (Chemelli *et al.*, 1999), as do mice models in which OX neurons are genetically destroyed (Hara *et al.*, 2001). Cerebrospinal fluid (CSF) samples are virtually devoid of OxA in approximately 95% of human narcoleptics with cataplexy (Nishino *et al.*, 2000). Taken together, these data indicate that the lack of either orexins or OX_2 results in a narcoleptic phenotype. Post-mortem studies on humans have revealed a marked loss of PPO mRNA and orexins (Peyron *et al.*, 2000; Thannickal *et al.*, 2000), but narcolepsy-related genetic alterations are not really seen in prepro-orexin or orexin receptor genes

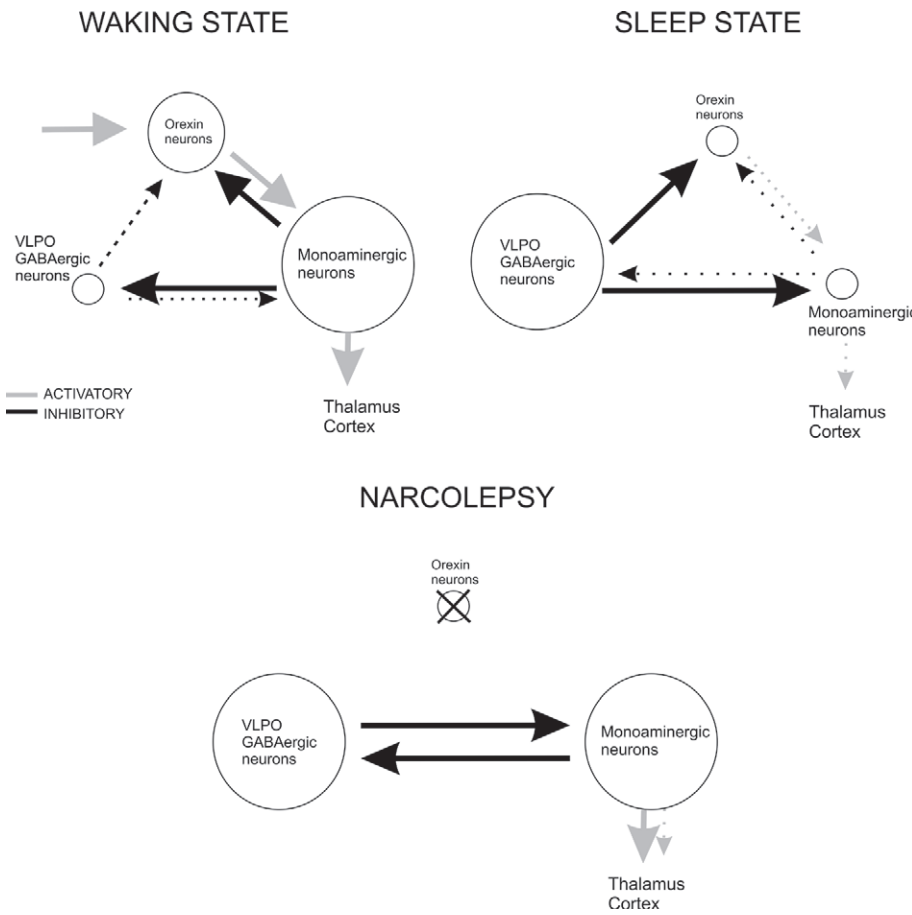

Figure 5. The 'Wake/sleep –triangle' and mechanisms how OX neurons stabilize the wake/sleep states. The OX neurons, the monoaminergic neurons in the brain stem waking center, and the GABAergic neurons in the ventro-lateral preoptic nucleus (VLPO) are connected to each other in a triangular manner.

In the wake state VLPO and monoaminergic waking center are reciprocally inhibiting each other. OX neurons send excitatory stimuli to the waking center which, in turn, maintain the inhibitory stimulus to the VLPO. The waking center also sends inhibitory stimuli to the OX neurons fine-tuning their activity.

In the sleep state the VLPO neurons are activated and send inhibitory signals to OX neurons and the waking center to maintain sleep.

In narcolepsy the loss of OX neurons leads to destabilization of the waking state and monoaminergic signaling output. The balance between the wake state and sleep state can change rapidly and patient falls asleep inappropriately.

The grey arrows indicate activating signaling and the black arrows inhibitory signaling. Solid and broken arrows indicate the relative strength and weakness of the stimulus while sizes of the spheres indicate the relative activity of the indicated brain area. The picture is adapted from Sakurai T. *et al.*, 2010.

(Sakurai *et al.*, 2010). The concomitant loss of prepro-dynorphin (Crocker *et al.*, 2005) and the above data suggest that OX neurons are partly or completely lost as a part of the narcolepsy etiology (Sakurai *et al.*, 2010). Narcolepsy correlates with certain HLA alleles (Kadotani *et al.*, 1998), indicating that an autoimmune response might be responsible for OX neuron loss Kornum *et al.* (2011). When OX neurons are lost, the balance in the triangular complex between OX neurons, the sleep-inducing VLPO neurons, and wake-signaling monoaminergic neurons is changed. The communication between the VLPO and waking center continues without stabilizing signaling, and the phenotypic outcome

is inappropriate, flipping from wakefulness to sleep and *vice versa* (Sakurai *et al.*, 2010).

1.4.3.4. The role of orexins and OX_1 as regulators of apoptosis

In addition to regulation of metabolism and arousal, orexins relay potential apoptotic signals via OX_1 and OX_2 receptors in CHO expression models, neuroblastoma cells, and colon carcinoma cells (Ammoun *et al.*, 2006b; Rouet-Benzineb *et al.*, 2004; Voisin *et al.*, 2011; Voisin *et al.*, 2006). OX_1 is expressed in every tested colon cancer cell line, as well as primary tumors and metastases, but not in normal colonocytes or cells derived from patients with irritable bowel syndrome (Voisin *et al.*, 2011). The molecular mechanism of apoptosis elicited by OX_1 seems to be independent of an increase in $[Ca^{2+}]_i$ (Ammoun *et al.*, 2006b; Voisin *et al.*, 2011), and relies on p38 MAPK (Ammoun *et al.*, 2006b) and/or Src family kinases and SHP-2 tyrosine phosphatase (El Firar *et al.*, 2009). This signaling activates the mitochondrial apoptosis pathway, including cytochrome c release and the activation of caspases 3 and 7 (Rouet-Benzineb *et al.*, 2004). The possibility to utilize aberrant expression of OX_1 against colon carcinoma could enable the development of a novel type of therapy, given that the few theoretical obstacles (e.g. receptor desensitization) could be overcome (Laburthe *et al.*, 2011).

1.5. The endocannabinoid system

The endocannabinoid system consists of endocannabinoids (eCBs), their GPCR-family receptors and the enzymatic machinery that synthesizes and degrades eCBs (Di Marzo, 2009). The hemp, *Cannabis sativa*, has for long been known to contain several psychoactive compounds. The most important and best characterized of these is Δ^9-tetrahydrocannabiol (THC), which was isolated and described by Gaoni and Mechoulam in 1964. Subsequent intensive research led to the elucidation of the target of THC via the key discovery that small concentrations of cannabinoid drugs inhibited AC (Howlett, 1984; Howlett *et al.*, 1984). These findings led to the suggestion of a signal transduction pathway being involved in the mechanisms of action of THC, in contrast to the earlier belief in altered membrane permeability induced by THC. Further evidence for the involvement of G-protein coupling came in later studies in which the requirement for Mg^{2+} and GTP and the sensitivity to pertussis toxin confirmed the $G_{i/o}$ connection (Howlett, 1985; Howlett *et al.*, 1986). The development of a stereoselective radiolabeled analogue [^{3}H]-CP55940 eventually led to the discovery of the main targets of THC, the cannabinoid receptors (Devane *et al.*, 1988). Cannabinoid receptor 1 (CB_1) was cloned from rats (Matsuda *et al.*, 1990) and from humans (Gerard *et al.*, 1990) and cannabinoid receptor 2 (CB_2) from HL60 promyelotic cells (Munro *et al.*, 1993).

1.5.1. Endocannabinoid receptors and signal transduction

Cannabinoid receptors belong to the rhodopsin branch of the GPCR superfamily (Pertwee *et al.*, 2010). The distribution of CB_1 and CB_2 in the body is strikingly different: CB_1 is almost exclusively expressed in neurons of the central nervous system (CNS) (Egertova *et al.*, 2000; Herkenham *et al.*, 1990; Howlett *et al.*, 2002; Matsuda *et al.*, 1993), while CB_2 is mainly found in immune cells and peripheral tissues (Munro *et al.*, 1993). CB_1 is also expressed in the liver, adipose tissue, reproductive organs, muscle, bone, and cardiac and vascular tissues, while some CB_2 expression is found in the brain microglia (Pertwee *et al.*, 2010). The existence of a third cannabinoid receptor is under debate (reviewed in Kano *et al.*, 2009).

Rat CB_1 consists of 473 amino acids, the human analogue of 472 and that in the mouse of 473 amino acids (Howlett *et al.*, 2002). The sequence homology of CB_1 between these three species is 97–99%. In humans, two N-terminal splice variants are known with different ligand-binding properties (Ryberg *et al.*, 2005). Cannabinoid receptors are associated with the $G_{i/o}$ family of G-proteins ($G_{i1,2,3}$ and $G_{o1,2}$), and AC inhibition has been seen in most tissues and cells investigated (Howlett *et al.*, 2002). In the presence of pertussis toxin, CB_1 has been shown to stimulate AC, indicating that CB_1 may be able to couple G_s in the absence of G_i (Glass *et al.*, 1997). CB_1 activation leads to the activation of the MAPK (ERK1/2) cascade (Bouaboula *et al.*, 1995; Wartmann *et al.*, 1995). Depending on the cell type and the stimulus, the mechanisms leading to MAPK phosphorylation (and activation) can vary. In CHO cells, the pathway consisting of PI3K, protein kinase B (PKB/Akt), Raf-1, and MAPK/ERK kinase (MEK) is utilized for MAPK phosphorylation (Galve-Roperh *et al.*, 2002). In the N1E-115 neuroblastoma cell line, a CB_1-induced reduction in cAMP and PKA activity reduced inhibitory c-Raf phosphorylation and stimulated MEK activation (Davis *et al.*, 2003).

CB_1 has been shown to activate p38 MAPK in hippocampal slice preparations (Derkinderen *et al.*, 2001) and human endothelial cells (Liu *et al.*, 2000). In CHO cells, CB_1 activates JNK and p38 (Rueda *et al.*, 2000). The generation of nitric oxide (NO) upon CB_1 stimulation has been documented in various cell types (Jones *et al.*, 2008; Prevot *et al.*, 1998; Stefano *et al.*, 1996), while in others, CB_1 reduces NO levels (Cabral *et al.*, 2001; Hillard *et al.*, 1999). Signaling through CB_1 has also been shown to regulate ion channels. CB_1 activates inward-rectifying K^+ channels (Kir) and inhibits N-, P/Q- and L-type voltage-gated Ca^{2+} channels, mostly by direct Gβγ interaction with the channel (Howlett *et al.*, 2002).

Endocannabinoids are also able to activate other endogenous receptors. Potential target receptors ('non-CB' receptors) include GPR55, opioid receptors, peroxisome proliferator-activated receptors (PPARs), and vanilloid type TPR channels (Kukkonen, 2011; Pertwee *et al.*, 2010).

1.5.2. Metabolism of endocannabinoids

The discovery of cannabinoid receptors started intensive research to elucidate their endogenous ligands. The first ligand isolated was surprisingly a lipid instead of a peptide, as predicted. The molecule was an amide of arachidonic acid and ethanolamide, *N*-arachidonylethanolamide, and was named as an anandamide from a word meaning bliss in the Sanskrit language (Devane *et al.*, 1992). It soon became evident that endogenous ligands were all lipid derivatives containing arachidonic acid. 2-Arachidonylglycerol (2-AG) was found in 1995 (Sugiura *et al.*, 1995), 2-arachidonylglyceryl ether (noladin ether) in 2001 (Hanus *et al.*, 2001), and virodhamine in 2002 (Porter *et al.*, 2002) (Figure 6.). In general, endocannabinoids are produced on demand at the site of need and are not usually stored in vesicles like classical neurotransmitters (Di Marzo, 2009).

1.5.2.1. Biosynthesis

Anandamide biosynthesis in the neurons is a two-step process. Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT) transfers a fatty acid from the sn1-position of a glycerophospholipid to the primary amine group in phosphatidylethanolamine (PE) creating *N*-acyl PE (NAPE). Next, a specific NAPE-PLD hydrolyses NAPE to anandamide and PA (Okamoto *et al.*, 2007). Ca-NAT is yet to be cloned, but NAPE-PLD was cloned in 2004 (Okamoto *et al.*, 2004). The generation and analysis of NAPE-PLD-deficient mice (Leung *et al.*, 2006) indicates the existence of an alternative pathway for anandamide biosynthesis. According to the hypothesis, NAPE is double-deacylated and the resulting glycerophosphate-NAE is hydrolyzed to glycerol-3-phosphate and anandamide (Simon *et al.*, 2006). This reaction is inhibited by MAFP,

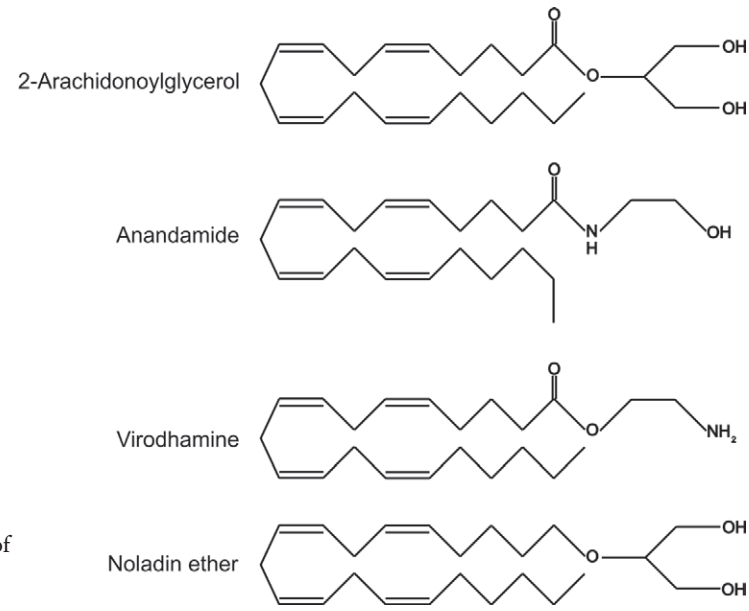

Figure 6. Chemical structures of four common endocannabinoids.

indicating the involvement of a serine hydrolase. Based on functional-proteomic analysis, α/β-hydrolase-4 (ABH4) is proposed to be the lipase converting NAPE to GP-NAE. GP-NAE-cleaving activity in mouse brain and testis preparations has also been described (Simon *et al.*, 2006). Together, ABH4 and 'GP-NAE phosphodiesterase' could constitute a NAPE-PLD-independent pathway for anandamide synthesis. Interestingly, a Ca^{2+}-independent NAT was recently discovered (Jin *et al.*, 2007), complicating the determination of the enzymes involved in anandamide biosynthesis.

2-AG is most likely produced via two separate metabolic pathways (Piomelli, 2003). The main pathway involves the PI-specific PLCβ and generation of 1,2-diacylglycerol (DAG), in which arachidonic acid is usually in the sn2 position. DAG has two metabolic fates. It can be converted to 2-AG, a monoacylglycerol, by sn1-specific DAG lipase (DAGL). So far, only two DAGLs have been cloned, DAGLα and DAGLβ (Bisogno *et al.*, 2003). Both isoforms are sn1-specific, expressed in a wide variety of tissues and abundant in the brain. Interestingly, only DAGLα-knockout mice showed a marked reduction in 2-AG levels, both in the basal state and after stimulation, and deficient retrograde synaptic suppression (Gao *et al.*, 2010; Tanimura *et al.*, 2010). Alternatively, DAG can be phosphorylated to phosphatidic acid by DAG kinase (DAGK). This ends the DAG signaling, but initiates other cascades related to the PA itself (see section 1.4.2.4.2 on PLD and PA). The alternative pathway for 2-AG synthesis starts with the phospholipase A_1 (PLA_1)-catalyzed production of 2-arachidonyl-lysophospholipid, which can be further hydrolyzed to 2-AG by 1-lyso-PLC activity (Piomelli, 2003). Neuronal 2-AG production can be initiated by increasing $[Ca^{2+}]_i$. This was first shown in cultured rat hippocampal slices after high frequency stimulation. 2-AG synthesis was blocked by pretreatment with tetrodotoxin (TTx) and the removal of the external Ca^{2+} (Stella *et al.*, 1997).

1.5.2.2. Degradation

Anandamide signaling is mainly terminated by fatty acid amide hydrolase (FAAH) (Cravatt *et al.*, 1996; Deutsch *et al.*, 1993; reviewed in Deutsch *et al.*, 2002). FAAH is an integral membrane protein with relatively broad substrate selection. It hydrolyzes NAEs, other bioactive fatty acid amides, and 2-AG, but anandamide appears to be the preferred substrate (Ueda *et al.*, 1995). FAAH is mainly expressed in the liver, followed by the brain, small intestine and testis (Deutsch *et al.*, 2002). Anandamide levels in FAAH-knockout mice are highly elevated (Cravatt *et al.*, 2001), confirming the central role of FAAH in anandamide degradation. Anandamide can also be degraded by the NAE-hydrolyzing acid amidase (NAAA) (Tsuboi *et al.*, 2005), which is localized in lysosomes instead of the plasma membrane. The tissue expression of the NAAA also differs from that of FAAH. NAAA mRNA is mostly expressed in the prostate, leukocytes, spleen, liver, kidneys, and pancreas (Ueda *et al.*, 2010), indicating possibly distinct physiological roles for these two anandamide-degrading enzymes.

Multiple enzymatic species are able to hydrolyze 2-AG *in vitro*, including FAAH (Goparaju *et al.*, 1998), neuropathy target esterase (NTE) (van Tienhoven *et al.*, 2002), and monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002). However, about 85% of the brain 2-AG is hydrolyzed by MAGL (Blankman *et al.*, 2007). The remaining 15% was found to be hydrolyzed by the previously uncharacterized enzymes ABHD6 and ABHD12 (α/β-hydrolase domain containing 6 and 12, respectively), suggesting that these three enzymes are the regulators of 2-AG levels *in vivo* (Blankman *et al.*, 2007).

Endocannabinoids are also metabolized by lipoxygenases (12-LOX and 15-LOX), cyclooxygenases (COX-2), and cytochrome P450 (Rouzer *et al.*, 2011). The physiological significance of this interaction is unknown, but in theory the cross-talk between endocannabinoid and eicosanoid systems could occur by several ways. Endocannabinoids could be hydrolyzed to provide AA precursor for eicosanoid biosynthesis. Oxygenated endocannabinoids could directly activate eicosanoid or endocannabinoid receptors, or they could be hydrolyzed and then bind to these receptors. Alternatively, there could be distinct receptor(s) for oxygenated endocannabinoids, and finally, oxygenation can function as a mechanism to terminate endocannabinoid signaling. There are experimental data supporting the role of COX-2 in the termination of endocannabinoid signaling (Rouzer *et al.*, 2011). Some pharmacological evidence of a putative receptor also exists (Rouzer *et al.*, 2011). However the interactions of endocannabinoid and eicosanoid systems remain poorly defined and further studies are required to clarify the issue.

Although anandamide was the first endocannabinoid discovered, the current view is that it is 2-AG and not

anandamide that is the primary endocannabinoid and CB_1 ligand in the CNS. The 2-AG concentration in the CNS is about 800 times higher than that of anandamide (Sugiura *et al.*, 1995), and 2-AG exhibits full agonist behavior toward CB receptors, while anandamide only functions as a partial agonist (Hillard, 2000).

1.5.3. Physiology of the endocannabinoid system

The endocannabinoid system (ECS) has been connected to the regulation of several physiological processes, including food intake and energy balance, at both central and peripheral levels (Di Marzo *et al.*, 2005). It has been known for centuries that cannabis consumption induces feeding and a preference for palatable food. Systemic stimulation of CB_1 by THC or pharmacological analogues or endocannabinoids stimulates feeding (Williams *et al.*, 1999; Williams *et al.*, 1998) and the blockade of CB_1 by rimonabant (SR141716A), the first selective CB_1 antagonist discovered (Rinaldi-Carmona *et al.*, 1994), suppresses the agonist-induced effect and reduces the overall food intake, even in starved animals (Colombo *et al.*, 1998). The observation that CB_1-knockout mice consume less food (Di Marzo *et al.*, 2001) supports the concept of eCBs as regulators of feeding. Hypothalamic ECS is activated transiently after fasting, resulting in an increased food intake (Di Marzo *et al.*, 2005). The effects of eCBs in the CNS are directed toward the brain areas regulating energy homeostasis, especially the orexinergic systems in the lateral hypothalamus and paraventricular hypothalamic nucleus (Kirkham *et al.*, 2002), and mesolimbic areas involved in hedonistic or motivational food intake (e.g. the desire for food) relayed by the nucleus accumbens and ventral tegmental area (Di Marzo *et al.*, 2005; Quarta *et al.*, 2011).

Endocannabinoids are known to act as retrograde regulators of synaptic transmission (Kano *et al.*, 2009; Ohno-Shosaku *et al.*, 2011). Endocannabinoids are synthesized in postsynaptic neurons in response to a depolarization-induced increase in $[Ca^{2+}]_i$. Cytosolic eCBs are transported out via the putative eCB transporter to act on CB_1 receptors expressed in presynaptic and/or nearby neurons. CB_1 signaling inhibits voltage-gated Ca^{2+} channels and activates Kir channels, and thus efficiently attenuates presynaptic depolarization and subsequent neurotransmitter release (Kano *et al.*, 2009). The outcome of retrograde modulation can also be the activation of relevant neurons via depolarization-induced suppression of inhibition (DSI) when CB_1-expressing neurons are GABAergic (Kano *et al.*, 2009). It is likely that retrograde modulation exerts the regulatory effects of eCBs in the CNS (Figure 7.).

ECS also participates in the regulation of energy homeostasis in the peripheral tissues (Silvestri *et al.*, 2011). During food deprivation, the synthesis of anandamide and 2-AG is increased in the small intestine and levels return to the baseline after feeding. Similar alteration has been shown to occur in the hypothalamus (Matias *et al.*, 2006). In adipose tissue, CB_1 stimulation induces the differentiation of preadipocytes, uptake of glucose and activation of several enzymes involved in anabolic fatty acid metabolism, including lipoprotein lipase and fatty acid synthase (Matias *et al.*, 2008b). Endocannabinoids also affect energy metabolism in the liver, skeletal muscle and pancreas (Matias *et al.*, 2008a). Endocannabinoids have additionally been implicated in the regulation of the brain–gut axis, a neuronal and humoral system connecting the CNS and GIT in a reciprocal manner (Storr *et al.*, 2007). During feeding, the GIT secretes the antiorexigenic hormones choleocystokinin (CCK) and peptide YY (PYY) into the circulation (Suzuki *et al.*, 2010). They act on the area postrema within the brain stem and the arcuate nucleus in the hypothalamus, both areas unprotected by the blood–brain barrier (Suzuki *et al.*, 2010). Endocannabinoids are suggested to act through the CB_1 located in the cell bodies of vagal afferent neurons in nodose ganglia (Burdyga *et al.*, 2004). CB_1 levels in the nodose ganglion are varied in relation to the nutritional status. They are upregulated during fasting and downregulated after feeding (Burdyga *et al.*, 2010), probably by a CCK-mediated mechanism (Burdyga *et al.*, 2004). Interestingly, the inhibitory action of CCK is reverted by OxA, ghrelin and anandamide (Burdyga *et al.*, 2010). BAT appears to be under endocannabinoid control. A systemic blockade of CB_1 with rimonabant increases BAT activity (Quarta *et al.*, 2010), and the effect is mediated by sympathetic innervations of BAT.

Recent progress in ECS physiology has revealed its key role in the pathophysiology of obesity (Di Marzo *et al.*, 2005; Quarta *et al.*, 2011). According to the current view, obesity is a complex disease involving disrupted homeostatic signaling, the accumulation of excessive fat and chronic low-grade inflammation (Weisberg *et al.*, 2003). The ECS appears to be hyperactive in obesity, which manifests as increased feeding and desire for food and accelerated lipogenesis.

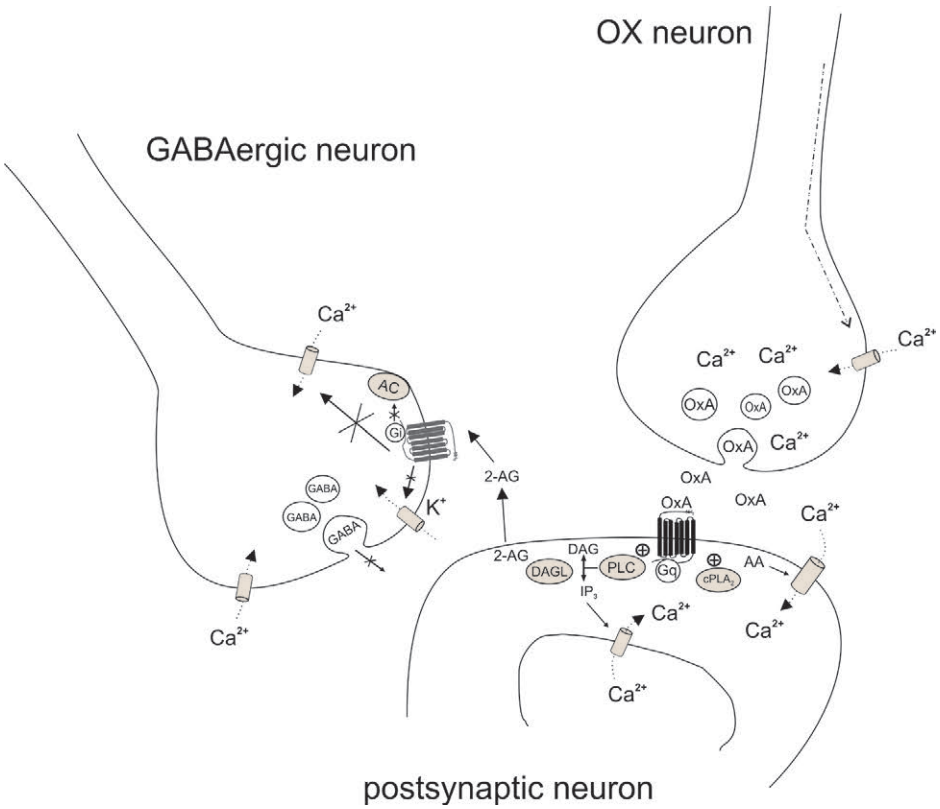

Figure 7. Orexins participate in the retrograde regulation of the synaptic transmission. Arriving action potential initiates the release of OxA via Ca^{2+} -controlled manner. OxA binds to the OX_1 (black) on the postsynaptic neuron leading to increase in intracellular Ca^{2+} and the production of 2-AG via PLCβ/DAGL pathway. The 2-AG then diffuses out of the cell and binds to CB1 receptor (grey) on nearby GABAergic neuron. CB_1 signaling leads to the attenuation of the GABA release and subsequent alleviation of the inhibitory GABA-signaling upon the target neuron. The overall outcome is enhanced activity of the OX_1-bearing neuron.

Dysfunction of insulin and leptin signaling in obesity is likely to play a part in ECS hyperactivity. Leptin is a known regulator of cEBs in the CNS (Di Marzo *et al.*, 2001) and in white adipose tissue (Buettner *et al.*, 2008). Insulin also down-regulates the levels of eCBs in adipocytes (D'Eon *et al.*, 2008; Murdolo *et al.*, 2006). The ECS is additionally implicated in the generation of the inflammatory status related to obesity by disturbing intestinal permeability and possibly by directly interacting with the immune system via CB_2 receptors (reviewed in Cluny *et al.*, 2012). Endocannabinoids have been shown to directly alter the localization of the key components in epithelial tight junctions and work in concert with gut microbiota (Muccioli *et al.*, 2010). An overactive ECS and high-fat diet together lead to the leakage of lipopolysaccharides from the gut (Cani *et al.*, 2007; Cluny *et al.*, 2012) and activation the innate immune system through toll-like receptors (Lumeng *et al.*, 2011), leading to chronic inflammation.

The endocannabinoid system can therefore be considered as an evolutionary adaptation aimed maximizing the outcome from a meal by optimizing nutrient intake and storage, and by reducing energy expenditure. In the modern age, when food is rarely scarce, this very efficient system is easily disturbed, resulting in obesity and associated metabolic disorders such as type II diabetes, which has reached epidemic proportions in countries with Western lifestyle.

2. AIMS OF THE STUDY

Stimulation of OX_1 orexin receptor has been shown to activate PLC and PLD (Johansson *et al.*, 2008; Johansson *et al.*, 2007). The original aim of this study was to investigate the involvement of PLA_2s in orexin receptor signaling, to develop techniques to assess PLA_2 activity and later examine the possible connection to endocannabinoid production. The specific aims therefore were to:

1) To investigate the involvement of PLA_2 in orexin receptor signaling;
2) To confirm which PLA_2 subspecies are activated and which other enzymes might be activated;
3) To develop a more practical and rapid assay for PLA_2 activity measurement;
4) To investigate the possible connection between OX_1 and endocannabinoid synthesis;
5) To develop an artificial cell-cell communication assay to measure the biological relevance of the 2-AG produced.

3. MATERIALS AND METHODS

3.1. Cell culture

Chinese Hamster Ovary (CHO) cells expressing human OX_1 receptor (CHO-hOX_1) were used in Papers I, II and IV, and CHO cells expressing human CB_1 receptor (CHO-hCB_1) in Paper II. The cells were grown in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 2 mM UltraGlutamin, 100 U/ml penicillin and 80 U/ml streptomycin. G418 (0.4 mg/ml) was used as a selection marker for CHO-hOX_1 and zeocin (0.25 mg/ml) for CHO-CB_1.

HEK293 cells expressing human OX_1 receptors and wild-type HEK293 cells were used in Papers III and IV, respectively. Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum (FCS), 2 mM UltraGlutamin, 100 U/ml penicillin and 80 U/ml streptomycin. In HEK293-OX_1 cells, hygromysin (0.05 mg/ml) was used as a selection marker.

Jurkat E6-1 T cell-derived lymphoma cells and HEL 92.1.7 erythroblast leukemia cells were grown in RPMI-1640, supplemented with 10% FCS, 2 mM UltraGlutamin, 100 U/ml penicillin and 80 U/ml streptomycin. The medium was renewed every two or three days (Paper IV).

All cell lines were grown in a humidified incubator at +37 °C and in 5% CO_2. Adherent cells were continued on plastic cell culture dishes with a bottom area of 56 cm^2, in 6-well plates (for TLC, Paper III) or 24-well plates (for AA release and cell-cell communication assay; Papers I, II and III). Suspension cells were continued in 200-ml cell culture flasks (Paper IV).

3.2. Conventional arachidonic acid and oleic acid release

CHO-hOX_1 cells at a density of 30 000 cells per well were plated on a 24-well plate and grown overnight in regular medium. The next day, the cells were labeled with ^{3}H-AA (0.1 µCi/well) overnight. Prior to the experiment, the wells were washed twice with 250 µl HBM (Hepes-buffered medium) containing lipid-free BSA (0.24 mg/ml), 200 µl HBM+BSA was added, and the cells were activated with 50 µl of HBM containing 5× concentrations of OxA or thapsigargin. The 24-well plates were immediately transferred to an incubator for 7 minutes, after which 200 µl of medium was collected into Eppendorf tubes, centrifuged briefly, and 100 µl of supernatant was transferred to a scintillation vial along with 3 ml of scintillation cocktail. The samples were analyzed for radioactivity with a liquid scintillation counter.

3.3. Filtration assay for arachidonic acid release

In order to speed up the throughput and make AA analysis less laborious, we developed a semi-high-throughput assay based on a filtration technique (Paper IV). Briefly, CHO cells were grown in dishes and loaded with [^{3}H]-AA 16 h before the experiments, as described in the section "Conventional arachidonic acid release" (3 µCi/dish). The next day, the cells were washed with HBM containing 2.4 mg/ml lipid-free BSA, detached with PBS containing 0.68 mM ethylenediaminetetraacetic acid (EDTA), counted and resuspended in HBM containing lipid-

free BSA. The cells were plated onto filter-bottomed 96-well plates (100 µl and 100 000 cells per well) containing the agonists. After 7-min incubation, the supernatants were collected on another 96-well plate by filtering using a vacuum manifold. Samples were collected into scintillation vials and the radioactivity assessed by scintillation counting.

3.4. cAMP measurement and the 2-AG reporter assay

cAMP was assessed utilizing a well-established method. CHO-hCB$_1$ cells were labeled with ^{3}H-adenine (5 µCi/ml) for 2 h before the experiment in normal culture medium. The cells were then detached from the dish, counted and resuspended to a concentration of 1×10^6 cells/ml in HBM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 minutes. Next, 100 µl aliquots (100 000 cells) were dispensed with a stepper pipette into a 96-well plate containing forskolin and/or various concentrations of CB$_1$ agonists and antagonists. After 10 minutes of incubation at +37 °C, the cells were centrifuged down, the supernatant discarded and cells were lysed with perchloric acid and rapidly frozen. After thawing, the plate was centrifuged again to pellet any debris, and 125 µl of supernatant was exposed to Dowex/Alumina chromatography for collection of ^{3}H-ATP+^{3}H-ADP and ^{3}H-cAMP fractions, which were then analyzed by scintillation counting.

The same method was applied for the 2-AG reporter assay utilizing CHO-CB$_1$ cells as the 2-AG production-dependent cAMP reporter. Briefly, CHO-hCB$_1$ cells were labeled with ^{3}H-adenine as above, detached and plated onto CHO-hOX$_1$ cells cultured on 24-well plates by gentle centrifugation. The cell pool was stimulated with forskolin (acting on CHO-hCB$_1$ cells) and OxA (acting on CHO-hOX$_1$ cells) and the cAMP production was determined after incubation for 10 min as above.

3.5. Ca^{2+} imaging

For Ca^{2+} measurements, CHO-hOX$_1$ cells were grown on 24-well plates on top of polyethyleneimine-coated glass coverslips overnight. Prior to the experiment the cells were loaded with 4 µM fura-2 acetoxymethyl ester for 20 min at +37 °C in HBM containing 1 mM probenecid, rinsed once with HBM and immediately placed in a perfusion chamber. Ca^{2+} measurements were performed at +35 °C using a Nikon TE2000 fluorescence microscope (20×/0.75 air objective) and Andor iXon 885 EM-CCD camera under the control of Nikon NIS Elements AR software with a 6D extension (Paper II). For Ca^{2+} imaging, the cells were excited with alternating 340 and 380 nm light (Sutter DG4 Plus) and the emitted light was collected through a 400-nm dichroic mirror and a 450-nm long-pass filter (Paper II). In Paper I, another imaging system was used in an equal manner. All chemical additions were made by constant perfusion (HBM + probenecid). When the inhibitors tetrahydrolipstatin (THL) and pyrrophenone were tested, the cells were pretreated with them for 20 min, and they were included in the perfusion medium as well. Regions of interest were defined in NIS software. Data were transferred to Microsoft Excel for visualization and quantization.

3.6. Thin layer chromatography

For TLC (thin layer chromatography) experiments, 250 000 CHO-hOX$_1$ cells were grown overnight on 6-well plates. The cells were labeled with ^{14}C-AA (0.2 µCi/ml) in cell culture medium (Ham's F12) 16 h prior to the experiments. ^{14}C-AA loading medium was removed, the cells were washed twice with HBM + S-BSA (2.4 mg/ml) and stimulated with OxA for 7 min. Supernatants from the cells were rapidly removed and spun down to remove detached cells. The lipids were extracted from the supernatant with a slightly modified Bligh and Dyer method. The final phase was collected and dried under a stream of N_2. The dried lipids were dissolved in chloroform and the samples run on TLC plates with ethylacetate:methanol (90:10). TLC plates were quantitated both by plate imaging and scintillation counting, both of which gave comparable results.

4. RESULTS AND DISCUSSION

4.1. AA release and PLA_2 activation (Papers I and II)

We observed a robust release of ^{3}H-radioactivity from ^{3}H-AA-labeled CHO-hOX_1 cells in response to OxA or OxB stimulation (Paper I). This "^{3}H-overflow" (as we coined it in Paper II to distinguish it from the actual AA release visualized using TLC) was orexin concentration-dependent. The ^{3}H overflow was readily inhibited by 10 μM MAFP (Paper I), but we were able to get potent inhibition even with submicromolar concentrations (Paper II). MAFP, although often used as a selective PLA_2 inhibitor, is an AA analogue and irreversible inhibitor of PLA_2 enzymes (and all other enzymes utilizing AA as a substrate). Partial inhibition was obtained with two other inhibitors of $iPLA_2$, R- and S-BEL; however, neither of these inhibitors may be very selective for $iPLA_2$. Due to the Ca^{2+}sensitivity of the response, although there could also be other explanations for this (Ammoun *et al.*, 2006a; Jäntti *et al.*, 2011; Johansson *et al.*, 2007), and the involvement of the ERK cascade (see below), $cPLA_2$ appeared to be a more likely player than $iPLA_2$. Thus, based on the results of Paper I, we could not firmly confirm any enzyme species responsible for the apparent AA release. However, the fact that the concentration–response curves observed were biphasic suggested that two different cascades could contribute to them.

In Paper II, the cascades were further examined. ^{3}H-AA overflow was shown, in a complementary manner, to be fully inhibited by pyrrophenone ($cPLA_2$ inhibitor) and THL (DAGL inhibitor). TLC experiments confirmed that indeed the AA overflow was composed of both 2-AG (produced from DAG by DAGL) and free AA (produced both from PL hydrolysis by $cPLA_2$ and from 2-AG hydrolysis by a MAGL-like activity). $cPLA_2$ activity seemed to take place at lower orexin concentrations than DAGL activity, suggesting an explanation for the biphasic concentration–response curves observed in Paper I. In Paper I we could also see that the potency of OxA to stimulate ^{3}H-overflow from ^{3}H-oleic acid-labeled cells was significantly lower than from ^{3}H-AA-labeled cells. In Paper II we could verify using THL that this indeed was due to the fact that ^{3}H-oleic acid was only released by the DAGL pathway, most likely by DAGL itself. Circumstantial evidence from the potency also supports the involvement of DAGL.

In paper I we also demonstrated that ^{3}H-AA overflow was attenuated by treatment with MEK1 inhibitors. MEK is the immediate upstream activator of ERK1/2. Both U0126 and PD98059 markedly reduced the ^{3}H-AA overflow in CHO-hOX1 cells. Phosphorylation of Ser505 in $cPLA_2$ by MAPK is known to be crucial for $cPLA_2$ activation (Lin *et al.*, 1993). On the other hand, OX_1 strongly activates ERK1/2 in CHO cells (Ammoun *et al.*, 2006a). It is thus tempting to speculate that coupling of OX_1 to MAPK represents a cross-talk between these two signaling pathway. We had interest in this pathway and we tried to explore it by two means. Firstly, we wanted to assess the phosphorylation by western blotting. Unfortunately, no direct antibodies were available for pSer505-$cPLA_2\alpha$ (and at the time we were not entirely sure that this was likely to be the isoform). Secondly, we tried to assess the involvement of Ser505 in the overexpression of a mutant of $cPLA_2\alpha$, the Ser505Ala mutant. We obtained this clone from another lab that had used it long ago. The plasmid could not be amplified, even in bacteria, and since no information was available on it, we did not continue along this line.

4.2. PLA_2 and ROC (Papers I, II and III)

One of the central aims of this thesis was the identification of the mechanisms of OX_1-mediated ROC activation in CHO-hOX_1 cells. We discovered that $cPLA_2$ activity was absolutely needed for the major part of the ROC response, as judged by the imaging data with pyrrophenone (Paper II) and earlier with MAFP (Papers I and III). The inhibition of DAGL activity by THL did not affect ROC influx (Paper II).

It is therefore likely that the products of the $cPLA_2$ reaction, AA or LPC or some of their metabolites, act as mediators of OxA-induced ROC activation in CHO cells. ARC and many TRP channels are regulated by AA, and LPA is likewise indicated in the regulation of some TRP channels (reviewed in Kukkonen, 2011). LPC is also indicated in activating non-store-operated Ca^{2+} channels in eosinophilic granulocytes (Zhu *et al.*, 2007). It is thus feasible that the ROC response elicited by OxA in CHO-hOX_1 cells is a result of LPC action and not that of AA. ROC inhibition by pyrrophenone does not reveal the downstream component, but only the enzymatic activity producing it. On the other hand, DAG has been shown to regulate TRPC channels in IMR-32 neuroblastoma cells transiently expressing OX_1 (Nasman *et al.*, 2006). If this was also the case

with CHO cells, THL treatment would have been expected to increase ROC influx, as THL should increase DAG levels. We did not see any significant differences between the control cells and THL-treated cells, or less inhibition after incubating pyrrophenone and THL together compared to pyrrophenone alone. This interpretation is not straightforward and has shortcomings. Increased levels of DAG followed by THL treatment would also activate PKC, and PKC is known to inhibit many TRPC channels (Trebak *et al.*, 2005; Venkatachalam *et al.*, 2003). It is also possible that the production of DAG was already saturated at 0.3 nM OxA in the control cells and the effect of THL may therefore have been beyond the detection limits of the experimental setup used. This subject is of interest and should be investigated in detail in a more suitable experimental protocol including a wider concentration range of OxA.

In HEK-293 cells expressing recombinant OX_1 receptors, exposure of the cells to OxA induced Ca^{2+} oscillations (Paper III). The oscillations required extracellular Ca^{2+}. The oscillations induced by a low concentration (1 nM) of OxA were investigated in detail. Previous studies with dominant-negative TRPC channel constructs have suggested that OxA-induced ROC activity relies at least in part on TRPC3/6 channels in CHO and IMR-32 cells (Larsson *et al.*, 2005; Nasman *et al.*, 2006). The strongest suppression of oscillations in HEK cells was obtained using the dominant-negative TRPC3 construct, pointing out the putative role of TRPC3 in OxA-induced oscillations. Mg^{2+} (10 mM) led to full inhibition in a reversible manner. MAFP fully attenuated Ca^{2+} oscillations induced by 1 nM OxA in HEK293 cells. Consequently, we measured ^{3}H-overflow from ^{3}H-AA-labeled HEK cells, and observed that this was significantly stimulated both at low and high OxA concentrations. The addition of exogenous AA restored oscillations in the presence of MAFP, although these became dissynchronized. It was thus concluded that at low OxA concentrations, Ca^{2+} oscillations occur in a manner dependent on Ca^{2+} influx via Mg^{2+}-sensitive ROC channels, but not SOCs (or, probably, Ca^{2+} release). In addition, AA release would be required for the response. However, MAFP is an irreversible inhibitor of PLA_2s (and several other AA-metabolizing serine hydrolases). It is therefore peculiar how a brief wash is able to remove MAFP-induced inhibition of Ca^{2+} influx. If AA was indeed important for the oscillatory response, MAFP might act as a direct antagonist for the AA binding site of, for instance, a channel protein from the extracellular side. AA perfusion could compete with MAFP removal from the channel, thus explaining the restoration of the Ca^{2+} influx. Dissynchronized oscillations might result because perfusion with 10 μM AA does not necessarily mimic the physiological concentrations of AA required for proper oscillations in HEK293 cells.

4.3. 2-AG release (Paper II)

As described above (***4.1.***), we were able to demonstrate in Paper II that the potent endocannabinoid 2-AG was also produced by OX_1 receptor activity in CHO-hOX_1 cells. 2-AG production was not only verified by the pharmacological evidence and TLC, but also utilizing the CB_1 receptor-based artificial cell-cell communication assay (also referred to as the 2-AG reporter assay). In this assay, we were able to show that the 2-AG produced in CHO-hOX_1 cells upon OX_1 receptor stimulation effectively exited the cells and stimulated CB_1 receptor in nearby cells (Paper II). This situation is basically similar to retrograde synaptic transmission occurring in the brain.

The finding was of interest, but somewhat expected. Orexin receptors strongly activate PLC and PLD, with an ample supply of DAG (Johansson *et al.*, 2008), and DAGL is also suggested to be activated by Ca^{2+} elevation. Thus, G_q-coupled GPCRs have been shown to be able to stimulate 2-AG production in previous studies (Kim *et al.*, 2002; Maejima *et al.*, 2001; Parrish *et al.*, 2006). In the work by Parrish and colleagues, the stimulation of 5-TH_{2A} in NIH3T3 cells led to the activation of PLC and an increase in $[Ca^{2+}]_i$, both of which were needed for 2-AG production. The centrality of Ca^{2+} for 2-AG production has also been noted in the comprehensive review of endocannabinoids by Kano *et al.* (2009). In our CHO cells, although both PLC and PLD were strongly activated by OX_1 receptor stimulation (Jäntti *et al.*, 2011; Johansson *et al.*, 2008; Johansson *et al.*, 2007; Lund *et al.*, 2000), mainly PLC contributed to the 2-AG release (Paper II). We could not detect any anandamide release from CHO-hOX_1 cells (not shown). This is interesting, since an increase in $[Ca^{2+}]_i$ is also considered to be important for anandamide biosynthesis (Piomelli, 2003). A likely explanation for this is that CHO cells do not express the enzymes needed for anandamide biosynthesis or release.

While physiological evidence points to the definitive collaboration of OX_1 and 2-AG (see ***4.4.***), it is difficult to prove with slice preparation that 2-AG is produced in OX_1-expressing neurons. Complex neuronal connections leave open the possibility that 2-AG is produced by other nearby neurons receiving simultaneous excitatory inputs or by neurons released from inhibitory inputs. Therefore, direct biochemical evidence from OX_1-expressing cell lines provides significant proof of the ability of OX_1 receptors to induce 2-AG biosynthesis and release.

4.4. Endocannabinoid system and orexin system connections

Indirect evidence for the connection of endocannabinoid and orexinergic systems was already provided in 2005. The work performed with OxB in dorsal raphe nucleus slice preparations demonstrated that OxB could inhibit glutamatergic synaptic currents in 5-HT neurons. This inhibition was mimicked by WIN 55,212-1, a synthetic CB_1 receptor agonist and blocked by AM-251, a known CB_1 antagonist, strongly pointing to endocannabinoid-relayed retrograde regulation (Haj-Dahmane *et al.*, 2005). Similar results were obtained in rat ventrolateral periaqueductal gray (vlPAG) slices (Ho *et al.*, 2011). OxA inhibited GABAergic nociception in vlPAG via a putative retrograde regulation mechanism.

Hilairet and coworkers explored the potential cross-talk of OX_1 and CB_1 receptors in CHO cells (Hilairet *et al.*, 2003). Three stable cell lines expressing OX_1, CB_1 and both receptors together were created. All constructs were well expressed on the plasma membrane and clear colocalization of OX_1 and CB_1 were observed in OX_1–CB_1 cells. A massive increase in the potency of OxA to activate ERK was observed in OX_1–CB_1 cells as compared to OX_1-expressing cells. The effect was blocked by the CB_1 antagonist rimonabant. Their conclusions was that OX_1–CB_1 receptors dimerized, as, according to the authors, the possibility of the presence of endogenous cannabinoids mediating the interaction was ruled out based on earlier work (Bouaboula *et al.*, 1997). The receptor dimerization model was later refined in (Ellis *et al.*, 2006) and in (Ward *et al.*, 2011). Both studies presented data supporting the idea of OX_1 and CB_1 forming heterodimers when coexpressed in the same cell. As in the study by (Hilairet *et al.*), Ellis and coworkers also documented an increase in OxA-induced ERK activation in OX_1–CB_1 coexpression in HEK293 cells. However, the potentiation was much less significant in HEK293 cells.

However, (Hilairet *et al.*) made a false assumption that their CHO OX_1–CB_1 expression system would be devoid of endocannabinoids. In the study by Bouaboula *et al.* (1997), only CHO-CB_1 cells and not CHO cells expressing both receptors were used. Also, the fact that introducing G_q-coupled receptors to the expression system and the following receptor stimulation could elevate endicannabinoid levels was not considered. Our finding that OX_1 is able to activate 2-AG production opens up a comprehensive alternative interpretation of their data. Both OX_1 and CB_1 have been shown to activate ERK upon ligand binding (Ammoun *et al.*, 2006a; Bouaboula *et al.*, 1995), and signaling cascades derived from OX_1 and CB_1 could thus converge at the MAPK level. In addition, recent evidence supports the view that endocannabinoids are able to act in an auto- or paracrine manner, and that the most likely explanation for the so-called constitutively active CB_1 receptor is indeed the presence of endogenously produced endocannabinoids (reviewed in Howlett *et al.*, 2011). We clearly demonstrated in our cell–cell communication assay that OX_1 stimulation is able to elevate 2-AG in biologically relevant quantities and even act via CB_1 receptors in nearby cells (Paper II). It is thus very likely that the increase in the potency of OX_1 for ERK phosphorylation at least partly results from 2-AG-mediated activation of CB_1 and subsequent signaling to ERK. The potentiation of ERK activation after coexpressing OX_1 and CB_1 may not therefore require receptor heterodimerization, as suggested by Ellis *et al. (2006)*, but a simple soluble substance, 2-AG, acting on CB_1 receptors and creating a feed-forward regulation resulting in the reported potentiation of OxA-relayed ERK activation. It is, however, conclusive that the presense or absence of receptor dimerization could not be verified or ruled out by this study.

The same chain of deduction is presented in Ellis *et al. (2006)*. The authors also failed to include the possibility of endogenous production of CB_1 substrate acting back on CB_1 receptor, which is the most logical explanation for the potentiation of ERK activation by OxA during the heterologous coexpression of OX_1 and CB_1. Their theory that heterodimerization of GPCRs could alter the pharmacological properties of the corresponding receptors is interesting.

Nevertheless, our finding of OX_1-induced 2-AG production (Paper II) raises questions about the suitability of the OX_1-CB_1 coexpression system used in Ellis *et al.(2006)* and Ward *et al.(2011)* to draw conclusions about alterations in the heterodimerization-induced pharmacological properties of GPCRs, even if the heterodimerization of the receptors was indeed verified.

4.5. AA release measurement methodology (Paper IV)

The measurement of AA release in a 24-well system is quite a laborious and time-consuming procedure. We therefore aimed to develop faster and easier methods to asses AA release (Paper IV). The assay was scaled down to 96-well plates giving four times more samples per plate. Another advantage of 96-well plates is the ability to design more complex experimental layouts. A further innovation was to

utilize filter-bottom plates and a vacuum manifold or centrifugation to speed up the most time-consuming wash steps in the protocol. The filter retains cells but allows the molecular components of the supernatant pass through. This also removes the centrifugation step needed in the conventional AA release protocol to remove detached cells.

Use of the filtration method also reduces the variation in the results. The cells are applied to the wells as a suspension. This ensures that an equal quantity of cells is more likely present in each well than if they were washed several times, as in the conventional AA release method. The use of suspended cells also overcomes the problem of detachment of cells during the various washes. This enables the use of fewer parallel samples, and more questions can be addressed per experiment without worrying about day-to-day variation between the experiments.

A requirement for this system is the use of cell suspensions. The detachment of adherent cells for the assay might affect their cellular physiology via altered cytoskeleton assembly and morphology. We nevertheless consider that if relatively simple pharmacological analyses of receptor functions are carried out, as described in Paper IV, the artifacts should not be too severe and the assay should remain scientifically reliable. The advantage in the use of the filter-based AA-release assay is the ability to also apply the method to naturally suspension-grown cells, such as blood cells and blood cell-derived cell lines. We tested both Jurkat E6-1 and HEL 92.1.7 cells for AA release and found them to respond to suitable stimuli and produce detectable amounts of AA.

One limitation of the filtration method is the one-time nature of the filtration. We varied the level of the vacuum and the relative centrifugal force, but the cells nevertheless became unresponsive after the first removal of fluids. We assume that some kind of minor damage or morphological change caused by the filtration were responsible for the unresponsiveness. The plasma membrane, however, appears to remain intact. We could not detect any major increase in radioactivity in the samples after a second filtration/centrifugation step, indicating that no excessive leakage occurred. The basal release in the filtration method was slightly increased compared to the conventional method. The reason for this is unknown, but might relate to the detachment of the cells for the assay.

The nature of the leaking substance (= basal release seen in the "^{3}H overflow" measurements) is unknown. According to the TLC data, this radioactivity originates neither from AA nor 2-AG. This is interesting, but proper investigation of this issue requires a mass-spectrometry approach.

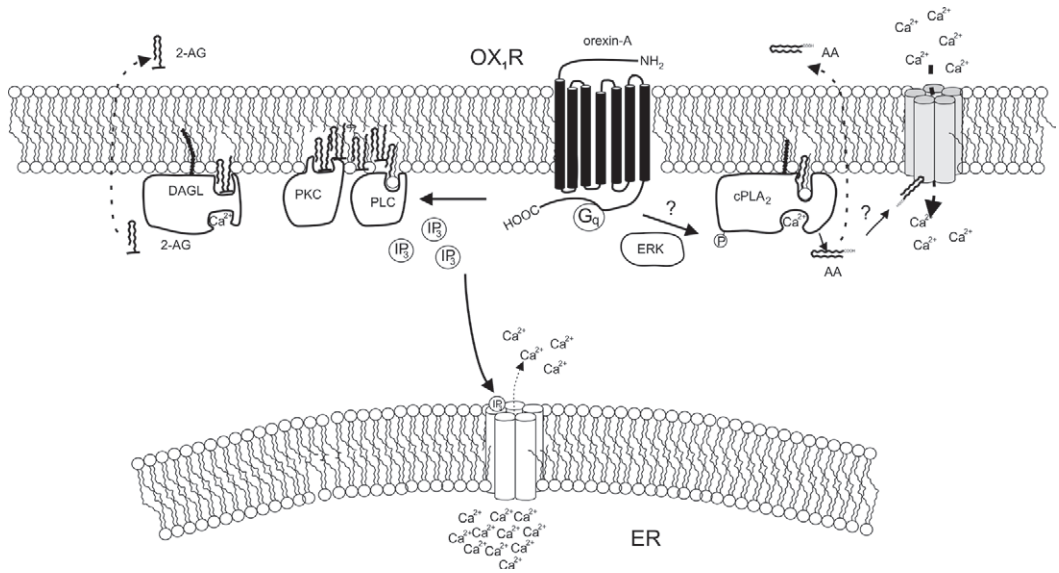

Figure 8. Summary of the intracellular signaling events upon OX_1 challenge. The binding of OxA to the OX_1 initiates Ca^{2+} entry to the cell cytoplasm from both intracellular stores (via PLCβ and IP_3 pathway) and extracellular sources via receptor-operated channel activated by an unknown $cPLA_2$ product (possibly AA). In addition, another lipid-derived signaling component, 2-AG, is produced upon the OX_1 stimulation. The increased intracellular Ca^{2+} concentration activates $cPLA_2$, but phosphorylation of $cPLA_2$ by OX1-activated ERK might also be needed for its full functionality.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Stimulation of OX_1 receptors with OxA was shown to activate robust AA release via a $cPLA_2$-mediated pathway in CHO-hOX_1 cells. $cPLA_2$ activity was also required in the OxA-driven ROC activity, possibly via TRP channels. This study, however, did not identify the exact molecular species responsible for ROC activation. In addition to AA, the other product of $cPLA_2$ reaction, a lysophospholipid (probably LPC), might act as a substrate for LPA production. LPA is known to activate some TRP channels. It is of interest to clarify this in detail. The molecular mechanism of $cPLA_2$ activation also remained unresolved, although AA release was strongly blocked by an inhibitor of MEK1, an enzyme that regulates ERK1/2, known to phosphorylate and activate $cPLA_2$. Further characterization of $cPLA_2$ is important to understand the mechanism by which OX_1 (and GPCRs in general) couples with phospholipases.

OxA binding to OX_1 also activated DAGL and the production of endocannabinoid 2-AG. This pathway is distinct from the pathway leading to AA release. CHO-hOX_1 cells are able to produce biologically relevant amounts of 2-AG, as noted in the cell–cell communication assay developed. Following this study, the OX_1 receptor joins various other G_q-coupled receptors able to induce endocannabinoid production. This result was somewhat expected, as GPCR signaling-induced endocannabinoids are known to be retrograde regulators of synaptic transmission. However, several redundant physiological functions of orexinergic and endocannabinoid systems, especially in the regulation of food intake, make the hypothesis of 2-AG acting as the combining molecule between these two central circuits plausible (Figure 5.).

Feed-forward signaling loops appear to be characteristic for OX_1 signaling. OX_1-induced AA release is dependent on Ca^{2+} influx (Paper I), and AA was shown to contribute to Ca^{2+} influx (Paper III). Similarly, OX_1-activated PLD1 (Jantti *et al.*, 2011) theoretically activates itself (see section 1.4.2.4.2.). Coexpression of OX_1 and CB_1 receptors in the same cells induces highly potent MAPK activation after OxA stimulation (Hilairet *et al.*, 2003). Finally, OX neurons also express receptors for orexins and are depolarized by orexin (Yamanaka *et al.*, 2010). It is tempting to speculate that the central role of orexins in maintaining arousal is based, at least in part, on the built-in feed-forward mechanisms of orexin receptor signaling pathways.

The filtration method developed can be used at a semi-high-throughput scale to assess the stimulus-activated release of AA (and other secreted compounds combined with a suitable detection system), with or without inhibitors of interest. It is very efficient and easy to perform. The only special requirements are the filter-bottomed 96-well plates and compatible vacuum manifold or centrifuge with 96-well plate adaptors. The equipment is inexpensive and the method easy to set up in a standard laboratory.

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I dedicate this thesis to my mother who passed away long time ago.

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