Studies on OX₁ orexin receptor coupling to arachidonic acid and endocannabinoid signaling

PAULI TURUNEN

Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki
Finland
And
Finpharma Doctoral Program, Drug Discovery Section

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“Carpe Diem Baby”
– Metallica
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The thesis is based on the following publications:


These publications are referred to in the text by their Roman numerals.

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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 OX₁ orexin receptor and OX₂ 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 OX₁ receptor. Stimulation of OX₁ leads to increased intracellular Ca²⁺ release via the classical phospholipase C/inositol-1,4,5-trisphosphate pathway, and subsequently also store-operated Ca²⁺ entry. In addition, OX₁ activation triggers receptor-operated Ca²⁺ entry by a mechanism not completely understood. Other intracellular signals originating from the OX₁ 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 A₂ (PLA₂) in OX₁ signal transduction was investigated in this thesis. Recombinant Chinese 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 cytosolic PLA₂α. AA release was highly dependent on extracellular Ca²⁺ and partly on the activation of ERK. The involvement of other PLA₂ enzymes was ruled out by pharmacological analysis. cPLA₂ activity was also shown to regulate receptor-operated Ca²⁺ 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-arachidonoyl glycerol (2-AG), an endocannabinoid, via the phospholipase C-diacylglycerol lipase pathway. OX₁-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 OX₁ receptor signaling were identified and the enzymatic species responsible were shown to be cPLA₂ 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.
<table>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>[Ca(^{++})](_i)</td>
<td>Cytoplasmic Ca(^{++}) concentration</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonyl glycerol</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>ARC</td>
<td>Arachidonic acid regulated channel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CB(_1)</td>
<td>CB(_1) cannabinoid receptor</td>
</tr>
<tr>
<td>CB(_2)</td>
<td>CB(_2) cannabinoid receptor</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CCP</td>
<td>Clathrin-coated pit</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAGK</td>
<td>Diacylglycerol kinase</td>
</tr>
<tr>
<td>DAGL</td>
<td>Diacylglycerol lipase</td>
</tr>
<tr>
<td>DMV</td>
<td>Dorsal motor nucleus of vagus</td>
</tr>
<tr>
<td>DRN</td>
<td>Dorsal raphe nucleus</td>
</tr>
<tr>
<td>eCB</td>
<td>Endocannabinoid</td>
</tr>
<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
</tr>
<tr>
<td>ENS</td>
<td>Enteric nervous system</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GP-NAE</td>
<td>Glycerophospho N-acetylenolamine</td>
</tr>
<tr>
<td>GRK</td>
<td>GPCR kinase</td>
</tr>
<tr>
<td>HBM</td>
<td>Hepes-buffered medium</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>Inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP(<em>R) (</em>)</td>
<td>Inositol-1,4,5-trisphosphate Receptor</td>
</tr>
<tr>
<td>LC</td>
<td>Locus coerulescens</td>
</tr>
<tr>
<td>LHA</td>
<td>Lateral hypothalamic area</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatic acid</td>
</tr>
<tr>
<td>MAFP</td>
<td>Methoxyarachidonyl fluorophosponate</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>NAAA</td>
<td>NAE-hydrolyzing acid amidase</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acyltransferase</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>OX(_1)</td>
<td>OX(_1) orexin receptor</td>
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<tr>
<td>OX(_2)</td>
<td>OX(_2) orexin receptor</td>
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<tr>
<td>OxA</td>
<td>Orexin-A</td>
</tr>
<tr>
<td>OxB</td>
<td>Orexin-B</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PEase</td>
<td>Palmitoyl ethanolamidase</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylincholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol(s)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP(_2)</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIPS(_K)</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PLA(_2)</td>
<td>Phospholipase A(_2)</td>
</tr>
<tr>
<td>PLC(_)</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PPO</td>
<td>Prepro-orexin</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G-protein signaling</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated channel</td>
</tr>
<tr>
<td>STIM</td>
<td>Stromal interaction molecule</td>
</tr>
<tr>
<td>SOC</td>
<td>Store-operated channels</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>THC</td>
<td>Δ(^9)-Tetrahydrocannabinol</td>
</tr>
<tr>
<td>THL</td>
<td>Tetrahydropipstatin</td>
</tr>
<tr>
<td>TMN</td>
<td>Tuberomammillary nucleus</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VLPO</td>
<td>Ventrolateral preoptic nucleus</td>
</tr>
<tr>
<td>VOC</td>
<td>Voltage-operated channels</td>
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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\textsuperscript{2+}-mobilizing signals, the “on-mechanisms” that rapidly leads to an increase in the intracellular concentration of calcium, [Ca\textsuperscript{2+}]\textsubscript{i}, b) the cellular processes regulated by Ca\textsuperscript{2+}, and finally c) the “off-mechanisms” that remove the Ca\textsuperscript{2+} from the cytoplasm and return the cell to the resting state. [Ca\textsuperscript{2+}]\textsubscript{i} in resting cells is held very low (around 100 nM) by stringent regulation of Ca\textsuperscript{2+} localization, energy-requiring pumps and transporters that transfer Ca\textsuperscript{2+} across cellular membranes, and Ca\textsuperscript{2+}-sequestering organelles or molecules (Clapham, 2007). A low cytoplasmic concentration prevents unwanted precipitation of calcium phosphates and establishes the framework for varying Ca\textsuperscript{2+} signal amplitude, timing, and localization (Clapham, 2007).

Extracellular fluid (ECF) contains Ca\textsuperscript{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\textsuperscript{2+} is commonly utilized in signal transduction cascades (often termed Ca\textsuperscript{2+} influx). In addition to ECF, Ca\textsuperscript{2+} can be mobilized from intracellular stores (Ca\textsuperscript{2+} release). Mitochondria and the endoplasmic reticulum (ER) are the most important cellular Ca\textsuperscript{2+} storage organelles. They participate in signaling and buffering of free cytoplasmic Ca\textsuperscript{2+} (Clapham, 2007). Cytoplasmic spikes in [Ca\textsuperscript{2+}]\textsubscript{i} concentrations are often referred to as Ca\textsuperscript{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\textsuperscript{2+} transients termed oscillations. Single Ca\textsuperscript{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\textsuperscript{2+} influx

Ca\textsuperscript{2+} influx mainly occurs via channels classified as ionotropic receptors (ligand gated ion channels), voltage-operated Ca\textsuperscript{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 (excitatory glycine N1-3 receptors), serotonin (5-HT\textsubscript{3} receptors) and ATP (P2X receptors). Some of these non-selective cation channels are also permeable for Ca\textsuperscript{2+} (Hayashi et al., 1996; Rathouz et al., 1994).

VOCs function as transducers converting changes in membrane potential into intracellular Ca\textsuperscript{2+} transients (Catterall, 2011). The ten known mammalian members are divided according to their α-subunit into three classes of Ca\textsubscript{1}, Ca\textsubscript{2} and Ca\textsubscript{3} (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\textsubscript{1}), N- (Ca\textsubscript{2,1}), P/Q- (Ca\textsubscript{2,2}), R- (Ca\textsubscript{2,3}), and T-type (Ca\textsubscript{3}) channels (Catterall, 2011). VOCs are Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} influx pathways that are not voltage-controlled or activated by emptying of ER Ca\textsuperscript{2+} stores (Tsien et al., 1990), but rather are activated upon separate receptor binding and subsequent intracellular signaling. Several intracellular secondary messengers have been implicated in ROC regulation, including inositol-1,4,5-trisphosphate (IP\textsubscript{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.
regulates basal Ca\(^{2+}\) levels (Brandman et al., 2007).

Various lipids and lipid derivatives are implicated in many ways an important signaling domain, and other signals are generated. 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\(^{2+}\)] completed the picture (Furuki et al., 1989; Spat et al., 1986). IP\(_3\) binds to the cytoplasmic tail of the specific IP\(_3\) receptors (IP\(_3\)Rs) 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 isofoms of IP$_3$ Rs are known with different tissue-dependent expression profiles. IP$_3$ Rs 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 (PLCγ1 and 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 PLCs are regulated by monomeric G-proteins of Ras and Rho families. PLC8 and ζ appear to be mainly regulated by an increase in [Ca$^{2+}$] (Kim et al., 1999).

Ga proteins of the G$_{α/β/γ}$ 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 (PMCA), extruding Ca$^{2+}$, and the SR/ER Ca$^{2+}$ ATPases (SERCA), 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 CB₁ and CB₂ belong to this group, as do orexin receptors OX₁ and OX₂.

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 M₁ receptor, about 30% of the receptors are in complexes at any given time point (Hern et al., 2010). Some GPCRs (such as the ß₂-adrenoceptor and GABAₐ 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 Ga 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 Ga subunit and can be further divided into four subclasses: Ga (Fung et al., 1981), Ga (Hildebrandt et al., 1983), Ga₉/₁₁ (Strathmann et al., 1990), and Ga₁₂/₁₃ (Strathmann and Simon, 1991; reviewed in Simon et al., 1991). The typical structure of Ga 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 Ga is palmitoylated at its N-terminus, and some also have myristoyl modification (Oldham et al., 2008). Gy subunits additionally contain lipid linkers. They can be farnesylated or geranilylated, ensuring the membrane association of the complex (Dupre et al., 2009).

1.3.2.1. Ga 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 Ga family proteins include the ability to stimulate adenylyl cyclase (AC) and activation by cholera toxin (Oldham et al., 2008).

1.3.2.2. Ga family

In some experiments, G-proteins appeared to inhibit rather than activate AC; this led to the discovery of Ga 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 Ga (“o” for other). The subgroup currently contains Ga, Ga, Ga, Ga, Ga, Ga, and Ga. Three additional members of this subgroup are specific to sensory organs: Ga participates in the bitter and sweet sensation in gustation, and Ga and Ga in phototransduction in the retina. All members except Ga are inactivated by pertussis toxin.

1.3.2.3. Ga family

Currently, the Ga family consists of Ga, Ga, Ga, Ga, Ga, Ga, Ga, Ga, and Ga. G-proteins (Mizuno et al., 2009; Oldham et al., 2008). GPCRs that connect to Ga (or simply G) G-proteins are known for their ability to temporarily increase the intracellular Ca²⁺ concentration. Upon receptor activation, Ga activates PLC (Taylor et al., 1991) and the
subsequently produced IP₃ binds to its receptors in the smooth ER membrane and opens Ca²⁺ channels, leading to an increase in [Ca²⁺]. (see section 1.2.2 for more detailed discussion on Ca²⁺ and Gα.) Other Gα-activated downstream signaling components include DAG-activated PKC, monomeric G-proteins and MAPK (Mizuno et al., 2009). Recently, Ga, has been shown to activate Ras independent of PLCβ (Liu et al., 2011).

1.3.2.4. Ga₁₂/₁₃ family
The members of this family are structurally distinct compared to other Ga families. Ga₁₂/₁₃ proteins have versatile functions. They are especially involved in targeting Rho via RhoGEFs (guanine nucleotide exchange factors) containing a Ga₁₂/₁₃-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., 2005). More than 30 Ga₁₂/₁₃-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 Ga (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 denaturing 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 Ca²⁺ 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 Gaβγ complex is associated with the receptor and GDP bound to the Ga 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 Ga subunit; the receptor functions as a GEF (Oldham et al., 2008). The nucleotide exchange leads to the dissociation of the trimeric Gaβγ complex from the cytoplasmic part of the GPCRs. Ga is further dissociated from Gaβγ 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 Ga 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 Ga 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 Ga 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 Ga 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 Gaβγ complex and transiently inactivates the receptor. β-Arrestins also guide GPCRs to CCP, where the complex is internalized by endocytosis. The internalized 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 OX1 and OX2 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 hcrtr.

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. OX1 contains 425 amino acids and the sequence homology between humans and rats is 91–98% (Sakurai et al., 1998). OX2R is slightly
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,R has a rather equal affinity for OxA and OxB, while OX,R 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 hypothalamic (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,R and OX,R (Marcus et al., 2001). OX,R mRNA is found in the ventromedial hypothalamic nucleus (VMH) and anterior part of the hypothalamus near the suprachiasmatic nucleus (Trivedi et al., 1998). OX,R 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,R is mostly found in the tuberomammillary 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 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.
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 \([\text{Ca}^{2+}]_i\) (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 G\(\beta\gamma\). 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, PKC\(_8\), 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 OX1

The kinase cascades initiated by OxA binding to OX1 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 OX1, 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. OX1 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 Ca2+ influx, PLC/PKC, Ras, Src, and PI3K (Ammoun et al., 2006a). Similar results have been recorded with the OX1 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 Ca2+ influx, p53, and caspases (Ammoun et al., 2006b).

1.4.2.4. Activation of phospholipases by OX1

Phospholipases are enzymes that hydrolyze glycerophospholipids (Murakami et al., 2011). Their nomenclature originates from enzymatic specificity. PLA1 releases fatty acid bound to the sn1 position in the glycerol backbone, while PLA2 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, tuberomammillary nucleus.) Picture is adapted from Ohno K and Sakurai T, 2008.
1.4.2.4.1 Phospholipase A₂

1.4.2.4.1.1 Overview

Our knowledge of phospholipase A₂ (PLA₂) has increased tremendously within the last two decades. PLA₂s 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). PLA₂s 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 PLA₂s 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 PLA₂ of bee venom. The discovery of intracellular PLA₂ made a systematic nomenclature essential (Schaloske et al., 2006). To date, at least 30 different PLA₂ are known in mammals. Based on their structure, catalytic mechanism and localization, PLA₂s are categorized into six classes, most of which contain several isoforms (Murakami et al., 2011).

1.4.2.4.1.2 Secretory PLA₂ family

PLA₂s derived from animal venoms are currently known as group I or secretory PLA₂ (sPLA₂). 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 sPLA₂ 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 Ca²⁺ 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 sPLA₂ has been discovered (reviewed by Hansaki et al., 2002). PLA₂-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 PLA₂s or GIVAs

Cytoplasmic PLA₂s or cPLA₂s (group IV A or GIVA) have six members, including the prototypic cPLA₂α. Other members include PLA₂β, PLA₂γ, PLA₂δ, PLA₂ε, and PLA₂ζ (these are occasionally referred to as IV A–F, respectively). From an evolutionary perspective, cPLA₂s are relatively new and appeared alongside vertebrate evolution and the development of eicosanoid signaling (Murakami et al., 2011). Cytoplasmic PLA₂s 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 PLA₁, PLA₂, lysophospholipase and acyltransferase activities (Murakami et al., 2011). All members (except cPLA₂γ) of the cPLA₂ family have a C2 domain in their N-terminus that directs the enzyme to the plasma membrane in a Ca²⁺-dependent manner. The only member showing specificity towards arachidonic acid is cPLA₂α.

1.4.2.4.1.3.1 Regulation of cPLA₂α activation

The activation mechanism of cPLA₂α is quite well known. Increasing [Ca²⁺] leads to cPLA₂ translocation.

![Figure 4](Image.png) 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 cPLA₂ and different lipid composition of the organelle membranes. The C2 domain of cPLA₂ binds to PC, unlike the C2 domain in PKC, which prefers phosphatidylserine. This has been verified in a clever study in which the C2 domains of cPLA₂α and PKCα were switched (Stahelin et al., 2003). Translocation profiles changed accordingly, and the chimeric cPLA₂α with C2-PKCα shifted to the plasma membrane, while PKCα with C2-PLA₂α moved to the perinuclear membrane. Translocation to the perinuclear membrane brings cPLA₂ close to COX enzymes; indeed, prostaglandin metabolism in cPLA₂α containing C2-PKCα is diminished (Murakami et al., 2003). Interestingly, both chimeric cPLA₂α 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 [Ca²⁺] 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). cPLA₂α has a putative lysine-rich binding site for PIP₃; this lipid is an important activator of the enzyme (Tucker et al., 2009).

1.4.2.4.1.3.2. cPLA₂s 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 cPLA₂α. 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 cPLA₂α 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 cPLA₂ 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 cPLA₂ activity help to form the positive curvature needed in tubule formation. Indeed, various methods to inhibit cPLA₂ (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. cPLA₂β features an additional Jim-C-domain, making it larger than other family members. It possesses an approximately 1500 times higher lysophospholipase activity than PLA₂ activity. It is constitutively located on mitochondrial membranes. Ubiquitously expressed cPLA₂γ 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 cPLA₂γ due to prominent lysophospholipase and transacylase activities (Yamashita et al., 2009). cPLA₂δ shows both PLA₁ and PLA₂ activities and it translocates to perinuclear membranes, but with much slower kinetics than cPLA₂α (Ohto et al., 2005). cPLA₂ε and cPLA₂ζ are found in the thyroid and ε also in muscle and the heart (Ohto et al., 2005). cPLA₂ε has weak enzymatic activity, but cPLA₂ζ has been shown to release AA or OA and to show some selectivity for PE over PC (Ghosh et al., 2007).

Ca²⁺-independent intracellular PLA₂s are known as iPLA₂s, 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 PLA₂β (Balboa et al., 1997). Several splice variants of iPLA₂β are known, but only two are active in vivo. The structural hallmark of iPLA₂β 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$_2$ shares a similar catalytic mechanism with cPLA$_2$, but does not display any specificity towards arachidonic acid in the sn2 position or for the phospholipid head group (Balsinde et al., 2005). This versatile enzyme also possesses transacetylase and lysophospholipase activities (Winstead et al., 2000). iPLA$_2$ 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., 2003), 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$_3$. This leads to the activation of PKC and an increase in [Ca$^{2+}$].

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 phophosphatic acid phosphohydrolases (Carman et al., 2006; Sciorra et al., 1999) or to LPA by PLA$_2$, 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).

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. PKCa) (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 ghrelin (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 administered 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 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 in pancreatic α- and β-cells (Ouedraogo et al., 2003). OxA has been shown to inhibit glucagon secretion by down-regulating preproglucagon via Ca2+ 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 tuberomammillary 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. 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,R (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, 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
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 OX₁ as regulators of apoptosis

In addition to regulation of metabolism and arousal, orexins relay potential apoptotic signals via OX₁ and OX₂ 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). OX₁ 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 OX₁ seems to be independent of an increase in \([\text{Ca}^{2+}]_{\text{i}}\) (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 OX₁ 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 Δ⁹-tetrahydrocannabinol (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., 1986). The development of a stereoselective radiolabeled analogue \([^{3}\text{H}]\)-CP55940 eventually led to the discovery of the main targets of THC, the cannabinoid receptors (Devane et al., 1988). Cannabinoid receptor 1 (CB₁) was cloned from rats (Matsuda et al., 1990) and from humans (Gerard et al., 1990) and cannabinoid receptor 2 (CB₂) from HL60 promyelocytic 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 CB₁ and CB₂ in the body is strikingly different: CB₁ 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 CB₂ is mainly found in immune cells and peripheral tissues (Munro et al., 1993). CB₁ is also expressed in the liver, adipose tissue, reproductive organs, muscle, bone, and cardiac and vascular tissues, while some CB₂ 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 CB₁ 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 CB₁ 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 G₁₀ family of G-proteins (G₁₀,₂,₃ and G₁₀,₄), and AC inhibition has been seen in most tissues and cells investigated (Howlett et al., 2002). In the presence of pertussis toxin, CB₁ has been shown to stimulate AC, indicating that CB₁ may be able to couple G₁₀ in the absence of G₁₂ (Glass et al., 1997). CB₁ activation leads to the activation of the MAPK (ERK1/2) cascade (Bouaboula et al., 1995; Wattmann 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 CB₁-induced reduction in cAMP and PKA activity reduced inhibitory c-Raf phosphorylation and stimulated MEK activation (Davis et al., 2003).
CB1 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, CB1 activates JNK and p38 (Rueda et al., 2000). The generation of nitric oxide (NO) upon CB1 stimulation has been documented in various cell types (Jones et al., 2008; Prevot et al., 1998; Stefano et al., 1996), while in others, CB1 reduces NO levels (Cabral et al., 2001; Hillard et al., 1999). Signaling through CB1 has also been shown to regulate ion channels. CB1 activates inward-rectifying K+ channels (Kir) and inhibits N-, P/Q- and L-type voltage-gated Ca2+ 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-Arachidonoylglycerol (2-AG) was found in 1995 (Sugiura et al., 1995), 2-arachidonoylglyceryl 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. Ca2+-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,
indicating the involvement of a serine hydrolase. Based on functional-proteomic analysis, a/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$_2$ (PLA$_2$)-catalyzed production of 2-arachidonyl-lyso phospholipid, which can be further hydrolyzed to 2-AG by 1-lyso-PLC activity (Piomelli, 2003). Neuronal 2-AG production can be initiated by increasing $[\text{Ca}^{2+}]_i$. 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 (a/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
that CB1-knockout mice consume less food (Di Colombo, 1998). The observation and reduces the overall food intake, even in starved animals (Colombo et al., 1994), suppresses the agonist-induced effect of CB1 antagonist discovered (Rinaldi-Carmona et al., 1999; Williams et al., 1998) and the blockade of CB1 by rimonabant (SR141716A), the first selective CB1 ligand in the CNS. The 2-AG concentration of CB1 by THC or pharmacological analogues or endocannabinoids stimulates feeding (Williams et al., 1999; Williams et al., 1998) and the blockade of CB1, by rimonabant (SR141716A), the first selective CB1 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 CB1-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 (Kano et al., 1998). CB1-expressing neurons in the cell bodies of vagal afferent neurons in nodose ganglia (Burdyga et al., 2010), probably by a CCK-mediated mechanism (Burdyga et al., 2010), BAT activity (Quarta et al., 2010), and the effect is mediated by sympathetic innervations of BAT.

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 CB1, by THC or pharmacological analogues or endocannabinoids stimulates feeding (Williams et al., 1999; Williams et al., 1998) and the blockade of CB1, by rimonabant (SR141716A), the first selective CB1 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 CB1-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 (Kano et al., 1998). CB1-expressing neurons in the cell bodies of vagal afferent neurons in nodose ganglia (Burdyga et al., 2010), probably by a CCK-mediated mechanism (Burdyga et al., 2010), BAT activity (Quarta et al., 2010), and the effect is mediated by sympathetic innervations of BAT.

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 CB1 receptors expressed in presynaptic and/or nearby neurons. CB1 signaling inhibits voltage-gated Ca2+ 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 CB1-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, CB1 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 cholecystokinin (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 CB1, located in the cell bodies of vagal afferent neurons in nodose ganglia (Burdyga et al., 2004). CB1 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 CB1 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.
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 (Buettnner 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 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 OX₁ 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 PLA₂s in orexin receptor signaling, to develop techniques to assess PLA₂ activity and later examine the possible connection to endocannabinoid production. The specific aims therefore were to:

1) To investigate the involvement of PLA₂ in orexin receptor signaling;
2) To confirm which PLA₂ subspecies are activated and which other enzymes might be activated;
3) To develop a more practical and rapid assay for PLA₂ activity measurement;
4) To investigate the possible connection between OX₁ 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 OX₁ receptor (CHO-hOX₁) were used in Papers I, II and IV, and CHO cells expressing human CB₁ receptor (CHO-hCB₁) 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-hOX₁ and zeocin (0.25 mg/ml) for CHO-CB₁.

HEK293 cells expressing human OX₁ 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-OX₁ cells, hygromycin (0.05 mg/ml) was used as a selection marker.

Jurkat E6-1 T cell-derived lymphoma cells and HEL 92.1.7 erythoblast 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% CO₂. Adherent cells were continued on plastic cell culture dishes with a bottom area of 56 cm², 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-hOX₁ 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 3H-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 [3H]-AA (0.1 µCi/dish) 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-

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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-hCB₁ cells were labeled with ³H-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 1×10⁶ 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 CB₁ 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 ³H-ATP+³H-ADP and ³H-cAMP fractions, which were then analyzed by scintillation counting.

The same method was applied for the 2-AG reporter assay utilizing CHO-CB₁ cells as the 2-AG production-dependent cAMP reporter. Briefly, CHO-hCB₁ cells were labeled with ³H-adenine as above, detached and plated onto CHO-hOX₁ cells cultured on 24-well plates by gentle centrifugation. The cell pool was stimulated with forskolin (acting on CHO-hCB₁ cells) and OxA (acting on CHO-hOX₁ cells) and the cAMP production was determined after incubation for 10 min as above.

3.5. Ca²⁺ imaging

For Ca²⁺ measurements, CHO-hOX₁ 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 acetoxyethyl ester for 20 min at +37 °C in HBM containing 1 mM probenecid, rinsed once with HBM and immediately placed in a perfusion chamber. Ca²⁺ 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 Ca²⁺ 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-hOX₁ cells were grown overnight on 6-well plates. The cells were labeled with ¹⁴C-AA (0.2 µCi/ml) in cell culture medium (Ham’s F12) 16 h prior to the experiments. ¹⁴C-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 N₂. 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 PLA₂ activation (Papers I and II)

We observed a robust release of ³H-radioactivity from ³H-AA-labeled CHO-hOX₁ cells in response to OxA or OxB stimulation (Paper I). This “³H-overflow” (as we coined it in Paper II to distinguish it from the actual AA release visualized using TLC) was orexin concentration-dependent. The ³H 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 PLA₂ inhibitor, is an AA analogue and irreversible inhibitor of PLA₂ enzymes (and all other enzymes utilizing AA as a substrate). Partial inhibition was obtained with two other inhibitors of iPLA₂, R- and S-BEL; however, neither of these inhibitors may be very selective for iPLA₂. Due to the Ca²⁺-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), cPLA₂ appeared to be a more likely player than iPLA₂. 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. ³H-AA overflow was shown, in a complementary manner, to be fully inhibited by pyrrophenone (cPLA₂ 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 cPLA₂ and from 2-AG hydrolysis by a MAGL-like activity). cPLA₂ 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 ³H-overflow from ³H-oleic acid-labeled cells was significantly lower than from ³H-AA-labeled cells. In Paper II we could verify using THL that this indeed was due to the fact that ³H-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 ³H-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 ³H-AA overflow in CHO-hOX₁ cells. Phosphorylation of Ser505 in cPLA₂ by MAPK is known to be crucial for cPLA₂ activation (Lin et al., 1993). On the other hand, OX₁ strongly activates ERK1/2 in CHO cells (Ammoun et al., 2006a). It is thus tempting to speculate that coupling of OX₁ to MAPK represents a cross-talk between these two signaling pathways. 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-cPLA₂ α (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 cPLA₂ α, 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. PLA₂ and ROC (Papers I, II and III)

One of the central aims of this thesis was the identification of the mechanisms of OX₁-mediated ROC activation in CHO-hOX₁ cells. We discovered that cPLA₂ 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 cPLA₂ 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 Ca²⁺ channels in eosinophilic granulocytes (Zhu et al., 2007). It is thus feasible that the ROC response elicited by OxA in CHO-hOX₁ 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 OX₁ (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 OX1 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 disynchronized. It was thus concluded that at low OxA concentrations, Ca\(^{2+}\) oscillations occur in a manner dependent on OxA 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\(_2\)s (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 \(\mu\)M 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 OX1 receptor activity in CHO-hOX1 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-hOX1 cells upon OX1 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\(_i\)-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 OX1 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-hOX1 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 OX1 and 2-AG (see 4.4.), it is difficult to prove with slice preparation that 2-AG is produced in OX1-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 OX1-expressing cell lines provides significant proof of the ability of OX1 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 CB1 receptor agonist and blocked by AM-251, a known CB1 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 OX1 and CB1 receptors in CHO cells (Hilairet et al., 2003). Three stable cell lines expressing OX1, CB1, and both receptors together were created. All constructs were well expressed on the plasma membrane and clear colocalization of OX1 and CB1 were observed in OX1−CB1 cells. A massive increase in the potency of OxA to activate ERK was observed in OX1−CB1 cells as compared to OX1-expressing cells. The effect was blocked by the CB1 antagonist rimonabant. Their conclusions was that OX1 and CB1 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 CB1 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 OX1 stimulation is able to elevate 2-AG in biologically relevant quantities and even act via CB1 receptors in nearby cells (Paper II). It is thus very likely that the increase in the potency of OX1 for ERK phosphorylation at least partly results from 2-AG-mediated activation of CB1, and subsequent signaling to ERK. The potentiation of ERK activation after coexpressing OX1 and CB1 may not therefore require receptor heterodimerization, as suggested by Ellis et al. (2006), but a simple soluble substance, 2-AG, acting on CB1 receptors and creating a feed-forward regulation resulting in the reported potentiation of OxA-mediated ERK activation. It is, however, conclusive that the presence 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 CB1 substrate acting back on CB1 receptor, which is the most logical explanation for the potentiation of ERK activation by OxA during the heterologous coexpression of OX1 and CB1. Their theory that heterodimerization of GPCRs could alter the pharmacological properties of the corresponding receptors is interesting.

Nevertheless, our finding of OX1-induced 2-AG production (Paper II) raises questions about the suitability of the OX1-CB1 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.

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**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 \( \text{OX}_1 \) receptors with OxA was shown to activate robust AA release via a \( \text{cPLA}_2 \)-mediated pathway in CHO-hOX1 cells. \( \text{cPLA}_2 \) 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 \( \text{cPLA}_2 \) 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 \( \text{cPLA}_2 \) 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 \( \text{cPLA}_2 \). Further characterization of \( \text{cPLA}_2 \) is important to understand the mechanism by which \( \text{OX}_1 \) (and GPCRs in general) couples with phospholipases.

OxA binding to \( \text{OX}_1 \) also activated DAGL and the production of endocannabinoid 2-AG. This pathway is distinct from the pathway leading to AA release. CHO-hOX1 cells are able to produce biologically relevant amounts of 2-AG, as noted in the cell–cell communication assay developed. Following this study, the \( \text{OX}_1 \) 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 \( \text{OX}_1 \) signaling. \( \text{OX}_1 \)-induced AA release is dependent on \( \text{Ca}^{2+} \) influx (Paper I), and AA was shown to contribute to \( \text{Ca}^{2+} \) influx (Paper III). Similarly, \( \text{OX}_1 \)-activated PLD1 (Jantti et al., 2011) theoretically activates itself (see section 1.4.2.4.2.). Coexpression of \( \text{OX}_1 \) and \( \text{CB}_1 \) 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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SUMMARY


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