

Molecular Determinants of Orexin Receptor–Ligand Interaction

Studies on Ligand Selectivity and Impact of Calcium

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List of the original papers

- I **Putula J**, Turunen PM, Jääntti M, Ekholm ME and Kukkonen JP (2011). Agonist ligand discrimination by the two orexin receptors depends on the expression system. *Neurosci Lett* 494: 57–60.
- II **Putula J**, Turunen PM, Johansson L, Näsman J, Ra R, Korhonen L and Kukkonen JP (2011). Orexin/hypocretin receptor chimaeras reveal structural features important for orexin peptide distinction. *FEBS Lett* 585: 1368–1374.
- III **Putula J** and Kukkonen JP (2012). Mapping of the binding sites for the OX₁ orexin receptor antagonist, SB-334867, using orexin/hypocretin receptor chimaeras. *Neurosci Lett* 506: 111–115.
- IV **Putula J** and Kukkonen JP (2013). Calcium affects OX₁ orexin/hypocretin receptor responses both via orexin binding and signal transduction machinery. Submitted manuscript.

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Abstract

Hypothalamic neuropeptides orexin-A and orexin-B, and their receptors OX₁ and OX₂ were found in 1998 as regulators of appetite. Later, they have been shown to also regulate, for instance, arousal states, stress reactions, and the reward system. Orexin peptides and receptors have been researched, especially in the field of drug discovery; however many relevant biochemical properties remain partly unsolved, including the factors determining orexin ligand binding properties and ligand selectivity. The study of these factors was pursued in this thesis.

Ligand selectivity was first studied here with chimaeric orexin receptors. With these chimaeras we mapped some of the molecular determinants of orexin receptors that are needed for the selectivity of orexin agonists and OX₁-specific antagonist. The second quarter of the orexin receptor seems to be the most important area both for agonist and antagonist selectivity. However, for antagonist selectivity, the third quarter also seems to have a role. Orexin receptor binding properties were studied by determining the impact of extracellular calcium concentration on orexin ligand binding. Calcium has been known to have an important role in orexin signal transduction as stimulation of orexin receptors causes a strong elevation of intracellular calcium, and reduction of extracellular calcium attenuates that calcium increase. In addition, other orexin receptor signalling, mediated by certain phospholipases and kinases, for instance, is attenuated. It remains unknown, however, how calcium causes these effects. With [¹²⁵I]-orexin-A, a clear decrease in binding was observed after reduction of the extracellular calcium concentration. Proper ligand binding is an important part of signal transduction, and indeed we saw a similar reduction also in the activities of phospholipase C (PLC) and adenylyl cyclase (AC). The concentration-relationship of calcium was identical for radioligand binding, PLC activation, and AC stimulation, while AC inhibition was more strongly attenuated. When the driving force for calcium influx was reduced with high-K⁺ medium, the orexin-A-induced PLC activity was more strongly reduced than orexin-A binding. In addition, inhibition of the orexin receptor-operated calcium channels had a more pronounced effect on the PLC activity than on the binding. It is thus suggested that reduction of extracellular calcium concentration has a dual effect on orexin receptor signalling, both by inhibition of orexin binding and attenuation of the enzymatic activity.

Orexin-B has higher binding affinity for OX₂ than OX₁ receptor. Commercially available orexin-B variant, [Ala¹¹, D-Leu¹⁵]-orexin-B, has been reported to display a higher selectivity for OX₂ than the native orexin-B. However, we observed that [Ala¹¹, D-Leu¹⁵]-orexin-B showed much lower OX₂-selectivity than originally reported. In addition, the selectivity of both forms of orexin-B was dependent on the cell line. These findings may be caused by biased agonism of the orexin receptor, meaning that the orexin receptor can be found in multiple conformations, each of which can interact differently with an agonist. This result extends our knowledge of orexin ligand binding properties, and the phenomenon should be considered, for instance, when novel agonists for orexin receptors are screened.

Abbreviations

[Ca ²⁺] _e	Extracellular calcium concentration
[Ca ²⁺] _i	Intracellular calcium concentration
2-AG	2-arachidonylglycerol
aa	Amino acid
AA	Arachidonic acid
AC	Adenylyl cyclase
Almorexant	(2 <i>R</i>)-2-[(1 <i>S</i>)-6,7-dimethoxy-1-(2-[4-(trifluoromethyl)phenyl]ethyl)-3,4-dihydroisoquinolin-2(1 <i>H</i>)-yl]- <i>N</i> -methyl-2-phenylethanamide
AT ₁	Angiotensin II type 1 receptor
BAT	Brown adipose tissue
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
CB ₁	Cannabinoid receptor type 1
Ch	Chimaera
CHO	Chinese hamster ovary
CNS	Central nervous system
cPLA ₂	Cytosolic phospholipase A ₂
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
DMEM	Dulbecco's Modified Eagle Medium
DORA	Dual orexin receptor antagonist
EMPA	<i>N</i> -ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulphonyl)-amino]- <i>N</i> -pyridin-3-ylmethyl-acetamide
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FDA	U.S Food and Drug Administration
FRET	Fluorescence/Förster resonance energy transfer
G _α	α subunit of the G-protein
G _{βγ}	βγ subunit complex of the G-protein
GABA	γ-aminobutyric acid
GAP	GTPase-activating/-accelerating protein
GEF	Guanine nucleotide exchange factor
GF	Glass fiber
GFP	Green fluorescent protein
GIRK	G-protein-coupled inwardly rectifying potassium
GPCR	G-protein-coupled receptor
G-protein	Guanine nucleotide-binding protein
GRK	G-protein-coupled receptor kinase
GSK	GlaxoSmithKline

GTPase	Guanine triphosphatase
HBM	Hepes-buffered medium
HCS	High-content screening
HEK	Human embryonic kidney
HTS	High-throughput screening
IP ₃	Inositol-1,4,5,-trisphosphate
JAK	Janus kinase
JNJ-10397049	1-(2,4-dibromophenyl)-3-[(4 <i>S</i> ,5 <i>S</i>)-2,2-dimethyl-4-phenyl-1,3-dioxan-5-yl]urea
KBM	K ⁺ -based medium
MAFP	Methyl arachidonyl fluorophosphonate
MAPK	Mitogen-activated protein kinase
NaBM	Na ⁺ -based medium
NPY	Neuropeptide Y
OX ₁	OX ₁ orexin receptor
OX ₂	OX ₂ orexin receptor
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
POMC	Proopiomelanocortin
PVDF	Polyvinylidene fluoride
RAMP	Receptor activity-modifying protein
REM	Rapid eye movement
RGS	Regulator of G-protein signalling
RT-PCR	Reverse transcription polymerase chain reaction
ROC	Receptor-operated calcium channel
SB-334867	1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride
SOC	Store-operated calcium channel
SPA	Scintillation proximity assay
STAT	Signal transducer and activator of transcription
Suvorexant	[(7 <i>R</i>)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2 <i>H</i> -1,2,3-triazol-2-yl)phenyl]methanone
TEA	Tetraethylammonium (chloride)
TM	Transmembrane helix
TRPC (channel)	Transient receptor potential (channel) of the canonical subfamily
YFP	Yellow fluorescent protein

1. Introduction

Cells must communicate with each other in order to support the functions of multicellular organisms. For efficient communication, cells need proteins called receptors. Receptors can be situated at the cell surface or inside the cell and they can bind signalling molecules, called ligands. Ligands can be, for instance, peptides, amino acids or fatty acids, and each receptor can only bind a receptor-specific ligand. If the bound ligand is an agonist, the receptor is activated and it can further activate specific intracellular signalling pathways. These pathways may be simple and straightforward or more complicated, including the involvement of second messengers or cross-communication of different pathways. The pathway ultimately transduces the signal to targets, like ion channels or enzymes. After receiving the signal, the behaviour of the target changes, for instance ion channels are opened or enzymes are activated.

Cell communication is crucial throughout the lifespan of an organism. Cell signalling already occurs during fertilisation, when sperm cells must sense chemoattractants in order to reach the egg cell. In a developing embryo, stem cells communicate with each other to determine which cell type they will differentiate to. Altogether, cells are constantly receiving signals that determine whether the cell should survive, divide or die.

There would be no multicellular organisms on earth without cell communication. Cells constantly receive signals from each other and from the environment, and these signals are passed to target molecules through signalling pathways. Cell signalling research is not only important to help us understand the communication by natural signalling molecules, like hormones, but to also understand the signalling by exogenous molecules, like drugs. In order to better understand pathological processes, we need to be able to detect what occurs on the cellular level and to understand the language of the cell: cell signalling.

2. Review of the literature

2.1. *G-protein-coupled receptors*

G-protein-coupled receptors (GPCR) constitute the largest membrane receptor superfamily with more than 800 different genes. Members of the GPCR family span from hormone to sensory receptors and also include receptors, for instance, for neuropeptides, chemokines, lipid mediators, nucleotides, and calcium ions (Pierce et al., 2002). Thus GPCRs are responsible for the regulation of most of the physiological processes in our body (Rosenbaum et al., 2009). As GPCRs form the most extensively studied receptor group, they are also the most common target of therapeutic drugs (Pierce et al., 2002; Civelli et al., 2013).

2.1.1. **GPCR structure**

GPCR structure is characterised by seven transmembrane helices (TMs) that are linked by alternating intra- and extracellular loops. TMs are arranged as a closed “barrel” with TM7 adjoining TM1, a structure that is stabilised by hydrogen bonds and salt bridges (Ji et al., 1998). Thus, the TMs form the core of the receptor. Extracellular amino and intracellular carboxyl tails also

characterise the structure of GPCR. Amino and carboxyl tails and intra- and extracellular loops, the intracellular loop linking TM5 and TM6 in particular, are the most variable structures within the GPCR family (Ji et al., 1998; Kobilka, 2007).

The first crystal structure solved for a GPCR was of bovine rhodopsin, which was published in 2000 (Palczewski et al., 2000). The crystal structures of several other GPCRs have been solved since (see for instance Cherezov et al., 2007; Wu et al., 2010; Shimamura et al., 2011; Hanson et al., 2012). For the GPCRs with still unknown crystal structure, parts of the structure, like the location of the ligand binding domain, can be predicted with mutagenesis studies, like site-directed mutagenesis (Kristiansen, 2004; Kobilka, 2007). Small organic agonists, like monoamines and odorants, seem to bind to the TMs of the GPCR, whereas peptide hormones and proteins utilise the amino terminal tail and extracellular sequences (Kobilka, 2007). The size of the ligand does not always correlate with the location of the binding site. For instance, glycoprotein hormones, glutamate, and Ca^{2+} , despite of being of very different sizes, all bind to the amino terminal tail of their receptors.

2.1.2. Signalling through GPCRs

GPCRs are named according to the ability of the activated receptor to signal through coupling to a guanine nucleotide-binding protein (G-protein). These G-proteins are membrane-bound heterotrimeric proteins consisting of α , β , and γ subunits (Wess, 1997). When inactive, $G\alpha$ subunit binds GDP. Ligand binding-triggered activation of the GPCR leads to coupling of the receptor with the G-protein and exchange of the $G\alpha$ -bound GDP for GTP. That exchange leads to the dissociation of the G-protein from the receptor. $G\alpha$ -GTP has decreased affinity for other G-protein subunits and thus a tight $G\beta\gamma$ complex is released from the $G\alpha$. Both the $G\alpha$ subunit and the $G\beta\gamma$ complex signal by activating or inhibiting effectors, of which there is a large number. These putative effectors include, for instance, ion channels and enzymes that further regulate the production, release and degradation of intracellular second messengers and thereby amplify the signal (Simon et al., 1991; Pierce et al., 2002; Milligan and Kostenis, 2006). The GDP/GTP binding pocket of $G\alpha$ has guanosine triphosphatase (GTPase) activity and it can hydrolyse the terminal phosphate of GTP (Wess, 1997; Wettschureck et al., 2004; Milligan and Kostenis, 2006). This hydrolysis leads to replacement of GTP with GDP and reassociation of the $G\beta\gamma$ complex with the $G\alpha$ subunit, terminating the G-protein signalling. However, the intrinsic GTPase activity of the $G\alpha$ subunit is not very pronounced, but is enhanced by other proteins (see, **2.1.5. Regulation of GPCRs**).

G-protein subunits do not always dissociate, but instead the subunits can undergo rearrangement. These non-dissociable G-proteins are found, for instance, in mammalian, yeast and plant cells (Klein et al., 2000; Bunemann et al., 2003; Adjobo-Hermans et al., 2006). G-protein may also be precoupled to the receptor. This kind of precoupling is found for, at least, muscarinic, dopamine and adenosine receptors, and it may contribute to a more rapid regulation of the effector proteins after receptor activation (Nobles et al., 2005).

Even though heterotrimeric G-proteins function in the plasma membrane, near the GPCRs, they can also be found in the membranes of intracellular organelles. For instance, membranes of endoplasmic reticulum (ER), Golgi apparatus, and endosomes are shown to include G-proteins of the stimulatory and inhibitory classes (G_s and G_i , respectively) (Helms, 1995). Actually, it is confirmed that G-proteins are not constantly located in the plasma membrane, but instead they reside in these intracellular membranes from where they can shuttle to the plasma membrane

(Chisari et al., 2007). In addition, intracellular G-proteins have functions in membrane trafficking, endocytosis and secretion, like protein transport from Golgi to apical membranes or stimulation of exocytosis (Helms, 1995; Chisari et al., 2007).

2.1.3. Alternative signalling pathways of GPCRs

GPCRs are sometimes called seven transmembrane receptors, heptahelical receptors or serpentine receptors to underline the fact that their signalling is not limited to the heterotrimeric G-proteins. For instance, metabotropic glutamate receptor and type 1 angiotensin II receptor (AT₁) use both G-protein-dependent and -independent signalling (Heuss et al., 1999; Wei et al., 2003). It is suggested that G-protein-independent signalling of AT₁ may activate an anti-apoptotic pathway in the heart (Rajagopal et al., 2005; Zhai et al., 2005).

How G-protein-independent signalling occurs remains partly unknown. Studies of G-protein-independent signalling prove challenging because suitable controls for the effectiveness of G-protein blocking are lacking (Heuss and Gerber, 2000). Some potential signalling mechanisms have been found, however, including one that involves G-protein-coupled receptor kinases (GRKs) and β -arrestin, which are described in more detail below (see, **2.1.5.3. GPCR kinases and arrestin**) (Rajagopal et al., 2005). Another mechanism, which actually seems to be active in the case of AT₁ receptor, includes the Janus kinase (JAK) pathway (Ritter and Hall, 2009). Binding of angiotensin II to the AT₁ receptor, and association of JAK with tyrosine protein phosphatase non-receptor type 11, causes JAK association with the AT₁ receptor. That promotes JAK phosphorylation and activation. JAK can further phosphorylate members of signal transducer and activator of transcription (STAT) family. STATs are transcription factors, which, once phosphorylated, dimerise and translocate into the cell nucleus, where they exert gene regulation to control cell growth.

Another special signalling feature of GPCRs originates from the fact that the receptors can have multiple conformations in the active (ligand bound) state (Kenakin, 2010). Each of these conformations can selectively interact with a different ligand. Different conformations of the receptor can eventually activate different signalling pathways. This phenomenon has multiple names like “biased agonism”, “functional selectivity”, “ligand-selective agonism” or “ligand-induced selective signalling”. It could have potential use in selective pharmacology as drugs could be used to only activate the pathway of interest (Kenakin, 2012).

Sometimes GPCRs can signal in the absence of a ligand. These receptors are called constitutively active and their constant activity is caused by mutations (Parnot et al., 2002). GPCRs with constitutively active mutations adopt a structure of the active receptor, and thus they are able to couple with the G-protein. Opioid and β_2 -adrenergic receptors were one of the earliest GPCRs with detected constitutive activity (Cerione et al., 1984; Costa and Herz, 1989). However, now constitutively active mutants have been found for many other GPCRs as well, including, for instance, α_1 -adrenergic, rhodopsin, and muscarinic receptors (Cohen et al., 1992; Scheer et al., 1996) (Spalding and Burstein, 2001). Constitutively active receptor mutants are associated with several disorders like night blindness, hyperthyroidism, and Leydig cell adenoma (Dryja et al., 1993; Parma et al., 1993; Liu et al., 1999).

2.1.4. Classification of G-proteins

Heterotrimeric G-proteins are classified into four families according to the $G\alpha$ subunits (Alexander et al., 2011). The families are $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12}$, and they all contain multiple isoforms of α subunits with different structures, expression profiles and, partially, functions as well. Because of the high number of different $G\alpha$ subunit isoforms, only the best known and the most studied isoforms of each G-protein family are described below. Figure 1 summarises the signalling pathways of each G-protein family.

2.1.4.1. $G\alpha_q$ family

$G\alpha$ subunits of the $G\alpha_q$ family activate β isoforms of phospholipase C (PLC β) (Neves et al., 2002). The $G\beta\gamma$ complex can also stimulate PLC β , and thus its activation is not restricted to the G-proteins of the $G\alpha_q$ family (Rebecchi and Pentylala, 2000). PLC catalyses the hydrolysis of phosphatidylinositols, like phosphatidylinositol-4,5-bisphosphate (PIP₂), to diacylglycerol (DAG) and inositol trisphosphate (IP₃). PIP₂ is not only a precursor of DAG and IP₃ but also works as a cellular regulator on its own (Suh and Hille, 2005). PIP₂ can, for instance, directly regulate several ion channels and participate in actin cytoskeleton remodelling, membrane targeting and vesicle trafficking (van den Bout and Divecha, 2009). The second messenger IP₃ causes elevation of intracellular calcium in two different ways. It directly binds to IP₃ receptors in the ER and activates the release of intracellularly stored calcium (Neves et al., 2002; Berridge, 2009). That indirectly activates store-operated calcium channels (SOCs) in the plasma membrane, which leads to calcium influx. DAG has multiple roles in the cell. It activates protein kinase C (PKC), which is involved in many cellular functions like receptor desensitisation, regulation of transcription and regulation of cell growth (Newton, 1995; Neves et al., 2002). DAG also activates some transient receptor potential (TRP) channels of the canonical subfamily (TRPC) most likely by direct binding to them (Soboloff et al., 2007; Kukkonen, 2011). As PKC is an inhibitor of most TRPC channels, DAG has a dual role in the regulation of these channels. In the Golgi apparatus, DAG is involved in vesicle budding (Kearns et al., 1997).

Another member of the $G\alpha_q$ family is $G\alpha_{11}$. $G\alpha_{11}$ -coupled receptors are involved in mediating the effects of almost all vasoconstriction-causing hormones (Wettschureck et al., 2004). As vascular resistance is one of the factors determining blood pressure, reduction of the resistance could be one approach to be used in the treatment of arterial hypertension. Thus blocking the signalling of $G\alpha_{11}$ in smooth muscle cells could be an effective method to treat hypertension (Wettschureck et al., 2004).

The remaining members of the $G\alpha_q$ family are $G\alpha_{14}$ - and $G\alpha_{15/16}$ -proteins. $G\alpha_{15}$ is murine and $G\alpha_{16}$ human homologue of the same protein (Simon et al., 1991). $G\alpha_{14}$ is expressed primarily in stromal and epithelial cells whereas $G\alpha_{15/16}$ is found in lymphocytes and myeloid cells. These members can also activate PLC β , but as can be predicted from their differing tissue distribution, different members of $G\alpha_q$ family may have also some distinct functions. In vascular smooth muscle cells, $G\alpha_q$ and $G\alpha_{14}$, but not $G\alpha_{15}$, can activate caspase 3 to cause cell death (Peavy et al., 2005). The role of $G\alpha_{15}$ is still partly unclear, but it may be involved, for instance, in hematopoietic cell growth and differentiation (Giannone et al., 2010). $G\alpha_{16}$ is reported to have an ability to activate the transcription factor STAT3, which was earlier thought to be controlled via cytokine receptors (Wu

et al., 2003). However, as mentioned above, now it is known that also G-protein-independent signalling of the angiotensin II receptor potentiates STAT signalling (Ritter and Hall, 2009).

2.1.4.2. $G\alpha_s$ family

$G\alpha_s$ subunits stimulate adenylyl cyclase (AC) which catalyses the conversion of ATP to cAMP (Pierce et al., 2002; Wettschureck et al., 2004; Zhang and Xie, 2012). Constant stimulation of AC activity can be achieved with cholera toxin, which catalyses ADP-ribosylation (and activation) of $G\alpha_s$ (Milligan and Kostenis, 2006). cAMP acts as a second messenger in the cell via protein kinase A (PKA) and other effectors (Pierce et al., 2002; Wettschureck et al., 2004; Zhang and Xie, 2012). cAMP has a ubiquitous role in many physiological functions, and homozygous $G\alpha_s$ knockout mice die early under embryonic development (Yu et al., 2001; Wettschureck et al., 2004).

Another G-protein belonging to the $G\alpha_s$ subfamily is $G\alpha_{olf}$. Like $G\alpha_s$, $G\alpha_{olf}$ also has the ability to activate AC (Offermanns and Simon, 1998). $G\alpha_{olf}$ is mainly expressed in specialised sensory cells, especially in olfactory epithelium (Simon et al., 1991). However, it is also found in striatum where it transduces dopamine receptor signals (Zhuang et al., 2000). Homozygous $G\alpha_{olf}$ knockout mice cannot smell odours, suggesting that $G\alpha_{olf}$ is needed for olfactory signal transduction (Belluscio et al., 1998).

2.1.4.3. $G\alpha_i$ family

In contrast to $G\alpha_s$, $G\alpha_i$ inhibits the activity of AC (Taussig et al., 1993). However, all members of the $G\alpha_i$ family are not involved in AC inhibition, and there are multiple AC isoforms, which are not all affected by $G\alpha_i$ (Stegborn et al., 2005; Sadana and Dessauer, 2009). $G\beta\gamma$ signalling to, for instance, AC and G-protein-coupled inwardly rectifying potassium (GIRK) channels is thought to mainly originate from G_i -proteins (Ikeda et al., 2002). $G\beta\gamma$ of the G_i -protein also stimulates PLC β (Neves et al., 2002); however $G\alpha_q$ is first required to preactivate it. This kind of crosstalk between different G-protein subtypes may have an important role in the refinement of signals (Chan et al., 2000). In addition to the crosstalk between G_i - and G_q -proteins, a $G\alpha_q$ -independent, Ca^{2+} influx-mediated pathway for the G_i -protein-mediated stimulation of PLC, is speculated (Murthy et al., 2004).

G_o - and G_z -proteins are also members of the $G\alpha_i$ family. As G_o is the most abundant G-protein expressed in the brain, it is also the main source of the brain's $G\beta\gamma$, which has an important role, for instance, in the activation of GIRK channels (Exner et al., 1999; Khan et al., 2013). Opening of the GIRK channels causes efflux of potassium ions, which leads to membrane hyperpolarisation: the mechanism used to reduce neuronal excitability and heart rate. One notable effect via GIRK channels is by opioids (Ikeda et al., 2002). A number of receptors, including α_2 adrenergic, D_2 dopamine and M_2/M_4 muscarinic receptors, can couple to G_o - and G_i -proteins. Functions of $G\alpha_o$ are still partly unclear but they seem to differ from the functions of the $G\alpha_i$. It is known that G_o -coupled muscarinic acetylcholine receptors mediate parasympathetic regulation of the heart (Wettschureck et al., 2004). $G\alpha_o$ has also been shown to have a role in the prevention of PKA translocation into the nuclear compartment (Ghil et al., 2006).

The function of the primarily neuronal $G\alpha_z$, has not been fully identified; however, it is known to inhibit AC. $G\alpha_z$ has exceptionally slow GTPase activity. There is, however, a $G\alpha_z$ -specific GTPase-activating protein (GAP) expressed together with G_z -protein, and it is partly

responsible for the hydrolysis of the GTP (Simon et al., 1991; Fields, 1998; Wettschureck et al., 2004).

Signalling of G_i-coupled GPCRs can be inhibited with pertussis toxin. Pertussis toxin catalyses ADP-ribosylation of all G α_i members, except G α_z , by preventing the interaction between G-protein and the GPCR. Thus G α_i remains in the inactive GDP-bound state (Milligan and Kostenis, 2006). However, there is also evidence for ADP-ribosylation-independent actions of pertussis toxin (Mangmool and Kurose, 2011).

2.1.4.4. *G α_{12} family*

The G α_{12} subfamily contains two members called G₁₂ and G₁₃. GPCRs known to couple to G₁₂- and G₁₃-proteins include receptors for acetylcholine, serotonin, vasopressin and dopamine, just to name some (Siehler, 2009). G α_{12} and G α_{13} can activate guanine nucleotide exchange factors (GEFs) which in turn activate Rho family G-proteins (Aittaleb et al., 2010). Rho family members are monomeric G-proteins and, like trimeric G-proteins, they also cycle between GTP-bound active and GDP-bound inactive states (Siehler, 2009). In the signalling of G α_{12} and G α_{13} , RhoGEFs, like p115-RhoGEF, homology-RhoGEF and leukemia-associated RhoGEF, promote enrichment of the GTP-bound form of RhoA, which regulates cytoskeleton proteins and can indirectly regulate the number of actin and myosin fibres. Overexpression of G α_{12} and G α_{13} is involved, for instance, in cancer and cardiovascular diseases (Siehler, 2009; Kozasa et al., 2011).

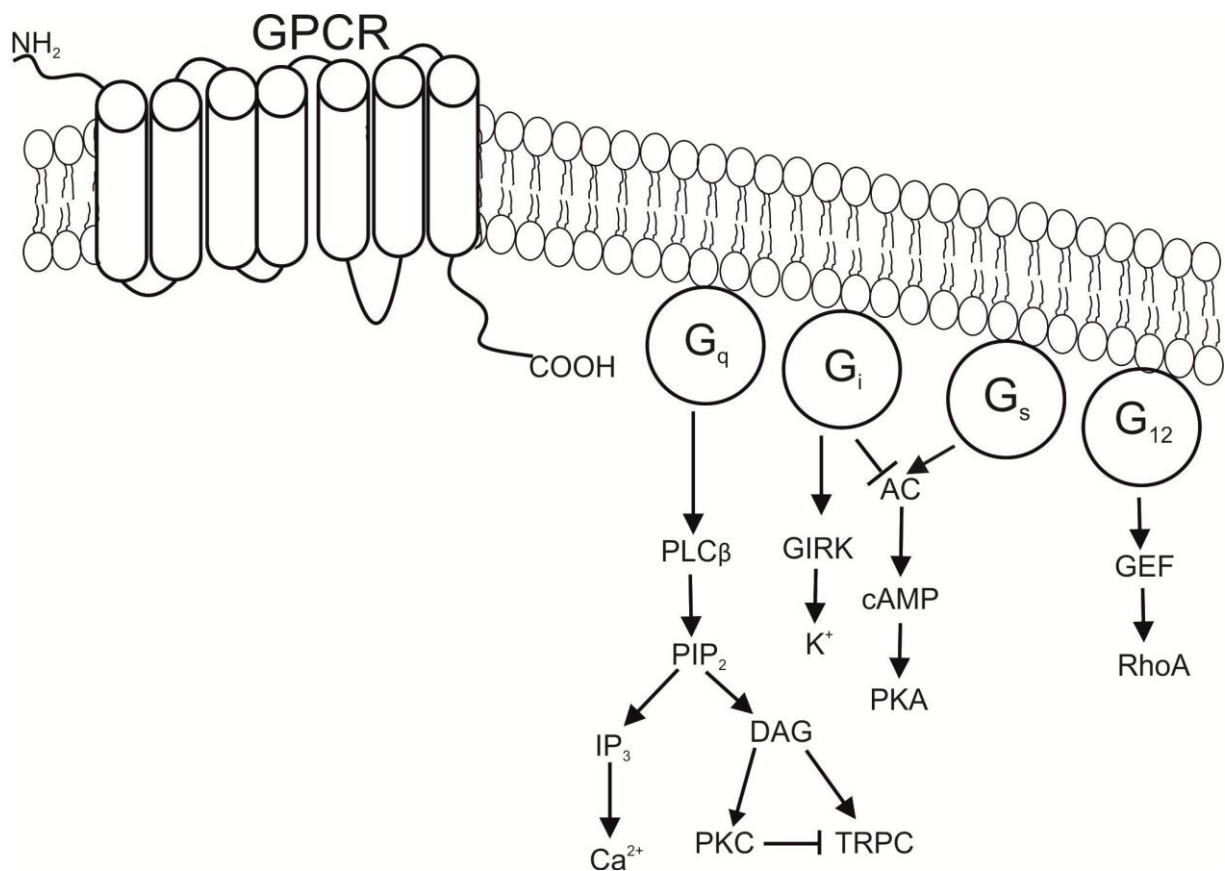


Figure 1. Simplified picture of the main signalling pathways of the four G-protein families. Arrows indicate activation and blunt end arrows inhibition. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; COOH, carboxyl-terminus of the receptor; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; GIRK, G-protein-coupled inwardly rectifying potassium; GPCR, G-protein-coupled receptor; IP₃, inositol triphosphate; NH₂, amino-terminus of the receptor; PIP₂, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLCβ, β isoform of phospholipase C; TRPC, transient receptor potential (channel) of the canonical subfamily.

2.1.5. Regulation of GPCRs

GPCRs are tightly regulated in both the short- and long-term manner throughout their life cycle (De Vries et al., 2000). Long-term regulation includes regulation via gene transcription, translation and post-translational modifications, like phosphorylation, palmitoylation or acetylation. Trafficking of the synthesised receptor to the cell membrane is also highly regulated. On the cell surface, the receptor interacts with short-term regulating proteins, which may, for instance, modulate receptor oligomerisation or change the ligand affinity or -selectivity of the receptor (De Vries et al., 2000; Millar and Newton, 2010).

2.1.5.1. *Dimerisation and oligomerisation*

It is speculated that GPCRs need to form homo- or heterodimers or -oligomers during their biosynthesis in the ER to be expressed on the cell surface (Hill, 2006). Homo- or hetero-oligomerisation also offers new possibilities for GPCR regulation. Some receptors activate certain signal pathways as homodimers but will show different signalling as heterodimers. This phenomenon is called “heteromer-directed signalling specificity” (Millar and Newton, 2010; Zhang and Xie, 2012). This has been found to occur with dopamine D₁ and D₂ receptors that couple to G_s- and G_i-proteins, respectively, as monomers but to G_q as heterodimers (Lee et al., 2004). Heteromer-directed signalling specificity is also believed to be involved in dysfunction of the receptor in certain pathological states, and thus dimerisation-affecting drugs could have a therapeutic use (Zhang and Xie, 2012).

2.1.5.2. *Regulators of G-protein signalling*

GTPase-activating (or -accelerating) proteins (GAPs) promote the hydrolysis of G α -bound GTP to GDP and thus termination of the G-protein signalling. Regulators of G-protein signalling (RGSs) are proteins working as GAPs at least in the signalling of G-proteins of G α_q , G α_i , and G α_{12} families, through binding to their G α subunits (De Vries et al., 2000; Zhong and Neubig, 2001; Siehler, 2009; Magalhaes et al., 2012). In addition to binding to G α subunit, direct interaction between RGS proteins and receptors has been observed (Zhong and Neubig, 2001). Binding of any RGS to G-proteins of G α_s family has not been found; actually, Asp229 of G α_s has been shown to structurally prevent the interaction (Natochin and Artemyev, 1998). However, type V AC, stimulated by G α_s , has been shown to enhance the GTPase activity of G α_s and it may thus work as a GAP for G α_s (Scholich et al., 1999). Similarly, an effector of G $_q$ signalling, PLC β , works as GAP by promoting the hydrolysis of G α_q -bound GTP (Biddlecome et al., 1996). In addition to the GAP activity, RGS proteins also have two other ways to turn off signalling of G-proteins. Firstly, they can physically prevent binding of effectors, like PLC β , to G-proteins. Secondly, after GTP hydrolysis, they can increase the affinity of G α subunits for the G $\beta\gamma$ complex and thereby enhance the formation of the inactive heterotrimer (De Vries et al., 2000). Independent of G-proteins, RGS proteins can target protein kinase A and regulate GIRK currents. In addition to negative regulation of signalling, also positive, activity-enhancing functions of RGSs have been shown for RGS4, which can potentiate α_2 -adrenergic receptor-stimulated GTP γ S binding to the α -subunit of a G-protein (Zhong and Neubig, 2001).

There are more than 20 different RGS proteins identified in mammals. All of these proteins contain a conserved RGS box region, which is needed for their GAP activity. Many of the RGS proteins have membrane-targeting signal sequence in their N-terminus to localise them near their targets. RGS proteins also have other domains that vary between different members of the RGS family and these are needed to link RGS proteins to other signalling pathways and proteins (De Vries et al., 2000). These extra domains are involved, for instance, in the regulation of protein localisation and intracellular trafficking (Zhong and Neubig, 2001; Magalhaes et al., 2012).

2.1.5.3. *GPCR kinases and arrestin*

The first proteins found to interact with GPCRs and to regulate uncoupling of GPCRs from G-proteins were GPCR kinases (GRKs) and arrestins. GRKs are composed of three functional

domains: the amino-terminal domain, which is homologous to RGS proteins; the central catalytic domain; and the carboxyl-terminal, membrane-targeting domain. GRKs phosphorylate serine and threonine residues in the third intracellular loop and carboxyl-terminal domain of activated GPCRs and thus promote desensitisation of GPCRs (Hill, 2006; Magalhaes et al., 2012). GRKs can also mediate phosphorylation-independent desensitisation of GPCR. For instance, GRK2 and GRK3 can mediate uncoupling of $G_{\alpha_{11}}$ from the receptor. GRKs also regulate endocytosis of GPCRs to allow their dephosphorylation and resensitisation (Magalhaes et al., 2012).

For many GPCRs, GRK-mediated phosphorylation alone is insufficient to cause desensitisation of the receptor. Instead, binding of β -arrestin proteins, together with GRK-mediated phosphorylation, is needed for full desensitisation and endocytosis of the receptor (Magalhaes et al., 2012). Some GPCRs bind β -arrestin with low affinity; they are dephosphorylated, resensitised and rapidly recycled back to the plasma membrane. Receptors that bind β -arrestins with high affinity are internalised as tight receptor- β -arrestin-complexes to endosomes. Their recycling to plasma membrane happens very slowly or not at all (Magalhaes et al., 2012).

β -arrestins were first identified for their ability to desensitise and thus reduce GPCR signalling (Lefkowitz, 1998). However, β -arrestins are now also known, for instance, as endocytotic adaptor proteins, which target the receptors for clathrin-mediated endocytosis, and as mediators of G-protein independent signalling (Lefkowitz and Shenoy, 2005; Shukla et al., 2011).

2.1.5.4. *Receptor desensitisation*

Prolonged binding of an agonist to a GPCR may not cause continued response. Instead it can lead to desensitisation of the receptor (Hill, 2006). Desensitisation is most extensively studied for the β_2 -adrenoceptor, and its desensitisation can be divided into agonist occupancy-dependent desensitisation and PKA-dependent desensitisation (Benovic et al., 1985; Hill, 2006). A weak stimulation of the β_2 -adrenoceptor stimulates AC and thus increases the level of cAMP, which activates PKA (Hill, 2006). PKA mediates desensitisation of the β_2 -adrenoceptor through phosphorylation but it can only cause partial uncoupling of the receptor from its G-protein. Agonist-dependent desensitisation may be used to achieve stronger desensitisation. In high agonist occupancy, GRKs can mediate phosphorylation of the receptor, eventually preventing the interaction between the receptor and the G-protein, and cause receptor internalisation (Hill, 2006).

Sometimes receptor phosphorylation works as a molecular switch, which exchanges the coupling of the receptor between different G-protein subtypes. For instance, PKA-dependent desensitisation of β_2 -adrenoceptor has been shown to switch the coupling of the receptor from G_s to G_i (Daaka et al., 1997).

2.1.5.5. *Receptor activity-modifying proteins*

Receptor activity-modifying proteins 1 and 2 (RAMP1 and RAMP2, respectively) regulate the expression of receptors at the cell surface and modulate the structure of the receptors resulting in altered ligand specificity (Hill, 2006; Magalhaes et al., 2012). RAMPs were first found to modulate the activity and pharmacology of calcitonin receptor and calcitonin receptor-like receptor of the *secretin* family GPCRs (McLatchie et al., 1998). Some studies have revealed, however, that RAMPs may also be involved in the regulation of other receptor types. At least some receptors of

the *glutamate* family are regulated that way (for more information of *secretin* and *glutamate* families, see below) (Magalhaes et al., 2012).

2.1.6. Classification of GPCRs

The GPCR superfamily can be classified into five different families based on their homology in sequence, structure and phylogeny. The families are *rhodopsin*, *secretin*, *glutamate*, *adhesion*, and *frizzled/taste2* family (Lagerstrom and Schioth, 2008).

The *rhodopsin* family, with its approximately 670 members, is the largest of these families and it also contains the highest number of pharmacologically targeted receptors (Pierce et al., 2002; Kobilka, 2007; Lagerstrom and Schioth, 2008; Rosenbaum et al., 2009). Receptors of the *rhodopsin* family can be further divided into α , β , γ , and δ groups (Lagerstrom and Schioth, 2008). The α group contains many important drug targets like histamine, dopamine and adrenergic receptors. The β group contains peptide-binding receptors, like receptor for gonadotropin-releasing hormone. Even though this group includes some important drug targets, peptide receptors present challenges regarding drug discovery given that peptide ligands are large and flexible, and thus the molecular features, which are important for the optimal interaction with a receptor, are difficult to identify. Smaller γ and δ groups include receptor for mediators like opioids and odorants, respectively (Lagerstrom and Schioth, 2008).

The *secretin* family contains receptors for peptide hormones, like the gastrointestinal peptide hormone secretin, glucagon, growth hormone-releasing hormone and corticotropin-releasing hormone (Lagerstrom and Schioth, 2008). All members of this family signal through coupling to G_s and thus mediate increase of intracellular cAMP (Pierce et al., 2002). In addition, all the receptors of the family have a similar extracellular hormone-binding domain and conserved cysteine residues in first and second extracellular loops (Lagerstrom and Schioth, 2008).

The relatively small *glutamate* family contains receptors, for instance, for glutamate, γ -aminobutyric acid (GABA), Ca^{2+} , and sweet and umami tastes. All receptors of this family have a large amino-terminal tail, which is needed for ligand binding (Pierce et al., 2002; Lagerstrom and Schioth, 2008).

All *adhesion* family members show an extended amino terminus, which is thought to be required for cell–cell contact (Millar and Newton, 2010). Members of the *adhesion* family share homology in sequence with the members of *secretin* family but are very different with respect to the N-terminal domain (Lagerstrom and Schioth, 2008). Most of the receptors of the *adhesion* family remain orphan, meaning that they do not have known endogenous ligands (Millar and Newton, 2010).

The *frizzled/taste2* family can be further divided into groups of frizzled, smoothed, and taste2 receptors (Lagerstrom and Schioth, 2008). The most studied ligands of frizzled receptors are Wnt glycoproteins. Frizzled receptors play an important role in the regulation of cellular polarity, embryonic development, synapse formation and regulation of proliferation (Huang and Klein, 2004). Smoothed receptors are structurally related to frizzled receptors. They are involved in many developmental processes in the Hedgehog signalling pathway. Mutations in both frizzled and smoothed receptors are detected in cancers and thus drugs targeting these receptors could have therapeutic use (Taipale et al., 2000; Nagayama et al., 2005; Lagerstrom and Schioth, 2008). Taste2-family receptors bind bitter-tasting compounds, like cycloheximide (Chandrashekar et al., 2000). A single taste receptor cell expresses multiple receptors of taste2 group and can thus sense

many structurally unrelated, bitter-tasting compounds; however, many receptors of the taste2 family are still orphan (Lagerstrom and Schioth, 2008).

Most of the putative GPCRs are identified based on the homology of their DNA sequences to sequences of known GPCRs and/or phylogenetic algorithms. Receptors identified this way may not have an endogenous ligand and they are called orphan receptors (Civelli et al., 2013). It is good to keep in mind that not all of the putative receptors identified finally couple to a G-protein. Still, about 25% of suggested human GPCRs remain orphan (Summers, 2012). As GPCRs have potential as drug targets, however, they are the most studied receptor family for pharmaceutical intervention. This will finally lead to discovery of ligands for orphan receptor, a process called deorphanisation (Wilson et al., 1998; Kobilka, 2007). The rhodopsin family includes the highest number of orphan receptors (currently 67) (Civelli et al., 2013).

2.2. GPCRs as drug target

There are several reasons why GPCRs are one of the most studied drug targets in the pharmaceutical industry. One central reason is that GPCRs constitute one of the largest gene families and they regulate many physiological functions. GPCRs also have a long history in drug discovery and they are already targets for many drugs world-wide. Actually, GPCRs are targets for more than 30% of all currently available drugs, like opioid analgesics, antihistamines, antidepressants and many cardiovascular drugs (Harrison and Traynor, 2003; Hill, 2006; Insel et al., 2012).

Drug discovery is a multidisciplinary process involving target identification and validation, identification and development of candidate drugs, preclinical safety and efficacy studies and finally clinical studies (Hughes et al., 2011). The whole process can take up to 15 years and only a very small number of candidate drugs are finally registered. The studies reported here mostly touch upon ligand development and methodology for ligand identification/screening. The approaches utilised for these in GPCR research will be outlined here.

Several different approaches may be used for ligand development. A classical method, utilised for instance in β -adrenoceptor antagonist development, is based on pharmacophore identification (Pitha et al., 1983). Pharmacophore means the molecular features of a chemical compound needed for the optimal interaction with the target of the compound and to cause its biological response (Yang, 2010). When several compounds, all of which bind to the same receptor, are studied, a pharmacophore can be identified. After identification, the pharmacophore is verified and utilised then to identify and develop novel ligands, for instance by actual or virtual means.

Chemical synthesis can be used to produce large sets of ligands to refine the binding affinity and selectivity and possibly even distinguish agonists and antagonists (Heckmann and Kessler, 2007). Other approaches may have to be used when there is no known natural ligand, the natural ligand is too complex or otherwise unsuitable, or when an allosteric binding site is the target. One such approach is to define putative ligands based on the receptor model (Schneider and Fechner, 2005). Models are often based on direct crystal structures, homology models of such and/or receptor mutagenesis studies (Kobilka, 2007). Another method would be to randomly screen with a more or less diverse library.

The technology used for drug screening is not a trivial choice. Many different methods are available with distinct advantages and disadvantages and costs. From a high-throughput

perspective, an ideal assay would be simple, homogeneous, reproducible, nonradioactive and easily adapted for robotics and microtiter plate format, and have a high signal-to-noise ratio (Thomsen et al., 2005). Methods used to screen ligands can be roughly divided into binding and functional assays. Sometimes, like in the case of biased ligands, several different assays utilising different pathways may need to be performed and the results combined to obtain enough information from a certain drug molecule (Kenakin and Christopoulos, 2013).

2.2.1. Binding assays

Historically, the first method used to study GPCR-binding compounds was receptor binding assay (Lefkowitz et al., 1970). While ligand screening often utilises equilibrium binding, kinetic measurements are also possible; however, only the equilibrium assays will be described here. Binding assay typically requires the use of a radioactive ligand, which has known binding parameters to the receptor of interest (Zhang and Xie, 2012). Radioligand is exposed to competition with the non-labelled ligands to be screened, and thus the inhibitory constant, K_i , for each ligand can be calculated. Fluorescence may be used as an alternative marking for the radioactivity (Cottet et al., 2011). This binding assay is usually rather inexpensive, but there are problems with it too. It requires a known ligand with suitable properties (Zhang and Xie, 2012). Furthermore, the method is not homogeneous as it requires the separation of bound and free ligand, which is usually achieved by filtration.

In homogenous binding assays, there is no need to separate or wash the unbound ligands before the measurement, and thus they are suitable for automation (de Jong et al., 2005). Homogenous assays can be based, for instance, on fluorescence polarisation, or fluorescence/Förster resonance energy transfer (FRET) between a labelled receptor and a labelled ligand (Allen et al., 2000; de Jong et al., 2005). These assays may be more suitable for particular systems and not others due to either methodological obstacles or high cost.

2.2.2. Functional assays

Binding assays only offer information regarding the most primary part of the ligand/receptor interaction, the binding itself. To further characterise the response and to study functional properties of the ligand, functional assays are needed (Zhang and Xie, 2012). Functional assays are particularly important to study the properties of agonists, and to distinguish agonist from antagonist. Assays include measurement of G-protein activation or G-protein-mediated events like second messenger generation or gene reporter activation (Chen et al., 2012; Zhang and Xie, 2012).

2.2.2.1. [³⁵S]GTP γ S binding assay

GTP γ S binding assay is used to determine the activation of GPCR by measuring the binding of GTP to G-protein (Harrison and Traynor, 2003; Zhang and Xie, 2012). Non-hydrolysable analogue of GTP, [³⁵S]GTP γ S, is used to radioactively label G-proteins as a measure of receptor activation. Being non-hydrolysable, [³⁵S]GTP γ S accumulates in the $G\alpha$, and as it remains membrane-bound, the membrane preparation can be filtrated and radioactivity from the filter counted, as is done for the radioactive receptor binding assay (Harrison and Traynor, 2003).

The method is most widely used with $G\alpha_i$ -coupled GPCRs as these G-proteins are the most abundant in many cell types and also their GDP to GTP exchange rate is faster than for other

subtypes (Harrison and Traynor, 2003; Zhang and Xie, 2012). The advantage of this method is that it measures the receptor-mediated response early after receptor activation and measurements are not subjected to amplification or other modulation that occurs in downstream signalling. That is why the method can also be used to make a difference between full, partial, and inverse agonist, neutral antagonist, and allosteric regulator. On the other hand, lack of amplification by downstream signalling usually leads to a low signal-to-noise ratio (Zhang and Xie, 2012). There are also non-radioactive GTP analogs that may be used in the same way (Frang et al., 2003).

2.2.2.2. *Second messenger assays*

GPCR signalling involves many quite easily detectable second messengers. The most widely used assays detect changes in the cellular levels of cAMP, IP₃, or Ca²⁺. Calcium assays are very popular in GPCR drug screening because of the good availability of Ca²⁺-binding fluorescent dyes, like Fluo-3 and Fluo-4 (Thomsen et al., 2005). Elevation of intracellular Ca²⁺ in cells labelled with these dyes can be easily detected with a fluorescence plate readers, like FLIPR. In addition to the fluorescent dyes, there are also some luminescence-based dyes, like the jellyfish photoprotein aequorin (Rudolf et al., 2003). Another reason for the popularity of the calcium assay format is that it can be used to study GPCRs coupling to several G-protein isoforms. Even though receptors coupling to G_q most readily elevate Ca²⁺, calcium assays are not restricted to these receptors only. Gα₁₆, a member of the Gα_q-family and thus an inducer of calcium signalling, can recognise and couple many receptors, which normally couple to G_s- or G_i-proteins (Milligan et al., 1996; Thomsen et al., 2005). Coexpression of Gα₁₆ has been used, for instance, in coupling of β₂ adrenergic and M₂ muscarinic receptors to calcium signalling (Offermanns and Simon, 1995). Chimaeric Gα_{q/11} or Gα₁₆-proteins, like Gα_{16/z}, can also be used to couple a wide range of receptors to calcium signalling (Milligan and Rees, 1999; Liu et al., 2003a). These approaches allow a wider use of the calcium assay. Still, there are some limitations in this assay format also. Slowly binding agonists are less suitable for the calcium assays as the Ca²⁺ response occurs rapidly and the negative feedback by IP₃ degradation and Ca²⁺ pumping are quickly turned on (Thomsen et al., 2005; Chen et al., 2012; Zhang and Xie, 2012).

IP₃ is rapidly hydrolysed into the bisphosphate, monophosphate, and finally to free inositol (Berridge, 2009). Treating the cells with Li⁺ inhibits inositol monophosphatase, the final enzyme of this cascade, and thus enables accumulation of IPs, IP₁ in particular. There are radiometric, like ³H-inositol-based, and non-radiometric, like fluorescent-based methods available to detect IP accumulation (Thomsen et al., 2005). Sometimes several methods have emerged to provide better assay formats for the HTS. For instance, scintillation proximity assay (SPA) has been combined with immobilised metal ion affinity chromatography, resulting in a fast and simple assay format for 96-well plates (Liu et al., 2003b). Slow kinetics of IP₁ accumulation makes it possible to use the method for slow-binding GPCR ligands (Chen et al., 2012; Zhang and Xie, 2012).

Accumulation of cAMP is studied with assay formats very similar to assays used to study accumulation of IP. Activated GPCRs coupled to G_s and G_i can stimulate or inhibit, respectively, AC, which catalyses the conversion of ATP to cAMP (Hadcock et al., 1990). Several cAMP assay formats are homogenous, and the detection is based, for instance, on radioactivity, luminescence, or fluorescence (Gabriel et al., 2003; Williams, 2004; Thomsen et al., 2005). For instance, accumulation of [¹²⁵I]-labelled cAMP can be directly detected with the SPA, once the label comes

into close proximity with the scintillant surface of the SPA plates (Williams, 2004). One non-radiometric alternative for studying cAMP accumulation is the use of ruthenium derivate-tagged cAMP, which is bound to special plates with anti-cAMP-antibody. After chemical and electrical stimulation, a light signal can be observed (Williams, 2004). Whichever assay format is used, cAMP hydrolysis by cyclic nucleotide phosphodiesterases usually has to be inhibited with phosphodiesterase inhibitor (Williams, 2004). In addition, an exogenous AC stimulus, usually forskolin, must be present when the inhibition of AC is measured (GPCRs coupling to G_i) (Seamon et al., 1981).

2.2.2.3. *Gene reporter assays*

Activated GPCRs regulate gene transcription of various genes via response elements for second messengers (Cheng et al., 2010). In reporter assays, these response elements are utilised to regulate expression of reporter proteins. Reporter proteins are usually enzymes linked with suitable substrates to colour, fluorescence or luminescence production like alkaline phosphatase, β -galactosidase, luciferase or green fluorescent protein (Zhang and Xie, 2012). Especially firefly luciferase is widely used as a reporter because of its sensitivity, wide linear range, stability and persistent response (Fan and Wood, 2007). Easy-to-set-up reporter assays are also popular in high-throughput screening (HTS) even though there are some concerns. The assays require long incubation times, and because signal is measured distal from the receptor activation, false positive results may occur. False results may also arise, for instance, from the interaction between studied chemical and the reporter protein or with protein synthesis machinery (Fan and Wood, 2007; Cheng et al., 2010; Zhang and Xie, 2012).

2.2.2.4. *In silico drug discovery*

The methods described above can be used for HTS. However, with constant improvement of computational methods, HTS can be more easily complemented with *in silico* screening. Such an approach can save both money and time as the number of experimentally tested compounds decreases, and compounds found with *in silico* screening have higher probability to also work in experimental approaches (Becker et al., 2004; Zhang and Xie, 2012).

Two *in silico* screening methods, named structure-based and ligand-based methods, are applied in GPCR drug discovery. Structure-based screening method requires a model of the structure of the target receptor (Zhang and Xie, 2012). The structure can be determined experimentally or with homological modelling (Shoichet and Kobilka, 2012). During the past few years, crystal structures of several GPCRs have been solved, which has improved homology-based models (Zhang and Xie, 2012). Screening is done so that the receptor structure and a library of molecules of interest are fitted together in a docking program. Hits are further tested, for instance, for their ability to compete with native ligands and for concentration-response relationships (Shoichet and Kobilka, 2012).

When the receptor structure is not available, as is the case for most GPCRs, ligand-based drug discovery can be used for *in silico* screening (Zhang and Xie, 2012). This method attempts to find new ligands through comparing their similarity on known ligands (Hessler et al., 2005). Ligand-based screening is usually combined with other methods to obtain sufficient information about biological activity of the hit compounds (Gruber et al., 2010; Zhang and Xie, 2012).

2.2.3. G-protein-independent functional assays

There are some G-protein-independent methods developed to study GPCRs. These methods offer the advantage to also study receptors with unknown signalling pathways, given that no information regarding G-protein subtype is needed. That is why they may be more suitable for deorphanisation of GPCRs (Zhang and Xie, 2012).

2.2.3.1. Receptor internalisation assays

High-content screening (HCS) is a method that combines fluorescence microscopy with automated image analysis (Bickel, 2010). Different kinds of HCS systems can be used to study GPCR internalisation. As described earlier (see, **2.1.5.3. GPCR kinases and arrestin**), GRKs and β -arrestin regulate GPCR desensitisation and endocytosis. This receptor internalisation process can be followed with a fluorophore-labelled ligand or an antibody, which is directed, for instance, against the extracellular domain of the receptor. The receptor can also be tagged with a fluorescent protein, like green fluorescent protein (GFP) (Zhang and Xie, 2012). When using fluorophore-labelled protein, no fluorescent dyes or secondary substrates are needed (Chen et al., 2012).

HCS has the advantage of being independent of associated G-protein subtype and signalling pathways, and thus it is a suitable method to deorphanise GPCRs. With the use of multiple fluorophores, several parameters can be detected simultaneously from one cell. Disadvantages are the high price of instruments, long incubation times and the fact that some receptors do not show much internalisation (Chen et al., 2012; Zhang and Xie, 2012). In addition, internalisation does not necessarily correlate with other signalling (Kenakin, 2013).

2.2.3.2. β -arrestin recruitment assays

In addition to HCS, there are also other β -arrestin-recruitment assays available. One of them is bioluminescence resonance energy transfer (BRET) (Zhang and Xie, 2012). BRET is based on energy transfer between *Renilla* luciferase-tagged protein, β -arrestin in this case, and receptor tagged with mutant variant of GFP, like yellow fluorescent protein (YFP) (Xu et al., 1999; Zhang and Xie, 2012). When these two tags come close together, bioluminescence emitted in *Renilla* luciferase excites YFP, which emits a detectable signal. BRET is calculated as the ratio of the emissions of *Renilla* luciferase and YFP. Although BRET is a suitable method for many GPCRs, it also has disadvantages. The studied receptors need to be labelled with a fluorophore (Zhang and Xie, 2012). Also, the responses are relatively small. In addition to BRET, there are also some β -arrestin recruitment assay kits commercially available. Detection in these kits is based on stimulation of reporter gene activity or chemiluminescent signal (Zhang and Xie, 2012). For instance, in the PathHunter enzyme complementation assay by DiscovereX, β -arrestin is linked with N-terminally truncated β -galactosidase while the GPCR of interest is tagged with this deleted part of the β -galactosidase (Olson and Eglen, 2007). Upon GPCR interaction with the β -arrestin, these two parts of the β -galactosidase come together activating the enzyme and generating a chemiluminescent signal upon exposure to the substrate (Zhang and Xie, 2012).

2.2.3.3. *Label-free cellular assays*

Ligand–receptor binding assays and functional assays described above are the main cellular assays used in drug discovery for GPCRs. During the past few years, however, new label-free cellular technology has become a competitor for traditional assays. Label-free assays are based on biosensors that convert the ligand binding-induced signals into quantifiable responses (Fang, 2011; Chen et al., 2012; Zhang and Xie, 2012). Detection can be based, for instance, on optical, electrical or magnetic signal (Daniels and Pourmand, 2007; Dey and Goswami, 2011; Colombo et al., 2012). Biosensors can also detect changes in cellular properties like adhesion and proliferation (Zhang and Xie, 2012).

Dynamic mass redistribution optical biosensor and impedance electric biosensor are the two most widely used biosensors (Chen et al., 2012). Receptor signalling often includes, for instance, protein trafficking or changes in the cell adhesion or morphology, which all lead to dynamic mass redistribution. In the optical biosensor-based assay, biosensors simply detect the changes in the mass distribution (Fang, 2011). Although the method was initially intended for binding assays, it has also been successfully used in cell morphological and GPCR signalling studies. Electric biosensors can measure impedance, which is considered to be a sum of cellular events (Fang, 2011; Zhang and Xie, 2012).

As already mentioned, biosensors offer high sensitivity and enable detection of several cellular events (Fang, 2011); however, they also have some disadvantages. Their high price limits broad applications. In addition, false positive or negative results can occur (Anderson et al., 2000; Cooper, 2002).

Regarding biosensors, another label-free cellular assay is based on changes in cell phenotype, particularly in aggregation of melanosomes in melanophores after changes in the activity of AC and PLC β (Graminski et al., 1993). Aggregation and deconcentration of melanosomes can be seen as light to dark changes in cells and this can be detected with a proper imaging and processing instrument. Difficulties persist, however, in melanophore technology when determining precise structure-activity relationships. Thus the method is not well suited for drug screening (Chen et al., 2012).

2.3. *Orexin/hypocretin*

In January 1998, Luis de Lecea, Greg Sutcliffe and their co-workers published a study about hypothalamus-specific mRNA that encodes peptides, which they suggested to work as neurotransmitters in the central nervous system (CNS) (de Lecea et al., 1998). These hypothalamus-produced peptides showed amino acid sequence similarity with the incretin hormone family and thus they were named hypocretins (*hypothalamic member of incretin family*). At that time, the physiological functions of hypocretins were only guessed, based on the comparison with other hypothalamic-produced neuropeptides. Productions of hypocretin and melanin-concentrating hormone, which is a promoter of food consumption, were mapped to the same area, and thus hypocretins were also speculated to regulate appetite.

Only a month after publication of this study, Masashi Yanagisawa and co-workers published a study about hypothalamus-specific mRNA that encodes two novel neuropeptides, both derived from the same precursor (Sakurai et al., 1998). They found that these neuropeptides bind to two orphan GPCRs and that they have an ability to stimulate food consumption in rats. Thus these

neuropeptides were termed orexins after the Greek word *orexis*, which means appetite. Two orexin peptides were called orexin-A and -B. Orphan receptors were named OX₁ and OX₂ receptors.

Now, 15 years later, there are thousands of research publications about orexins, and only between the years 2006 and 2010, there were more than 70 orexin receptor-targeting compounds patented (Coleman and Renger, 2010). Dysregulation of orexin system is involved in several pathological states like narcolepsy, Parkinson's disease and addiction (Scammell and Saper, 2005). Thus orexin research is, and will be, a field, which researchers will find of interest for some time to come.

2.3.1. Structure of orexin peptides

Both orexin-A and orexin-B are derived from the same prepro-orexin precursor through proteolytic processing (Sakurai et al., 1999). Prepro-orexin is a 131 amino acid (aa) long peptide, consisting of a secretory signal sequence, 31 aa long orexin-A and 28 aa long orexin-B. Mature orexin-A is post-translationally modified with an N-terminal pyroglutamic acid (Figure 3) (Sakurai et al., 1998). Orexin-A includes four Cys residues, which form two intrachain disulfide bonds. These Cys residues are conserved among different species. The amino acid sequences of orexin-A and orexin-B are rather conserved among mammals but are more diverse in lower vertebrates (Kukkonen, 2013b).

2.3.2. Orexin receptors

Orexin receptors, OX₁ and OX₂, belong to the GPCR receptor family. Orexin peptides and receptors were actually found when a large number of orphan GPCRs were screened for endogenous ligands (Sakurai et al., 1998). In humans, OX₁ and OX₂ receptors have 64% homology in their amino acid sequences (Kukkonen, 2013b). Orexin peptides have distinct binding affinities for the orexin receptors. Affinities of orexin-A are similar for both the OX₁ and OX₂ receptors while the binding affinity of orexin-B is higher for the OX₂ than OX₁ receptor (Sakurai et al., 1998). This may also be reflected in the potency of the peptides (see, *5.3. Impact of the expression system on orexin agonist discrimination*).

2.3.3. Distribution of orexin receptors and peptides

Orexin peptide and receptor distribution studies are based either on the antibodies, for instance for radioimmunoassay and immunohistochemistry methods, or on measuring the expression level of mRNA by *in situ* hybridisation or reverse transcription polymerase chain reaction (RT-PCR). Orexin antibodies have been observed to cross-react with other antigens and GPCR antibodies (including orexin receptor antibodies) (Kukkonen, 2013b). Thus the results achieved solely by methods based on the orexin antibodies, should be questioned, and thus they have been overlooked in this review.

Orexin peptide and receptor mRNAs are detected in the CNS and several peripheral tissues. In rodents, orexin peptides producing neurons are primarily expressed in the lateral hypothalamus and also in nearby regions, like posterior hypothalamus (de Lecea et al., 1998; Kukkonen et al., 2002; Stoyanova et al., 2010). From these regions, neurons project to other hypothalamic nuclei and forebrain, midbrain, and brain stem regions (Kukkonen et al., 2002). Orexin neuron projections are also found in the rat spinal cord, where orexins may be involved in the modulation of somatosensory information (Date et al., 2000). Of the peripheral tissues, prepro-orexin mRNA is

detected, for instance, in rat testis and human adrenal gland, pancreas and stomach (Johren et al., 2001; Nakabayashi et al., 2003).

High expression of OX₁ mRNA is detected in the rat hypothalamus, thalamus, and substantia nigra (Hervieu et al., 2001). In the peripheral tissues, OX₁ mRNA is expressed, for instance, in the human adipose tissue and testis and in the rat adrenal gland and pancreas (Kirchgessner and Liu, 1999; Lopez et al., 1999; Karteris et al., 2004; Digby et al., 2006). In the CNS, sites of OX₂ mRNA expression partly overlap with the expression sites of OX₁, for instance, in the hypothalamus and thalamus (Cluderay et al., 2002). However, their expression patterns are mainly distinct, which may indicate differential regulation of physiological effects by the orexin receptor subtypes (Tsuji and Sakurai, 2013). In the peripheral tissues, expression of OX₂ is mostly similar to the expression of OX₁ but there are some minor exceptions. In some peripheral tissues, like in the kidney and testis, only the expression of OX₁ mRNA is found, whereas in the lung, only OX₂ mRNA is expressed (Johren et al., 2001). The expression levels of orexin receptor subtypes may vary according to gender. In the adrenal gland, OX₂ mRNA expression levels are much higher in male than in female rats, and in the hypothalamus OX₁ expression levels are higher in female than in male rats (Johren et al., 2001). This indicates, for instance, that sex steroids may be involved in the regulation of orexin receptor expression levels.

2.3.4. Orexin receptor signalling

It is assumed that both orexin receptor subtypes couple to G_q. That assumption is based on the fact that activation of the orexin receptors induces an increase in the intracellular calcium concentration (Sakurai et al., 1998). This assumption is rather premature, however. It is not easy to determine G-protein coupling of a GPCR. That is, for instance, because of limited number of molecular tools, and also because disturbing the cellular environment in the measurements may ultimately prevent the signalling of the G-protein (Kukkonen, 2004; 2013b). Detecting changes in the activity of AC or PLC after receptor activation should not be directly concluded to be due to coupling of the receptor to certain G-protein, as both AC and PLC are regulated in many ways (Kukkonen, 2013b). Despite the complex and still partly uncharacterised signalling of the orexin receptors, some of the best known signalling properties of the orexins are summarised here and in the figure 2.

2.3.4.1. Elevation of the intracellular calcium concentration

Sakurai *et al.* originally showed the elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) after orexin receptor activation in Chinese hamster ovary (CHO) cells (Sakurai et al., 1998). Later orexin receptors have been shown to also have the same ability in other cell types, like in neuronal cells isolated from hypothalamus, cortex or spinal cord and in recombinant neuro-2a, PC12 and HEK-293 cell lines (Holmqvist et al., 2002; Kukkonen et al., 2002; Magga et al., 2006; Putula et al., 2011b). Elevation of $[Ca^{2+}]_i$ occurs in two different ways. Stimulation of the orexin receptor leads to activation of PLC, probably through receptor coupling to G_q, and PLC catalyses the formation of IP₃ (Uramura et al., 2001). IP₃ elevates intracellular calcium by releasing intracellular calcium from the ER and indirectly through activating store-operated calcium channels (SOCs) and thus causing calcium influx. On the other hand, increased $[Ca^{2+}]_i$ is achieved through opening of receptor-operated calcium channels (ROCs) that leads to influx of calcium through the channels. ROC activation does not require IP₃ but, instead, phospholipase A₂ (PLA₂) activity seems to be necessary for this (see, **2.3.4.3. Regulation of lipid signalling**). Activation of ROCs and SOCs is dependent

on the concentration of orexin-A, at least in recombinant cell models. At low orexin-A concentration, ROCs are primarily responsible for calcium elevation, and Ca^{2+} influx through ROCs somehow enhances PLC activation, which triggers Ca^{2+} release from the ER and activation of SOCs (Lund et al., 2000; Kukkonen and Åkerman, 2001; Johansson et al., 2007; Turunen et al., 2010; Turunen et al., 2012). At high orexin-A concentration, PLC activation is independent of ROCs (Lund et al., 2000; Kukkonen and Åkerman, 2001).

2.3.4.2. Regulation of the activity of adenylyl cyclase

Three different pathways for orexin receptors to regulate the activity of AC have been shown. Firstly, OX_1 can couple to G_s and thus stimulate AC and cAMP production (Holmqvist et al., 2005). Another way to stimulate AC is via coupling to G_q and activation of PKC, which is known to be a stimulator of some AC isoforms (Kawabe et al., 1994). In addition to stimulatory effects, inhibitory effects in AC regulation have also been seen (Holmqvist et al., 2005). That is probably mediated via OX_1 coupling to G_i -protein as the inhibition can be prevented with pertussis toxin. In some reports, no changes in cAMP levels after orexin receptor activation are detected at all (Magga et al., 2006). Altogether, the variation in results may be due to differences in cell types or experimental setups used. In addition, AC is regulated in multiple ways, not only by G_s - and G_i -proteins. It is likely, however, that both OX_1 and OX_2 receptors couple to $\text{G}\alpha_i$, $\text{G}\alpha_s$, and $\text{G}\alpha_q$ families of G-proteins, but that is only shown indirectly (Kukkonen, 2013a; b; Kukkonen and Leonard, 2013).

Stimulation of the activity of adenylyl cyclase is involved in glucocorticoid release in human adrenocortical cells (Mazzocchi et al., 2001). That release is mediated by orexin-A binding to OX_1 receptor, which activates adenylyl cyclase-dependent cascade. This may be one way orexins are involved in the maintenance of our energy homeostasis.

2.3.4.3. Regulation of lipid signalling

Several phospholipase isoforms, including PLA, PLC, and PLD, are activated after stimulation of orexin receptor in recombinant cell lines (Kukkonen, 2013b). Upon OX_1 receptor activation, release of arachidonic acid (AA) is detected in recombinant CHO cells (Turunen et al., 2010). AA is the major end product of PLA_2 activity and cytosolic PLA_2 (cPLA_2) in particular is involved in orexin receptor signalling (Turunen et al., 2012). There is a strong connection between the release of AA and elevation of $[\text{Ca}^{2+}]_i$, especially through ROCs. Reduction of the extracellular Ca^{2+} abolishes the orexin-mediated release of AA (Turunen et al., 2010). On the other hand, blocking of the PLA_2 activity, and thus AA release, with methyl arachidonyl fluorophosphonate (MAFP) or pyrrophenone, causes a notable inhibition in the orexin receptor-operated Ca^{2+} influx (Turunen et al., 2012). Thus it is speculated that AA release and Ca^{2+} influx create a positive feedback loop in the orexin receptor signalling (Turunen et al., 2010).

It is generally assumed that orexin receptors couple to G_q -protein and thus activate $\text{PLC}\beta$. It seems that at least two different isoforms of PLC are activated in orexin signalling cascades in the CHO cells (Johansson et al., 2008), but the identity of these is unknown. Similar to the activation of PLA, PLC activation is also regulated by Ca^{2+} influx, more prominently through ROCs. ROCs thus seem to have a very important role in orexin receptor signalling (Johansson et al., 2007).

Stimulation of OX_1 receptors activates PLD, most likely PLD1 isoform (Jäntti et al., 2011). PLD hydrolyses phosphatidylcholine to choline and phosphatidic acid (PA), which is an

intracellular messenger. PA indirectly activates the production of PIP₂, which is, on the other hand, needed for the PLD activity. PA can be converted to DAG, and thus PLD activation causes increase both in PA and DAG concentrations (Jenkins and Frohman, 2005). Activation of PLD1 after orexin receptor stimulation occurs via PKC. The exact targets of PLD in orexin receptor signalling are still unknown.

Even though most of the knowledge regarding orexin-mediated regulation of lipid signalling originates from studies with recombinant cell lines, there are, however, some studies that have been conducted in native neuronal cells. Orexin-induced PKC activation may mediate responses like Ca²⁺ signalling and cell depolarisation, in, at least, rat dopamine neurons or neurons in the prefrontal cortex or nucleus tractus solitarius (Uramura et al., 2001; Yang et al., 2003; Song et al., 2005).

Endocannabinoids are AA-containing lipid-derived messenger molecules (Pertwee, 2006). The best known endocannabinoids are 2-arachidonylglycerol (2-AG) and *N*-arachidonylethanolamine (anandamide). Orexin-A-induced activation of OX₁ receptor triggers the release of 2-AG from the PLC-produced DAG in a reaction catalysed by DAG lipase (DGL) (Turunen et al., 2012; Kukkonen, 2013b). Previous studies have proposed formation of heteromers between orexin receptors and endocannabinoid receptors (Ellis et al., 2006; Ward et al., 2011), however, current findings suggest that heteromerisation is not necessary. In a cell-cell communication assay, 2-AG released upon OX₁ receptor stimulation activates endocannabinoid signalling through binding to endocannabinoid receptor CB₁ in the same or neighbouring cells (Turunen et al., 2012; Jääntti et al., 2013). The role of orexinergic and endocannabinoid system connection in physiological functions is still partly unclear although some interesting findings of their co-operation have been made. Both orexins and endocannabinoids have a role as regulators of appetite, and orexin-A-induced appetite can be blocked with the CB₁ antagonist rimonabant (Crespo et al., 2008). Another overlapping function between orexinergic and endocannabinoid systems is seen in the regulation of analgesia (Kukkonen, 2013b). It is speculated that orexin signalling-induced 2-AG production in the periaqueductal gray matter, the site crucial for descending pain inhibition, results in CB₁ receptor activation and, finally, inhibition of GABAergic signalling and antinociception (Ho et al., 2011).

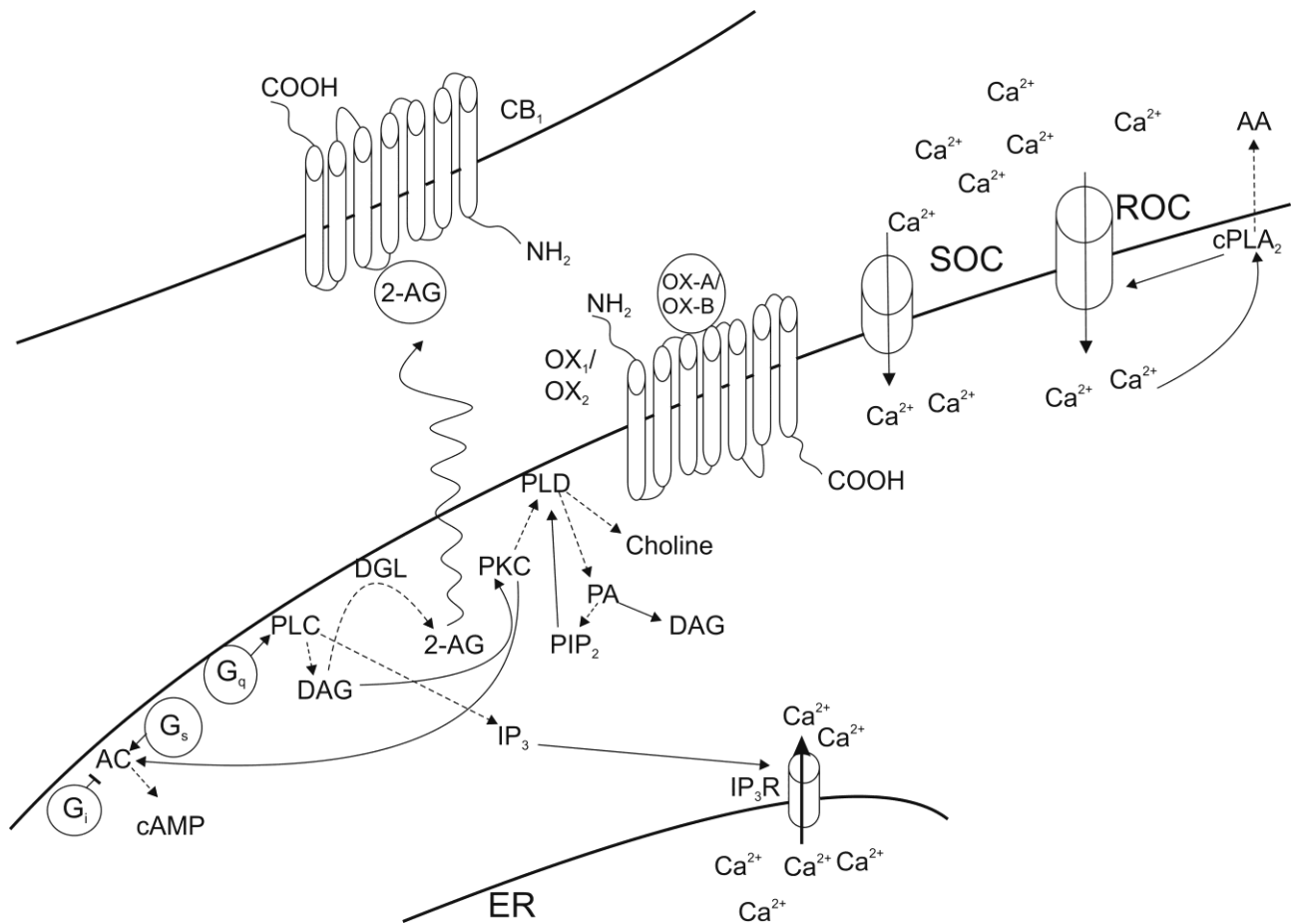


Figure 2. Schematic figure of orexin receptors signalling based on the signalling pathways described in the text. Arrows with solid line indicate activation of enzymes and arrows with dashed line indicate products of enzymatic reactions. Blunt end arrows indicate inhibition. The mechanism for the transport of 2-AG over the membrane is unclear, and thus it is indicated here with a wave line. 2-AG, 2-arachidonylglycerol; CB₁, cannabinoid receptor type 1; cPLA₂, cytosolic phospholipase A₂; DGL, diacylglycerol lipase; ER, endoplasmic reticulum; IP₃R, inositol triphosphate receptor; OX₁, OX₁ orexin receptor; OX₂, OX₂ orexin receptor; OX-A, orexin-A; OX-B, orexin-B; PA, phosphatidic acid; PIP₂, phosphatidylinositol-4,5-biphosphate; PLD, phospholipase D; ROC, receptor-operated calcium channel; SOC, store-operated calcium channel.

2.3.5. Physiological functions of orexins

As orexin peptide and receptor expression is detected in multiple peripheral tissues, orexins can be expected to have several different physiological functions. Some of the physiological functions for orexins were identified already at the time orexins were found and some later using genetically modified animal models (Sakurai et al., 1998; Kukkonen, 2013b).

2.3.5.1. Regulation of energy homeostasis

Sakurai and his co-workers were the first researchers to detect that central administration of orexin-A causes an increase in appetite in rats (Sakurai et al., 1998). That effect, as well as fasting-

stimulated appetite, can be blocked with the OX₁-specific antagonist SB-334867 (Haynes et al., 2000). Later, a link between orexin neuron activity and blood glucose level has been shown (Burdakov et al., 2005). When the blood glucose level is low, production of orexins is up-regulated, and during the high blood glucose level, orexin production decreases (Tsuneki et al., 2012). Thus orexins can stimulate appetite when necessary and that way participate in short-term regulation of energy homeostasis (Kukkonen et al., 2002). In addition to sensing glucose, orexin neurons also can sense dietary amino acids, and especially nonessential amino acids can potently activate orexinergic neurons (Karnani et al., 2011). In addition to stimulation of orexin neurons by low glucose level, orexin neurons can be excited by ghrelin, which is an appetite-stimulator, and inhibited by leptin, which can regulate energy homeostasis in multiple ways (Scammell and Winrow, 2011). Orexin neurons also directly interact with neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons, which are involved in stimulation and inhibition of feeding, respectively. The ability of orexin neurons to activate NPY neurons and inhibit POMC neurons partly causes the feeding-stimulating effect of orexin neurons (Muroya et al., 2004). However, all the responses seen with respect to regulation of energy homeostasis are from studies where orexin is administered to the animals. Thus we cannot be sure, whether the same physiological responses are seen after “native” orexin release.

2.3.5.2. Regulation of sleep-wakefulness cycle

A high activity of orexin neurons is detected in areas involved in the regulation of arousal states. That indicates a central role of orexins in the control of sleep-wakefulness cycle, and indeed stimulation of orexin neurons has been shown to increase arousal (Hagan et al., 1999). Orexins have been shown to excite several monoaminergic systems, including noradrenergic, dopaminergic, histaminergic and serotonergic neurons, and these neurons are speculated to mediate the arousal-promoting effects of orexins (Hagan et al., 1999; Nakamura et al., 2000; Liu et al., 2002; Yamanaka et al., 2002). The histaminergic neurons seem to be the most important target for the orexinergic system as it is reported that stimulation of histaminergic neurons via OX₂, is the main approach by which the orexinergic system promotes arousal (Yamanaka et al., 2002). Increased rapid eye movement (REM) sleep is seen in prepro-orexin knockout mice, which also show other symptoms of narcolepsy (Chemelli et al., 1999; Kukkonen, 2013b). As orexin has been shown to be a central regulator of arousal states, orexin receptor-targeting drugs could play an important role in the treatment of sleep disorders like narcolepsy and insomnia.

2.3.5.3. Regulation of thermogenesis

Brown adipose tissue (BAT) is the main heat producer in rodents and it has significant role in maintaining body temperature (Morrison et al., 2012; Kukkonen, 2013b). Thermogenesis in BAT is controlled by the sympathetic nervous system. In addition to thermoregulation, BAT is involved in the regulation of body weight (Morrison et al., 2012). Interestingly, orexin-deficient mice are obese although they eat less than control individuals, and that has been suggested to be caused by impaired thermogenesis in BAT (Hara et al., 2001; Sellayah et al., 2011). The impaired thermogenesis is speculated to be caused by the inability of BAT cells to differentiate in the absence of orexins (Sellayah et al., 2011). The defective brown adipocytes cannot, for instance, accumulate triglycerides. The impaired thermogenesis is not necessarily the only explanation for the obesity of

orexin-deficient mice, however, as the obesity is found to also be dependent on the genetic background and environmental factors (Hara et al., 2005).

2.3.5.4. Regulation of reward system

Narcolepsy, a neurological disorder likely caused in humans by reduction in the number of orexin-producing neurons, is typically treated with psychostimulants like amphetamine or methylphenidate (Mitler and Hayduk, 2002). These drugs typically cause strong addiction but, interestingly, in narcoleptic patients no addiction is usually detected (Tsujino and Sakurai, 2013). Orexin neurons receive projections from the ventral tegmental area and nucleus accumbens, both of which are important areas in the reward system and thus also in addiction (Harris et al., 2005). The orexinergic system has been shown to have an important role especially in food and drug rewards (Mahler et al., 2012).

Orexin receptor antagonists, like the OX₁-specific SB-334867 and the OX₂-specific JNJ-10397049, have been shown to cause reduction of ethanol self-administration in alcohol-preferring rats (Lawrence et al., 2006; Shoblock et al., 2011). Orexins are also involved in the alcohol relapse process, and alcohol seeking activates orexin neurons (Dayas et al., 2008). Blocking OX₁ with SB-334867 has been shown to reduce alcohol relapse (Dhaher et al., 2010). Taken together, orexin seems to have a role in the development of addiction. Prevention of the relapse to drug seeking thus offers a new target for orexinergic drugs (Perez-Leighton et al., 2013).

2.3.5.5. Other functions

Intracerebroventricular injection of orexin-A and -B induces stress-like symptoms, like increased heart rate and blood pressure (Shirasaka et al., 1999; Smith et al., 2002). Orexins can also increase typical stress behaviour in rats, and the behaviour can be blocked with SB-334867 (Duxon et al., 2001). This phenomenon may be based on the ability of orexins to regulate the release of several hormones involved in stress response (Kukkonen et al., 2002; Scammell and Winrow, 2011; Kukkonen, 2013b). These hormones include, for instance, noradrenalin, glucocorticoids, and vasopressin.

Orexin-A has also shown to be an effective anti-nociceptive and anti-hyperalgesic regulator at least in rodents. Intracerebroventricular or intravenous injection of orexin-A increases analgesia in the hotplate test and the effect can be blocked with the OX₁-specific antagonist SB-334867 (Bingham et al., 2001). Antinociceptive effect of orexin may occur through, or together with, adenosine or endocannabinoid signalling (Kukkonen, 2013b).

2.3.6. Pathological states associated with malfunction of orexinergic system

As the orexinergic system regulates many important physiological functions, it is easy to understand that malfunction of orexin-producing or orexin-responsive neurons may cause severe conditions. Some of these pathological conditions are more obvious, like narcolepsy, but some are less well studied, like the possible involvement of the orexinergic system in neurodegenerative disorders.

2.3.6.1. *Narcolepsy*

Narcolepsy is a sleep disorder characterised by excessive daytime sleepiness and rapid transition from wakefulness into REM sleep (Chemelli et al., 1999; Mitler and Hayduk, 2002). Narcoleptic patients may also express cataplexy attacks, meaning that they can suddenly lose their muscle tone, usually after some emotional situation like laughing. Some patients also suffer from parasomnia. The reported prevalence of narcolepsy varies between 0.02 and 0.18% in different countries (Mignot, 1998). In humans, narcolepsy is mostly a sporadic disease although in dogs familial forms have also been found (Lin et al., 1999; Kukkonen, 2013b).

Only a year after the discovery of orexins, there were two studies published that described the involvement of the orexinergic system in narcolepsy in animal models. Prepro-orexin knockout mice showed narcolepsy-like symptoms, like disturbances in REM sleep regulation (Chemelli et al., 1999). That was seen in the night time, which is normally the active time of mice. These mice also showed reduced activity and cataplectic attacks during the night time, when compared to control mice. Another study described that mutations in the OX_2 gene are associated with narcolepsy in dogs. Narcoleptic Labrador retrievers and Doberman pinchers were shown to have deletion of one exon (exon 4 in Doberman pincher and exon 6 in Labrador retriever) in their OX_2 gene (Lin et al., 1999). Deletions lead to a reading frame shift in the mRNA for the OX_2 gene and formation of a premature stop codon.

In human narcoleptics, a reduced level of orexin-A in cerebrospinal fluid can be detected (Nishino et al., 2000; Thannickal et al., 2000). That reduction is caused by death of orexin-producing neurons; up to 90% of orexin neurons may be lost (Thannickal et al., 2000). The reason for the orexin neuron death is still unclear, but certain human leukocyte antigen class II haplotypes are strongly associated with narcolepsy, indicating a possible autoimmune origin of narcolepsy (Chemelli et al., 1999; Kukkonen, 2013b).

2.3.6.2. *Neurodegenerative disorders*

The human prepro-orexin gene is located on chromosome 17q21, which is the site typically associated with neurodegenerative disorders. Especially dementia symptoms of neurodegenerative diseases, like Alzheimer's or Parkinson's disease, are linked to that chromosome (Wijker et al., 1996; Foster et al., 1997; Sakurai et al., 1998). Symptoms of Alzheimer's disease include cognitive disturbances and also narcolepsy-related symptoms like excessive daytime sleepiness and disturbed REM sleep. As in the human narcoleptics, and Alzheimer's disease patients, the numbers of orexin neurons and levels of peptides are significantly decreased (Fronczek et al., 2012). Orexin neurons are not the only neuron type degenerating in Alzheimer's disease, however, as other neurons that are also involved in arousal state regulation are damaged. In addition to Alzheimer's disease, orexin neuron loss has been seen in patients with Huntington's and Parkinson's diseases (Fronczek et al., 2007; Thannickal et al., 2007; Aziz et al., 2008).

2.3.6.3. *Obesity*

Obese humans are also reported to have a lowered level of orexin-A in plasma, even though that has only been shown with the purported unreliable orexin-A-specific antibodies (Adam et al., 2002). However, that may indicate that orexin has a role in energy expenditure. Indeed, mice with no orexin neurons are less spontaneously active than control mice (Chemelli et al., 1999). Orexin-

induced thermogenesis in BAT also increases energy expenditure (Morrison et al., 2012). Increased energy expenditure requires simultaneous activation of orexin receptors in different brain regions, but it remains unknown how this interaction between different regions happens. Interestingly, orexin peptide over-expression protects from the obesity caused by a high-fat diet (Funato et al., 2009). This effect is mediated in particular by OX₂ receptor signalling.

Shorter sleep time and sleep stability are linked with development of obesity in humans (Spiegel et al., 2009). Orexin receptor signalling through multiple brain regions affects our behaviour, like feeding, sleeping and activity. Together with sympathetic nervous activity, which also is regulated by orexins, these regulate our energy expenditure. Thus orexins seem to form a link between arousal and energy homeostasis (Van Cauter and Knutson, 2008).

2.3.7. Orexin receptor pharmacology

As described above, orexin-A has higher binding affinity for OX₁ receptor than orexin-B (Sakurai et al., 1998). One should keep in mind, however, that affinity of orexin peptides also depends on the expression levels of G-proteins and orexin receptors (Kukkonen et al., 2002). If the level of expressed G-proteins and receptors is low, the "productive" ligand-receptor complexes are more scarce. The detected low signal may thus incorrectly be concluded to be caused by low affinity or efficacy.

Molecular determinants for orexin peptide binding and functionality in the orexin receptors have been studied with chimaeric and point-mutated receptors and truncated and alanine-scanned peptides (Darker et al., 2001; Ammoun et al., 2003; Putula et al., 2011b; Tran et al., 2011; Putula and Kukkonen, 2012). The transmembrane helices two, three, and four seem to be the most crucial in determining the orexin receptor subtype selectivity for orexin agonists (Malherbe et al., 2010; Putula et al., 2011b; Tran et al., 2011). Apparently, the same areas are important for antagonist selectivity too (Malherbe et al., 2010; Tran et al., 2011; Heifetz et al., 2012; Putula and Kukkonen, 2012). These studies are more closely discussed in the results and discussion chapter. In orexin-A, the C-terminus is the most important part to contribute to the OX₁ receptor activation and thus binding as well (Darker et al., 2001; Ammoun et al., 2003). That also seems to be the case for the OX₂ receptor, but OX₂ seems to require fewer determinants for orexin-A binding as it is more resistant towards peptide truncation and other mutagenesis (Ammoun et al., 2003). The C-terminal part of the orexin-B is also the most important for the orexin-B-caused receptor activation (Lang et al., 2004).

2.3.7.1. Orexin receptor agonists

Possibilities for studies of orexin agonisms are limited because of the low number of available agonists. There are no published studies of behavioural responses to exogenously administered orexin peptides in humans. It is known, however, that intranasally administered orexin-A can increase cognition in nonhuman primates exposed to sleep deprivation (Deadwyler et al., 2007).

In narcoleptic mouse models, orexin-A administration has been shown to increase wakefulness and decrease cataplectic attacks (Mieda et al., 2004). Thus it is expected that orexin receptor agonists could also have medical use in the treatment of narcolepsy in humans. Neurodegenerative disorders with symptoms like sleep disturbances might be treatable with orexin agonists (see, **2.3.6.2. Neurodegenerative disorders**). Yet another potential use of orexin agonists could be in the treatment of cancer (Voisin et al., 2011). That is because orexin receptor activation

mediates apoptosis in colon cancer and neuroblastoma cells (Rouet-Benzineb et al., 2004; Ammoun et al., 2006b; Voisin et al., 2006). Also obesity, both in humans and in animals, might be treatable with an orexin agonist, as it has been shown that orexin peptide overexpression protects rodents from diet-induced obesity (Funato et al., 2009).

However, all the currently available agonists are peptides, which do not have good properties to be used as drugs. For instance, bioavailability by orally administered peptide drug is low because peptides are quickly inactivated by gastrointestinal enzymes and also large peptides penetrate intestinal membranes poorly (Morishita and Peppas, 2006). Intravenously injected peptides are quickly inactivated and often have poor CNS penetration. There is only one non-peptide orexin agonist reported in a patent application (Yanagisawa, 2010).

There are some synthetic, modified orexin peptides created to study the interaction between receptor and the ligand (Gotter et al., 2012). For instance, [Ala¹¹, D-Leu¹⁵]-orexin-B is a commercially available orexin agonist, suggested to show OX₂-selectivity (Asahi et al., 2003). Only a 10-fold difference in the relative potency between the receptors was seen as compared to orexin-A (Putula et al., 2011a). In addition, there is a small molecule allosteric OX₂ potentiator, which does not bind to the ligand binding pocket of the orexin receptor but may potentiate the response of the receptor to its cognate ligand (Lee et al., 2010).

2.3.7.2. OX₁ receptor-specific antagonists

Most of the orexin receptor antagonists are developed to treat insomnia (Coleman and Renger, 2010; Scammell and Winrow, 2011). Currently, the clinically used hypnotics are mainly based on enhancing the signalling of γ -aminobutyric acid (GABA) (Liu and Wang, 2012). These are involved in many brain functions, however, and interfering with their signalling causes unwanted side effects like confusion, tolerance, drug abuse and morning sedation (Scammell and Winrow, 2011). Using orexin receptor antagonists as hypnotics might involve fewer side effects (Scammell and Winrow, 2011). In addition to insomnia, orexin antagonists might find use, at least, in the treatment of eating disorders or addiction (Coleman and Renger, 2010; Scammell and Winrow, 2011; Gotter et al., 2012).

The pharmaceutical company GlaxoSmithKline (GSK) published the first OX₁ receptor-selective antagonist, 1-(2-methylbenzoxanzol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride (SB-334867), in 2001 (Smart et al., 2001). The binding affinity of SB-334867 favours OX₁ with a 50-fold difference compared to OX₂, and thus high doses of this antagonist may result in the blocking of both receptor subtypes (Smart et al., 2001; Scammell and Winrow, 2011). Sometimes SB-334867 is also detected to work as a weak agonist (Kukkonen, 2013b). Some other concerns include that SB-334867 has been reported to interact with at least seven other targets in addition to OX₁ receptor (Gotter et al., 2012). Recently SB-334867 was reported to be readily hydrolysed in acidic and basic conditions (McElhinny et al., 2012). However, as SB-334867 has favourable pharmacokinetic properties and easy availability commercially, it is a popular tool when studying orexin receptor signalling and animal behaviour *in vivo* (Gotter et al., 2012). It has been reported, for instance, to reduce alcohol consumption and food intake in rodents (Haynes et al., 2002; Jupp et al., 2011). In addition to SB-334867, GSK has also developed related OX₁-specific antagonists, like SB-408124, SB-410220, and SB-674042 (Scammell and Winrow, 2011).

2.3.7.3. *OX₂ receptor-specific antagonists*

Hoffmann-La Roche has published a highly OX₂-selective antagonist, *N*-ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulphonyl)-amino]-*N*-pyridin-3-ylmethyl-acetamide (EMPA), which shows up to 900-fold selectivity when compared to OX₁. It has reported to reduce physical activity in rodents, but no other physiological effects are published so far (Malherbe et al., 2009a). Johnson & Johnson has developed an OX₂-specific antagonist, 1-(2,4-dibromophenyl)-3-[(4*S*,5*S*)-2,2-dimethyl-4-phenyl-1,3-dioxan-5-yl]urea (JNJ-10397049), which displays about 600-fold selectivity for OX₂, and was shown to increase sleep in rats (McAtee et al., 2004; Dugovic et al., 2009). JNJ-10397049 has also been shown to reduce extracellular histamine concentration in the lateral hypothalamus, which may be the way the compound can promote sleep (Dugovic et al., 2009).

2.3.7.4. *Dual orexin receptor antagonists*

OX₁ knockout mice do not show signs of disturbed sleep regulation, but deletion of OX₂ causes increased sleepiness (Scammell and Winrow, 2011). When both receptor subtypes are deleted, mice display symptoms very similar to narcolepsy (Willie et al., 2003). Thus, it can be speculated that OX₂ receptor-specific antagonists could be fairly effective sleep promoters, but an antagonist, which blocks both receptor subtypes, could be the best way to treat insomnia (Scammell and Winrow, 2011). Actually, development of dual orexin receptor antagonists (DORAs) seems to be the highest priority in pharmaceutical companies, when it comes to finding new drugs targeting orexin receptors.

GSK has developed a DORA called SB-649868. It was shown to be an effective and well-tolerated sleep promoter in men with primary insomnia (Bettica et al., 2012). The drug entered phase II studies but its development was later halted because of toxicity (Gotter et al., 2012).

Actelion originally developed another promising DORA called (2*R*)-2-[(1*S*)-6,7-dimethoxy-1-(2-[4-(trifluoromethyl)phenyl]ethyl)-3,4-dihydroisoquinolin-2(1*H*)-yl]-*N*-methyl-2-phenylethanamide (almorexant). Later, GSK started to collaborate with Actelion in this project (Coleman and Renger, 2010). Almorexant promotes sleep when administered orally to healthy animals and humans during the active period of the circadian cycle (Brisbare-Roch et al., 2007; Malherbe et al., 2009b). In human trials, almorexant was shown to be a very effective sleep inducer in people with primary insomnia (Hoever et al., 2012). Development of almorexant continued until phase III, but was recently discontinued due to its tolerability profile (Gotter et al., 2012).

Merck has also produced some promising DORA compounds: DORA-1 and DORA-5 were reported to increase sleep in rodents (Gotter et al., 2012). The most promising DORA by Merck, and also the most promising orexin antagonist so far, is [(7*R*)-4-(5-Chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2*H*-1,2,3-triazol-2-yl)phenyl]methanone (suvorexant) (also known as MK-4305). Suvorexant is orally bioavailable and also has good brain penetration (Cox et al., 2010). In rodents, dogs and monkeys, it has been shown to reduce physically active time and increase REM sleep (Cox et al., 2010; Herring et al., 2012). Suvorexant is well-tolerated in humans and it has been shown to be an effective sleep inducer among people with primary insomnia (Herring et al., 2012; Sun et al., 2013). The phase III studies of suvorexant are now completed, and new drug application for suvorexant was recently under the review by U.S. Food and Drug Administration (FDA). However, FDA did not accept the application as such but wants to decrease the dosing of suvorexant (<http://www.mercknewsroom.com/press-release/research-and->

development-news/merck-receives-complete-response-letter-suvorexant-merck). In any case, suvorexant has the potential to become the first orexin receptor targeting drug on the market.

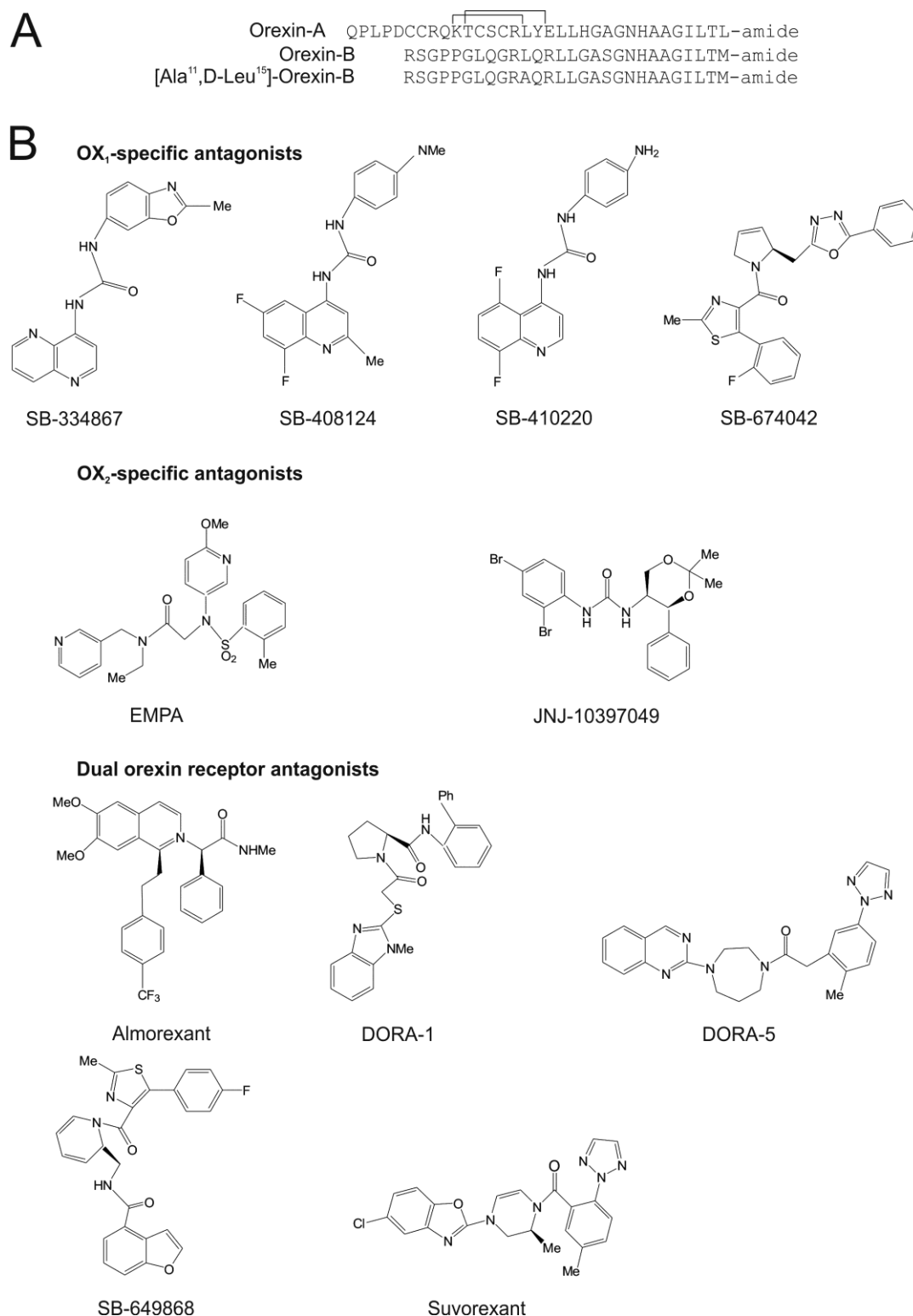


Figure 3. A) Alignment of orexin-A, orexin-B and [Ala¹¹, D-Leu¹⁵]-orexin-B amino acid sequences. Disulfide bonds of orexin-A are marked. The peptides are C-terminally amidated and the first N-terminal glutamine of orexin-A is cyclised to pyroglutamyl. The fifteenth amino acid of [Ala¹¹, D-Leu¹⁵]-orexin-B is substituted from L-leucine to D-leucine. B) Structures of the orexin receptor antagonists mentioned in the text.

3. Aims of the study

At the commencement of this doctoral thesis, there were no studies published on the molecular determinants of orexin receptors needed for ligand binding or selectivity. Thus, orexin receptor chimaeras were created to map the selectivity-conferring areas for orexin agonists and antagonist. In the later part of these studies the attempt was to optimise an orexin binding assay and use it, along with other methods, to study the impact of Ca^{2+} concentration to orexin ligand-receptor interaction and orexin signal transduction. The aims of the studies overall were:

- 1) To map the molecular determinants of the orexin receptor needed for the selectivity of orexin agonists orexin-A, orexin-B, and [Ala¹¹, D-Leu¹⁵]-orexin-B (Papers I and II).
- 2) To map the molecular determinants of the orexin receptors needed for the OX₁-selectivity of the antagonist SB-334867 (Paper III).
- 3) To study the impact of extracellular Ca^{2+} in the binding of orexin-A to OX₁ receptor and in the enzymatic activity of PLC and AC (Paper IV).

4. Material and methods

4.1. Cell culture (Papers I-IV)

CHO cells expressing human OX₁ (Papers I and IV) or human OX₂ receptor (Paper I) were cultured in Ham's F-12 medium supplemented with 100 U/ml penicillin, 80 U/ml streptomycin, 10% (v/v) fetal calf serum (FCS), 2.5 mM L-glutamine, and 10 mM Hepes (Hepes only in Paper IV). G418 (400 µg/ml) was used as a selection marker. HEK-293 human embryonic kidney cell line was used in Papers I, II, and III. HEK-293 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml penicillin, 80 U/ml streptomycin, 10% FCS, 2 mM UltraGlutamine, and 15 mM Hepes. Both CHO and HEK-293 cells were cultured at 37 °C in 5% CO₂ in an air-ventilated humidified incubator.

For the Ca^{2+} assay (Papers I and II), CHO and HEK-293 cells were cultured on 96-well polystyrene plates, which were pretreated for coating with polyethyleneimine (PEI; 25 µg/ml, 1 h, 37 °C) for HEK-293 cells. For the binding and SPA assays (Paper IV) CHO-OX₁ cells were cultured on 48-well plates or 96-well SPA plates similarly pretreated with PEI. For the IP and cAMP release assays CHO-OX₁ cells were culture on PEI-treated 48-well plates (Paper IV) and HEK-293 cells were cultured on 56-cm² dishes (Paper III).

4.2. Experimental media (Papers I-III)

The standard experimental medium in all studies is called Hepes-buffered medium (HBM; Papers I-III) or Na⁺-based medium (NaBM; Paper IV). Independent of the name, it has the same composition of 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 10 mM glucose, 2.5 mM probenecid, and 20 mM Hepes; pH adjusted to 7.4 with NaOH. For final experiments, HBM/NaBM was usually supplemented with 1 mM CaCl₂ (Papers I-IV) and sometimes with Ca^{2+} and/or EGTA to obtain lower Ca^{2+} concentrations (Paper IV). For the PLC assay, it was supplemented with 10 mM LiCl₂. In K⁺-based medium (KBM), all Na⁺ salts in NaBM were replaced with corresponding K⁺ salts; this medium was mixed with regular HBM in

a ratio 0.86:0.14, respectively, to yield a medium called High-K⁺-NaBM with a K⁺-concentration of 127 mM and 1 mM CaCl₂ (Paper IV). In TEA-NaBM, 70 mM of NaCl of HBM was replaced with tetraethylammonium chloride (TEA) and 1 mM CaCl₂ was added.

4.3. Construction of the orexin receptor chimaeras (Papers I–III)

In the first round of mutagenesis (creation of chimaeras Ch1–6, see figure 4), polymerase chain reaction (PCR) fragments of OX₁ and OX₂ receptors were created. Next, these fragments were combined to create two-part receptor chimaeras with the N-terminus of the receptor protein from the OX₁ and C-terminus of the receptor protein from the OX₂ receptor, and *vice versa*. Chimaeric receptors did not include amino acid changes except the stop codon was mutated to GGA to allow the fusion of the receptor with C-terminal GFP. In the second round of mutagenesis, four additional, three-part chimaeras (Ch7–10, Figure 4) were created utilising the native restriction sites in the chimaeras Ch1–6. OX₁-GFP and OX₂-GFP receptors were also reconstructed from the chimaeras Ch1–6. For additional information on the construction including the second round of chimaeras (Ch7–10), see Papers II and III (Putula et al., 2011b; Putula and Kukkonen, 2012).

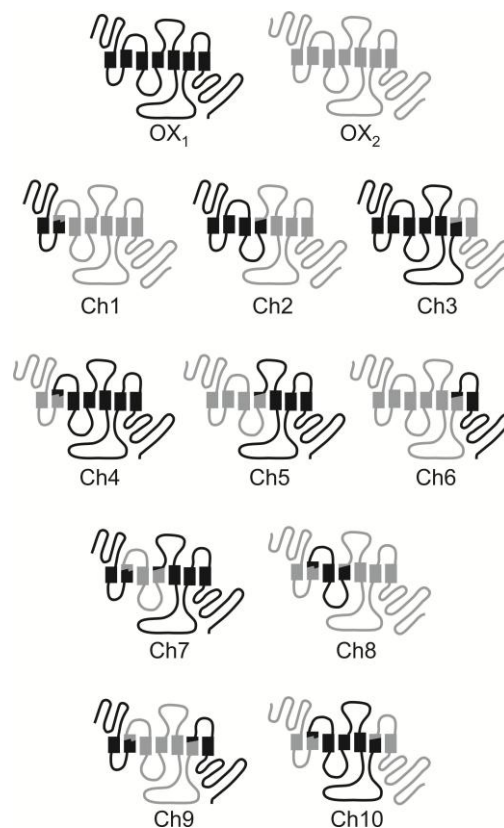


Figure 4. Orexin receptor chimaeras constructed from OX₁ and OX₂ receptors. Reprinted from FEBS Letters, 585(9), Putula J, Turunen PM, Johansson L, Näsman J, Ra R, Korhonen L and Kukkonen JP, Orexin/hypocretin receptor chimaeras reveal structural features important for orexin peptide distinction, 1368-1374, © 2011, with permission from Elsevier (adapted from Putula et al., 2011b).

4.4. Transfections (Papers I–III)

Fugene HD was used to transiently transfect HEK-293 cells with chimaeric orexin receptors. The cells were grown to 40–50% confluence on the 96-well plates (Papers I and II) or 56-cm² dishes (Paper III) and transfected with 0.1 µg DNA and 0.25 µl Fugene HD per well or 10 µg DNA and 25 µl Fugene HD per dish in Opti-MEM. The cells were transfected 48 h prior to the experiments. Transfection efficiency was checked with a fluorescence microscope before the experiments.

4.5. Ca²⁺ assay (Papers I and II)

FLIPR Calcium 4 Assay Kit was used for the calcium measurements in the CHO cells. FLIPR medium was prepared by dissolving FLIPR Calcium 4 Assay Kit in Hank's balanced salt solution supplemented with 20 mM Hepes and 5 mM probenecid. 50 µl of this solution was added to the CHO cells present in a 96-well plate in 50 µl of cell culture medium. The cells were incubated for one hour at 37 °C. For HEK-293 cells, fluo-4 acetoxymethyl ester was used. After 48 h of transfection, the cell culture medium was removed and 2 µM fluo-4 in HBM, supplemented with 1 mM CaCl₂, was added to the cells. The cells were incubated for one hour at 37 °C. After the incubation, the cells were washed twice with the same HBM. Both CHO and HEK-293 cells were measured with the FlexStation automated fluorescence plate reader at 37 °C utilising the wavelengths 485 nm (excitation) and 525 nm (emission). Each well was measured for 120 seconds and the ligand was added at 30 seconds. The data was analysed and visualised in Microsoft Excel.

4.6. IP release assay (Papers III and IV)

In Paper III, HEK-293 cells were transiently transfected with chimaeric orexin receptors, and then loaded with 5 µCi/ml of [³H]myo-inositol after 24 hours. For the chimaeras with low expression, 10 µCi/ml was used for the loading. After 24 h loading, cells were washed with phosphate-buffered saline (PBS), detached with PBS containing 0.2 g/l EDTA, spun down and resuspended in HBM, which contained 10 mM LiCl. After a 10 min incubation with the LiCl, the cells were dispensed to a 96-well plate, which already contained orexin-A (0–1000 nM) and SB-334867 (0–10 000 nM). The plate was stimulated for 30 minutes at 37 °C. Reactions were stopped by centrifugation (700 g, 1 min, 4 °C) and rapid removal of the medium. 100 µl of cold 0.4 M perchloride acid was added to each well and the plate was frozen down.

In Paper IV, intact CHO cells stably expressing OX₁ receptor were used. The cells, cultured on PEI-coated 48-well plates, were loaded with 3 µCi/ml of [³H]myo-inositol. After a 24-h loading, the medium was removed and cells were washed with PBS. Next, 100 µl of one of the following media, all containing 10 mM LiCl and 0.1% BSA, to make the assay comparable with the binding assay, was added to the cells: HBM/NaBM containing variable concentrations of CaCl₂ (100 nM–1 mM), HBM/NaBM + 1 mM CaCl₂ containing 10 µM SKF-96365 (SOC inhibitor), TEA-NaBM (ROC inhibitor, 70 mM final TEA-concentration) or high-K⁺-NaBM (the final K⁺-concentration was 127 mM). High K⁺ concentration depolarises the cells and thus reduces the driving force for Ca²⁺ influx. Addition of the LiCl-containing buffer was followed by 10-min incubation at 37 °C and then addition of orexin-A in 25 µl volume. The plate was incubated for 10-min at 37 °C. The reaction was stopped by rapid removal of the medium. 100 µl of cold 0.4 M perchloride acid was added to each well and the plate was frozen down.

After thawing the samples (both Papers III and IV), 50 µl of 0.36 M potassium hydroxide / 0.3 M potassium bicarbonate mixture was added to neutralise the samples. The plates were centrifuged for 10 min (400 g, 4 °C), and samples were loaded to BioRad AG 1-X8-anion-exchange chromatography columns. Columns were washed with 8 ml of water and 8 ml of mixture of 5 mM Na₂-tetraborate and 60 mM NH₄-formate. The total inositol phosphate fraction was eluted with 4 ml of mixture of 0.1M formic acid and 1M NH₄-formate. 8 ml of HiSafe 3 scintillation cocktail was added to the samples for counting with Wallac 1414 liquid scintillation counter.

4.7. Binding assay (Paper IV)

Optimisation of the binding assay was started by testing how [¹²⁵I]-orexin-A sticks to different filter materials. [¹²⁵I]-orexin-A was vacuumed through empty glass fiber (GF/A and GF/B), polyvinylidene fluoride (PVDF), mixed cellulose esters or nitrocellulose filters and radioactivity from the filters was determined with a Wallac Wizard 1480 γ-counter. The smallest amount of non-specific binding occurred in glass fiber and PVDF filters and thus different coatings were further tested for these filter materials only. 0.5% PEI, 0.1% bovine serum albumin (BSA), 5% milk, and 0.5% polyvinylpyrrolidone (PVP) + 0.1% Tween 20 were tested for coatings. The coating solution was sucked once through the filter and then added on top of the filters to keep them constantly wet. Coating was continued for 1 h at room temperature and the filters were washed once with HBM before applying 150 µl of 0.1 nM [¹²⁵I]-orexin-A. After filtering the samples, the filters were washed three times with 2 ml of HBM before moving to γ-counter tubes for measurement.

Suspended cells: One 10-cm dish of CHO-hOX₁ cells was detached and cells were pelleted by centrifugation (400 g, 5 min). The cell pellet was suspended in NaBM, containing 0.1% BSA. 50 µl of NaBM or SB-334867 (in the final concentration of 10 µM) and 50 µl of 0.02 nM [¹²⁵I]-orexin-A (supplemented with “cold” orexin-A to achieve the final orexin concentration of 0.2 nM) was added to multiwell plates or eppendorf tubes, depending on whether filter-bottom plates or individual membrane filters were used to separate orexin receptor bound label from the non-bound. The reaction was started by addition of 50 µl of cell suspension and the samples were incubated for 90 min at room temperature.

Intact cells: 24 h after plating the CHO-hOX₁ cells on PEI-coated 48-well plates, the cell medium was removed. 100 µl of one of the following buffers was added to the cells: NaBM supplemented with different concentrations of CaCl₂ (100 nM–1 mM), TEA-NaBM or high-K⁺-NaBM (see, 4.6. *Inositol phosphate release assay*), either with or without 10 µM SB-334867. The cell plate was incubated for a few minutes at 37 °C to warm it up. 50 µl of 0.02 or 0.025 nM [¹²⁵I]-orexin-A, supplemented with “cold” orexin-A to reach the final orexin concentrations of 1 or 10 nM, respectively, was added to the cells. The cells were incubated for 10 min at 37 °C. After that, the cells were rapidly washed once with cold NaBM. Finally, the cells were detached with 0.2 M NaOH and moved to γ-counter tubes for measurement.

4.8. Scintillation proximity assay (Paper IV)

Scintillation proximity assay (SPA) was tested in two different ways: with a conventional way, where the scintillation is measured directly from the wells without separation, and with a manner, where the incubation medium is carefully removed but the wells are not washed. The latter method

seemed to work better in our case as it yielded lower non-specific binding and higher signal-to-noise ratio. Also incubation time and temperature was easier to control as there was no need for overlapping incubation times.

CHO-OX₁ cells were cultured on a Cytostar-T SPA plate overnight. On the following day, the cells were washed with PBS and left in 50 µl of NaBM containing different CaCl₂ concentrations (100 nM–1 mM), 20 µM SB-334867 in NaBM or high-K⁺-NaBM. 50 µl of 0.02 or 0.025 nM ¹²⁵I-orexin-A, supplemented with “cold” orexin-A to get the final orexin concentrations of 1 or 10 nM, respectively, was added to the cells. The cells were incubated for 10 min at room temperature. For the conventional method, the plate was sealed with a clear cover tape and counted with Wallac Microbeta Trilux liquid scintillation counter. For the alternative method, the medium was quickly and completely removed after the incubation. The plate was then sealed for counting.

4.9. cAMP release assay (Paper IV)

CHO-hOX₁ cells were cultured on PEI-coated 48-well plates. In some experiments, signaling of the G_i-protein was inhibited with pertussis toxin. In these cases, a day after seeding the cells, the medium was replaced with medium, which contained 100 ng/ml of pertussis toxin. On the following day - or a day after seeding the cells when no pertussis toxin was used - the cells were loaded with 5 µCi/ml of [³H]-adenine for two hours. 0.5 mM isobutylmethylxanthine was dissolved in NaBM with the aid of bath sonication, and the NaBM was supplemented with different concentrations of CaCl₂ (10–1000 µM) and 0.1% BSA. 3 µM of the PKC inhibitor GF-109203X was also included into this buffer solution, to prevent the activation of AC by PKC and thus exclude the signaling of the G_q-protein (Holmqvist et al., 2005). After loading of the cells with [³H]-adenine, the medium was removed and the cells were washed once with PBS. 100 µl of NaBM, including isobutylmethylxanthine and GF-109203X, was added to the cells and the cells were incubated for 10 min at 37 °C. 25 µl of 10 µM forskolin, diluted in NaBM, or a mixture of orexin-A (0.1–1000 nM) and forskolin, was added to the wells. The cells were incubated for an additional 10 min at 37 °C. After that, the supernatant was discarded and 100 µl of cold 0.33 M perchloride acid was added instead. The plates were frozen down.

Dowex columns (Dowex-50W-X8, 200–400 mesh) were washed with 20 ml of water before use. After thawing the samples, the cell debris was centrifuged down (1100 x g, 10min), and the samples were loaded to the Dowex columns and 0.33 M perchloride acid was added to give a total volume of 1 ml. ATP and ADP fractions were eluted with 2 ml of water into scintillation vials. 5 ml of HiSafe 3 scintillation cocktail was added to the vials for counting with Wallac 1414 liquid scintillation counter. Alumina columns, prepared from neutral Al₂O₃, were washed with 8 ml of 0.1 M imidazole. After the ATP and ADP fractions from the Dowex columns were eluted, Dowex columns were placed on top of the washed alumina columns. AMP and cAMP were eluted into the alumina columns with 10 ml of water. cAMP was eluted from the alumina columns into big scintillation vials with 4 ml of 0.1 M imidazole. 8 ml of HiSafe 3 scintillation cocktail was added to the vials before counting. After use, Dowex columns were washed with 2 ml of 1 M hydrochloric acid and 8 ml of water and stored in 0.1 M hydrochloric acid. Alumina columns were washed with 10 ml of 0.1 M imidazole and stored in 0.1 M imidazole.

5. Results and discussion

5.1. Molecular determinants for orexin agonist distinction (Paper II)

Orexin receptors could serve as potential drug targets in the treatment of multiple pathological states like cancer, addiction or narcolepsy. However, the lack of information of the molecular determinants contributing to the ligand binding and functional activity hinders ligand development. We used chimaeric orexin receptors to map the orexin receptor areas important for orexin peptide distinction. We measured the elevation of intracellular Ca^{2+} after activating chimaeric orexin receptors with orexin-A, orexin-B, and [Ala¹¹, D-Leu¹⁵]-orexin-B. As orexin-A should show no selectivity between the OX₁ and OX₂ receptors, it served as an internal control.

Exchanging the first quarter of the receptor, from the amino terminus to TM2 (Ch1 and Ch4, figure 4), or the last quarter, from TM6 to the carboxyl-terminus (Ch3 and Ch6), caused no significant changes in the agonist profile of OX₁ or OX₂ receptors. In contrast, when the amino terminal half, from the amino terminus to TM4 (Ch2 and Ch5), of the receptor was changed, the agonist profile dramatically shifted to largely follow the profile of the donor of the N-terminus. These results suggested that the second quarter of the orexin receptor, from TM2 to TM4, contains the most important determinants of the agonist selectivity. This was confirmed with chimeras, where only the second quarter was changed (Ch7 and Ch8). Indeed, exchange of the region between these two transmembrane helices only, was enough to change the agonist profile of the receptors. TM2 and TM3 have been found to be crucial in determining the orexin receptor subtype selectivity for orexin peptides also by other groups. Especially residues Q134 in the TM3 of OX₂ receptor and V130 in the TM3 of OX₁ receptor are needed for the functional activity and binding of orexin peptides (Malherbe et al., 2010; Tran et al., 2011). Certain residues in TM5 were also important for the binding and functional activity of orexin-A (Tran et al., 2011).

The second quarter of the receptor is not the only determinant for agonist distinction as none of the chimaeras in this study displayed a fully similar agonist profile to OX₁. Interestingly, the “subtype-selective” agonists, orexin-B and [Ala¹¹, D-Leu¹⁵]-orexin-B, showed differences in their receptor activation profiles. That was readily seen with the chimaera, where the second quarter of the OX₂ was inserted in the OX₁ receptor (Ch7). That chimaera was indistinguishable from OX₂ with respect to orexin-B but showed reduced potency for [Ala¹¹, D-Leu¹⁵]-orexin-B. The opposite chimaera, where the second quarter of the OX₁ was inserted in the OX₂ receptor (Ch8), showed very low expression and thus the functionality of the chimaera is uncertain.

For the analysis of the results, it should be considered that the results relate to the potency of orexin-A, which is supposed to be non-selective for OX₁ and OX₂ receptors. This idea is partially based on comparison to orexin-B and partially on the apparently similar potency of orexin-A in cell lines expressing either receptor subtype. However, the results of Malherbe et al. (2010) suggest that similar expression level of OX₁ and OX₂ produces a similar orexin-A potency; thus its intrinsic efficacy may be similar for both receptor subtypes. Nevertheless, it is possible that in some of the chimaeras, the actual affinity/potency of orexin-A is affected distorting the comparison.

5.2. Molecular determinants of OX₁ antagonist SB-334867 binding (Paper III)

The same orexin receptor chimaera approach used in the Paper II was also used to characterise the molecular determinants involved in the functional activity of the OX₁ receptor-specific antagonist SB-334867. As the fluorescence plate reader was no longer accessible, inositol phosphate release was now used as a read-out. Similar to the agonists, the second quarter of the orexin receptor seems to be the most important area involved in the high affinity interaction with SB-334867. The third quarter, from TM4 to the TM6, also contributes in the binding, as was confirmed with the chimaeras Ch9 and Ch10.

For another OX₁ receptor-specific antagonist, SB-674042, and for non-selective orexin receptor antagonist, almorexant, the most crucial residues for OX₁ receptor binding are located in TM3, TM5, TM7, and in the second extracellular loop (Malherbe et al., 2010). Two residues in TM3, T135 and V138, were found to be most critical for the selectivity of the OX₂ receptor-selective antagonist EMPA. In another study, TM3 was found to be critical for orexin receptor interaction with small molecule antagonists (Tran et al., 2011). TM3 was more closely studied with homology modelling and site-directed mutagenesis followed by molecular dynamics simulations (Heifetz et al., 2012). Differences in the amino acid sequences of the TM3 in the OX₁ and OX₂ receptors affect the interactions of TM3 with other TMs. These structural differences in TM3 may underlie the differences in ligand binding between orexin receptors. Even though the selectivity-conferring areas of the receptor for antagonists binding are partly conserved, each antagonist seems to also interact with specific amino acids, as mutations of certain residues dramatically affect the binding and functionality of one antagonist but not the others (Malherbe et al., 2010; Tran et al., 2011; Heifetz et al., 2012).

The agonists (Paper II) and antagonist (Paper III) in our studies did not show similar results in all respects. With agonists, none of the chimaeras displayed a fully similar profile to OX₁, but with SB-334867, several chimeras showed binding of equally high affinity as OX₁. Instead, with the chimaeras it was difficult to obtain as low binding affinity as SB-334867 for the OX₂ receptor. This indicates that, in addition to the second quarter of the orexin receptor, there might be other areas also involved in the interaction of agonists and antagonists with the receptor.

5.3. Impact of the expression system on orexin agonist discrimination (Paper I)

Some interesting properties of orexin-B and [Ala¹¹, D-Leu¹⁵]-orexin-B were observed in the study described in Paper II. Firstly, [Ala¹¹, D-Leu¹⁵]-orexin-B (as compared to orexin-A) only showed 23-fold selectivity for OX₂ receptor in HEK-293 cells. This is far from the 400-fold selectivity originally reported (Asahi et al., 2003). Secondly, in CHO cells, the selectivity was only 14-fold. Even lower and also variable selectivity was seen with orexin-B, which showed 9- and 2.6-fold selectivity for OX₂ in HEK-293 and CHO cells, respectively. The most obvious reason for the difference between the cell lines is biased agonism, meaning that orexin receptor could have multiple conformations, each of which can differently interact with an agonist (Kenakin, 2012). Biased agonism of orexin is also suggested by alanine-scanned orexin peptides, which display differences in the activation of Ca²⁺ influx and release (Ammoun et al., 2003). Distinct

heteromerisation partners or signal transduction machinery, like diverse G-protein levels, can also impact the differences between cell lines (Kukkonen, 2013b).

5.4. Optimisation of an orexin binding assay (unpublished)

As part of a co-operative project to find novel orexin-receptor-binding molecules, pharmacological analysis of molecules selected by *in silico* screening is underway. Thus, the development of an orexin binding assay, which could be used for this project, was pursued here. One binding assay for orexin was already described at the time orexins were found (Sakurai et al., 1998). However, instead of intact cells, a new assay for suspended cells, based on filtering, was sought, as this could enable faster sample analysis. Both native orexin peptides can be bought labelled with ^{125}I , and both show high non-specific binding to different surfaces (previous findings). In previous findings, orexin-A is suggested to be less "sticky", and thus this was chosen to test. Also, it has better availability and applicability to both OX_1 and OX_2 receptors, in contrast to orexin-B. Binding of ^{125}I -orexin-A to plastic ware was reduced with the use of special low binding pipette tips (TipOne RPT low retention tips, Starlab) and by adding 0.1% BSA in the NaBM. Different materials (glass fiber, polyvinylidene fluoride, mixed cellulose esters and nitrocellulose) and coatings (non-fat milk, PEI, BSA, PVP-Tween) were tested in order to reduce binding to filters. Even though some materials, especially polyvinylidene fluoride filter coated with BSA, appeared promising, the method seemed to require so much hands-on work, that attached cells were ultimately used. CHO-h OX_1 cells attached on a multiwell plate also yielded better results than any of the filter materials tested. The ultimate protocol only differed from that originally described with respect to incubation time (10 min instead of 90 min), the amount of BSA (0.1% instead of 0.5%) and the number of washing steps (one step instead of three). Ten minutes does not yield equilibrium binding, but it also causes very little internalisation of the receptors (Jäntti et al., 2013).

5.5. Impact of Ca^{2+} on orexin binding and PLC activity (Paper IV)

Elevation of intracellular Ca^{2+} can be observed after orexin receptor activation (see, 2.3.4.1. *Elevation of the intracellular calcium concentration*) and reduction of extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$) reduces this elevation (Smart et al., 1999; Lund et al., 2000; Ammoun et al., 2003). This is seen as a shift in the concentration-response curve for orexin. This is not the only orexin response to be affected, but instead, for instance, PLA_2 , PLC, and extracellular signal-regulated kinase (ERK) responses are affected by reduced $[\text{Ca}^{2+}]_e$ (Lund et al., 2000; Ammoun et al., 2006a; Johansson et al., 2007; Turunen et al., 2010). Reduction of extracellular Ca^{2+} also reduces the concentration of intracellular Ca^{2+} (Lund et al., 2000; Ammoun et al., 2003) As, at least, PLC and c PLA_2 are known to require Ca^{2+} for their activity, their attenuated activation could be explained by reduced intracellular Ca^{2+} concentration (Kukkonen, 2013a). In addition to the enzymatic activity, extracellular Ca^{2+} may also be needed for orexin to bind to its receptor. Thus extracellular Ca^{2+} could regulate orexin signalling at multiple levels, i.e. at the level of orexin binding and, at some level of orexin receptor signalling. As this is still largely unclear, the impacts of Ca^{2+} on orexin-A binding to OX_1 receptor and on orexin-induced PLC activity were studied.

PLC activity and ^{125}I -orexin-A binding to OX_1 was first assessed under different $[\text{Ca}^{2+}]_e$ (100 nM – 1 mM). For the binding studies, we used both above described conventional binding

assay and SPA. Both orexin-A binding to OX₁ and elevation of PLC after OX₁ activation were significantly decreased after reduction of extracellular Ca²⁺. The concentration-relationship curves of Ca²⁺ were highly similar for both binding and PLC activity, indicating that reduced binding could fully explain the reduced PLC activity. It has previously been observed, however, that PLC activity can also be reduced with pharmacological inhibitors of ROCs and SOCs or by reducing the driving force for Ca²⁺ influx (Johansson et al., 2007). Inhibition of ROCs with TEA-NaBM had a stronger impact on PLC activity than inhibition of SOCs with SKF-96365. Even though this has been investigated earlier (Johansson et al., 2007), we now used a shorter 10 min activation time to match the assays. The impact of membrane depolarisation on orexin-A binding and PLC activity was tested with high-K⁺-NaBM. Depolarisation reduced PLC activity much more strongly than binding. In addition, inhibition of the ROCs with TEA-NaBM had a more pronounced effect on the PLC activity than on binding. Altogether, the results may indicate that extracellular Ca²⁺ plays a dual role with respect to the OX₁ receptor; Ca²⁺ is necessary for orexin-A binding to OX₁ receptor but also its influx is needed to act on PLC or other targets, which influences PLC activity.

PLC isoforms may be directly affected by Ca²⁺ (see, **2.3.4.3. Regulation of lipid signalling**). Therefore the impact of Ca²⁺ availability/influx is more difficult to analyse for orexin receptors, i.e. it is difficult to judge whether Ca²⁺ affect PLC directly or some orexin receptor signalling component, in addition to the binding. We therefore turned our focus on the AC regulation. It has previously been shown that AC is regulated in multiple ways (by G_s- and G_i-proteins and G_q → PLC → PKC pathway) in CHO cells expressing OX₁ receptor (see, **2.3.4.2. Regulation of the activity of adenylyl cyclase**). When the PKC response is eliminated by an inhibitor, pure G_s (low potency) and G_i (high potency) responses, which are not directly affected by Ca²⁺, can be seen. We thus measured the response via G_i (cells treated with forskolin and the PKC inhibitor, GF109203X). The response was even more strongly affected by reduction of [Ca²⁺]_e from 1 mM to 100 μM and 10 μM than the PLC response. That indicates that the impact of extracellular Ca²⁺ is not only carried by the binding or some direct effect on the orexin receptor target enzymes. We also assessed the G_s response (cells treated with pertussis toxin, forskolin, and GF109203X). Although the response cannot be seen at such low orexin-A concentrations as the binding, PLC stimulation, and AC inhibition, it also seemed to verify the conclusion by being strongly affected by reduction of [Ca²⁺]_e.

6. Conclusions and future directions

Some of the factors determining orexin receptor ligand selectivity and binding properties were studied in this thesis. First, we roughly mapped the molecular determinants needed for the orexin peptide distinction with chimaeric orexin receptors. We found the second quarter of the receptor, from the TM2 to TM4, to be the most important part of the orexin receptor needed for the agonist selectivity. However, none of the chimaeric receptors displayed fully similar profile to OX₁ indicating that the second quarter is not the only determinant. Similar to the OX₁-selective antagonist, SB-334867, the second quarter of the orexin receptor is the most crucial in determining the selectivity. The third quarter of the receptor was also shown to be able to contribute to the functional selectivity. The chimaeric receptor results should assist in the computer modelling of the orexin receptors. Valid computer models would improve drug discovery and thus increase the chances to find novel orexin receptor binding molecules.

We also studied the impact of extracellular calcium for orexin binding and signal transduction. We showed that reduction of extracellular calcium concentration clearly reduced the binding of ¹²⁵I-orexin-A to the OX₁ receptor and enzymatic activities of PLC and AC after OX₁ receptor stimulation. The exact target for Ca²⁺ in orexin receptor-peptide interaction should be more closely studied as this could offer helpful information also for drug discovery of orexin receptor targeting drugs. For instance, as probably at least two different PLC isoforms are involved in orexin signalling, the impact of Ca²⁺ should be studied separately for these isoforms. In addition, before the effects of extracellular Ca²⁺ on orexin binding and signalling can be fully distinguished, it is important to find more selective ways to inhibit the orexin receptor-operated calcium influx.

We found that the “OX₂-selective” agonist [Ala¹¹, D-Leu¹⁵]-orexin-B showed much lower selectivity for the OX₂ receptor than originally reported. In addition, the selectivity was dependent on the cell type and was lower in CHO than in HEK cells. This was concluded to be caused by signal trafficking. Therefore this “orexin subtype-selective” agonist should not be used for receptor subtype determination. Signal trafficking should also be considered when new orexin receptor agonists are screened.

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8. References

- Adam JA, Menheere PP, van Dielen FM, Soeters PB, Buurman WA, Greve JW (2002) Decreased plasma orexin-A levels in obese individuals. *Int J Obes Relat Metab Disord* 26:274-276.
- Adjobo-Hermans MJ, Goedhart J, Gadella TW, Jr. (2006) Plant G protein heterotrimers require dual lipidation motifs of Galpha and Ggamma and do not dissociate upon activation. *J Cell Sci* 119:5087-5097.
- Aittaleb M, Boguth CA, Tesmer JJ (2010) Structure and function of heterotrimeric G protein-regulated Rho guanine nucleotide exchange factors. *Mol Pharmacol* 77:111-125.
- Alexander SP, Mathie A, Peters JA (2011) Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* 164 Suppl 1:S1-324.
- Allen M, Reeves J, Mellor G (2000) High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. *J Biomol Screen* 5:63-69.
- Ammoun S, Holmqvist T, Shariatmadari R, Oonk HB, Detheux M, Parmentier M, Åkerman KE, Kukkonen JP (2003) Distinct recognition of OX1 and OX2 receptors by orexin peptides. *J Pharmacol Exp Ther* 305:507-514.
- Ammoun S, Johansson L, Ekholm ME, Holmqvist T, Danis AS, Korhonen L, Sergeeva OA, Haas HL, Åkerman KE, Kukkonen JP (2006a) OX1 orexin receptors activate extracellular signal-regulated kinase (ERK) in CHO cells via multiple mechanisms: The role of Ca²⁺ influx in OX1 receptor signaling. *Mol Endocrinol* 20:80-99.
- Ammoun S, Lindholm D, Wootz H, Åkerman KE, Kukkonen JP (2006b) G-protein-coupled OX1 orexin/hcrtr-1 hypocretin receptors induce caspase-dependent and -independent cell death through p38 mitogen-/stress-activated protein kinase. *J Biol Chem* 281:834-842.
- Anderson GP, King KD, Gaffney KL, Johnson LH (2000) Multi-analyte interrogation using the fiber optic biosensor. *Biosens Bioelectron* 14:771-777.
- Asahi S, Egashira S, Matsuda M, Iwaasa H, Kanatani A, Ohkubo M, Ihara M, Morishima H (2003) Development of an orexin-2 receptor selective agonist, [Ala(11), D-Leu(15)]orexin-B. *Bioorg Med Chem Lett* 13:111-113.
- Aziz A, Fronczek R, Maat-Schieman M, Unmehopa U, Roelandse F, Overeem S, van Duinen S, Lammers GJ, Swaab D, Roos R (2008) Hypocretin and melanin-concentrating hormone in patients with Huntington disease. *Brain Pathol* 18:474-483.
- Becker OM, Marantz Y, Shacham S, Inbal B, Heifetz A, Kalid O, Bar-Haim S, Warshaviak D, Fichman M, Noiman S (2004) G protein-coupled receptors: in silico drug discovery in 3D. *Proc Natl Acad Sci U S A* 101:11304-11309.
- Belluscio L, Gold GH, Nemes A, Axel R (1998) Mice deficient in G(olf) are anosmic. *Neuron* 20:69-81.
- Benovic JL, Pike LJ, Cerione RA, Staniszewski C, Yoshimasa T, Codina J, Caron MG, Lefkowitz RJ (1985) Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. *J Biol Chem* 260:7094-7101.
- Berridge MJ (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 1793:933-940.
- Bettica P, Squassante L, Zamuner S, Nucci G, Danker-Hopfe H, Ratti E (2012) The orexin antagonist SB-649868 promotes and maintains sleep in men with primary insomnia. *Sleep* 35:1097-1104.
- Bickle M (2010) The beautiful cell: high-content screening in drug discovery. *Anal Bioanal Chem* 398:219-226.

- Biddlecome GH, Berstein G, Ross EM (1996) Regulation of phospholipase C-beta1 by Gq and m1 muscarinic cholinergic receptor. Steady-state balance of receptor-mediated activation and GTPase-activating protein-promoted deactivation. *J Biol Chem* 271:7999-8007.
- Bingham S, Davey PT, Babbs AJ, Irving EA, Sammons MJ, Wyles M, Jeffrey P, Cutler L, Riba I, Johns A, Porter RA, Upton N, Hunter AJ, Parsons AA (2001) Orexin-A, an hypothalamic peptide with analgesic properties. *Pain* 92:81-90.
- Brisbare-Roch C, Dingemans J, Koberstein R, Hoeber P, Aissaoui H, Flores S, Mueller C, Nayler O, van Gerven J, de Haas SL, Hess P, Qiu C, Buchmann S, Scherz M, Weller T, Fischli W, Clozel M, Jenck F (2007) Promotion of sleep by targeting the orexin system in rats, dogs and humans. *Nat Med* 13:150-155.
- Bunemann M, Frank M, Lohse MJ (2003) Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci U S A* 100:16077-16082.
- Burdakov D, Gerasimenko O, Verkhratsky A (2005) Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons in situ. *J Neurosci* 25:2429-2433.
- Cerione RA, Codina J, Benovic JL, Lefkowitz RJ, Birnbaumer L, Caron MG (1984) The mammalian beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* 23:4519-4525.
- Chan JS, Lee JW, Ho MK, Wong YH (2000) Preactivation permits subsequent stimulation of phospholipase C by G(i)-coupled receptors. *Mol Pharmacol* 57:700-708.
- Chandrashekar J, Mueller KL, Hoon MA, Adler E, Feng L, Guo W, Zuker CS, Ryba NJ (2000) T2Rs function as bitter taste receptors. *Cell* 100:703-711.
- Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98:437-451.
- Chen L, Jin L, Zhou N (2012) An update of novel screening methods for GPCR in drug discovery. *Expert Opin Drug Discov* 7:791-806.
- Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F (2010) Luciferase Reporter Assay System for Deciphering GPCR Pathways. *Curr Chem Genomics* 4:84-91.
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258-1265.
- Chisari M, Saini DK, Kalyanaraman V, Gautam N (2007) Shuttling of G protein subunits between the plasma membrane and intracellular membranes. *J Biol Chem* 282:24092-24098.
- Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, Schiöth HB (2013) G protein-coupled receptor deorphanizations. *Annu Rev Pharmacol Toxicol* 53:127-146.
- Cluderay JE, Harrison DC, Hervieu GJ (2002) Protein distribution of the orexin-2 receptor in the rat central nervous system. *Regul Pept* 104:131-144.
- Cohen GB, Oprian DD, Robinson PR (1992) Mechanism of activation and inactivation of opsin: role of Glu113 and Lys296. *Biochemistry* 31:12592-12601.
- Coleman PJ, Renger JJ (2010) Orexin receptor antagonists: a review of promising compounds patented since 2006. *Expert Opin Ther Pat* 20:307-324.
- Colombo M, Carregal-Romero S, Casula MF, Gutierrez L, Morales MP, Böhm IB, Heverhagen JT, Prospero D, Parak WJ (2012) Biological applications of magnetic nanoparticles. *Chem Soc Rev* 41:4306-4334.
- Cooper MA (2002) Optical biosensors in drug discovery. *Nat Rev Drug Discov* 1:515-528.
- Costa T, Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* 86:7321-7325.

- Cottet M, Faklaris O, Zwier JM, Trinquet E, Pin JP, Durroux T (2011) Original Fluorescent Ligand-Based Assays Open New Perspectives in G-Protein Coupled Receptor Drug Screening. *Pharmaceuticals* 4:202-214.
- Cox CD, Breslin MJ, Whitman DB, Schreier JD, McGaughey GB, Bogusky MJ, Roecker AJ, Mercer SP, Bednar RA, Lemaire W, Bruno JG, Reiss DR, Harrell CM, Murphy KL, Garson SL, Doran SM, Prueksaritanont T, Anderson WB, Tang C, Roller S, Cabalu TD, Cui D, Hartman GD, Young SD, Koblan KS, Winrow CJ, Renger JJ, Coleman PJ (2010) Discovery of the dual orexin receptor antagonist [(7R)-4-(5-chloro-1,3-benzoxazol-2-yl)-7-methyl-1,4-diazepan-1-yl][5-methyl-2-(2H -1,2,3-triazol-2-yl)phenyl]methanone (MK-4305) for the treatment of insomnia. *J Med Chem* 53:5320-5332.
- Crespo I, Gomez de Heras R, Rodriguez de Fonseca F, Navarro M (2008) Pretreatment with subeffective doses of Rimonabant attenuates orexigenic actions of orexin A-hypocretin 1. *Neuropharmacology* 54:219-225.
- Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 390:88-91.
- Daniels JS, Pourmand N (2007) Label-Free Impedance Biosensors: Opportunities and Challenges. *Electroanalysis* 19:1239-1257.
- Darker JG, Porter RA, Eggleston DS, Smart D, Brough SJ, Sabido-David C, Jerman JC (2001) Structure-activity analysis of truncated orexin-A analogues at the orexin-1 receptor. *Bioorg Med Chem Lett* 11:737-740.
- Date Y, Mondal MS, Matsukura S, Nakazato M (2000) Distribution of orexin-A and orexin-B (hypocretins) in the rat spinal cord. *Neurosci Lett* 288:87-90.
- Dayas CV, McGranahan TM, Martin-Fardon R, Weiss F (2008) Stimuli linked to ethanol availability activate hypothalamic CART and orexin neurons in a reinstatement model of relapse. *Biol Psychiatry* 63:152-157.
- de Jong LA, Uges DR, Franke JP, Bischoff R (2005) Receptor-ligand binding assays: technologies and applications. *J Chromatogr B Analyt Technol Biomed Life Sci* 829:1-25.
- de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 95:322-327.
- De Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG (2000) The regulator of G protein signaling family. *Annu Rev Pharmacol Toxicol* 40:235-271.
- Deadwyler SA, Porrino L, Siegel JM, Hampson RE (2007) Systemic and nasal delivery of orexin-A (Hypocretin-1) reduces the effects of sleep deprivation on cognitive performance in nonhuman primates. *J Neurosci* 27:14239-14247.
- Dey D, Goswami T (2011) Optical biosensors: a revolution towards quantum nanoscale electronics device fabrication. *J Biomed Biotechnol* 2011:348218.
- Dhafer R, Hauser SR, Getachew B, Bell RL, McBride WJ, McKinzie DL, Rodd ZA (2010) The Orexin-1 Receptor Antagonist SB-334867 Reduces Alcohol Relapse Drinking, but not Alcohol-Seeking, in Alcohol-Preferring (P) Rats. *J Addict Med* 4:153-159.
- Digby JE, Chen J, Tang JY, Lehnert H, Matthews RN, Randeve HS (2006) Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B. *J Endocrinol* 191:129-136.
- Dryja TP, Berson EL, Rao VR, Oprian DD (1993) Heterozygous missense mutation in the rhodopsin gene as a cause of congenital stationary night blindness. *Nat Genet* 4:280-283.
- Dugovic C, Shelton JE, Aluisio LE, Fraser IC, Jiang X, Sutton SW, Bonaventure P, Yun S, Li X, Lord B, Dvorak CA, Carruthers NI, Lovenberg TW (2009) Blockade of orexin-1 receptors attenuates orexin-2 receptor antagonism-induced sleep promotion in the rat. *J Pharmacol Exp Ther* 330:142-151.

- Duxon MS, Stretton J, Starr K, Jones DN, Holland V, Riley G, Jerman J, Brough S, Smart D, Johns A, Chan W, Porter RA, Upton N (2001) Evidence that orexin-A-evoked grooming in the rat is mediated by orexin-1 (OX1) receptors, with downstream 5-HT_{2C} receptor involvement. *Psychopharmacology (Berl)* 153:203-209.
- Ellis J, Padiani JD, Canals M, Milasta S, Milligan G (2006) Orexin-1 receptor-cannabinoid CB1 receptor heterodimerization results in both ligand-dependent and -independent coordinated alterations of receptor localization and function. *J Biol Chem* 281:38812-38824.
- Exner T, Jensen ON, Mann M, Kleuss C, Nurnberg B (1999) Posttranslational modification of Galphao1 generates Galphao3, an abundant G protein in brain. *Proc Natl Acad Sci U S A* 96:1327-1332.
- Fan F, Wood KV (2007) Bioluminescent assays for high-throughput screening. *Assay Drug Dev Technol* 5:127-136.
- Fang Y (2011) Label-Free Receptor Assays. *Drug Discov Today Technol* 7:e5-e11.
- Fields TA (1998) Identification of a GTPase activating protein specific for the heterotrimeric G protein, Gz. *Cell Signal* 10:43-48.
- Foster NL, Wilhelmsen K, Sima AA, Jones MZ, D'Amato CJ, Gilman S (1997) Frontotemporal dementia and parkinsonism linked to chromosome 17: a consensus conference. Conference Participants. *Ann Neurol* 41:706-715.
- Frang H, Mukkala VM, Syysto R, Ollikka P, Hurskainen P, Scheinin M, Hemmila I (2003) Nonradioactive GTP binding assay to monitor activation of g protein-coupled receptors. *Assay Drug Dev Technol* 1:275-280.
- Fronczek R, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, Lammers GJ, Swaab DF (2007) Hypocretin (orexin) loss in Parkinson's disease. *Brain* 130:1577-1585.
- Fronczek R, van Geest S, Frolich M, Overeem S, Roelandse FW, Lammers GJ, Swaab DF (2012) Hypocretin (orexin) loss in Alzheimer's disease. *Neurobiol Aging* 33:1642-1650.
- Funato H, Tsai AL, Willie JT, Kisanuki Y, Williams SC, Sakurai T, Yanagisawa M (2009) Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. *Cell Metab* 9:64-76.
- Gabriel D, Vernier M, Pfeifer MJ, Dasen B, Tenailon L, Bouhelal R (2003) High throughput screening technologies for direct cyclic AMP measurement. *Assay Drug Dev Technol* 1:291-303.
- Ghil S, Choi JM, Kim SS, Lee YD, Liao Y, Birnbaumer L, Suh-Kim H (2006) Compartmentalization of protein kinase A signaling by the heterotrimeric G protein Go. *Proc Natl Acad Sci U S A* 103:19158-19163.
- Giannone F, Malpeli G, Lisi V, Grasso S, Shukla P, Ramarli D, Sartoris S, Monsurro V, Krampera M, Amato E, Tridente G, Colombatti M, Parenti M, Innamorati G (2010) The puzzling uniqueness of the heterotrimeric G15 protein and its potential beyond hematopoiesis. *J Mol Endocrinol* 44:259-269.
- Gotter AL, Webber AL, Coleman PJ, Renger JJ, Winrow CJ (2012) International Union of Basic and Clinical Pharmacology. LXXXVI. Orexin receptor function, nomenclature and pharmacology. *Pharmacol Rev* 64:389-420.
- Graminski GF, Jayawickreme CK, Potenza MN, Lerner MR (1993) Pigment dispersion in frog melanophores can be induced by a phorbol ester or stimulation of a recombinant receptor that activates phospholipase C. *J Biol Chem* 268:5957-5964.
- Gruber CW, Muttenthaler M, Freissmuth M (2010) Ligand-based peptide design and combinatorial peptide libraries to target G protein-coupled receptors. *Curr Pharm Des* 16:3071-3088.
- Hadcock JR, Ros M, Watkins DC, Malbon CC (1990) Cross-regulation between G-protein-mediated pathways. Stimulation of adenylyl cyclase increases expression of the inhibitory G-protein, Gi alpha 2. *J Biol Chem* 265:14784-14790.

- Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, Benham CD, Taylor SG, Routledge C, Hemmati P, Munton RP, Ashmeade TE, Shah AS, Hatcher JP, Hatcher PD, Jones DN, Smith MI, Piper DC, Hunter AJ, Porter RA, Upton N (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci U S A* 96:10911-10916.
- Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, Desale H, Clemons B, Cahalan SM, Schuerer SC, Sanna MG, Han GW, Kuhn P, Rosen H, Stevens RC (2012) Crystal structure of a lipid G protein-coupled receptor. *Science* 335:851-855.
- Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* 30:345-354.
- Hara J, Yanagisawa M, Sakurai T (2005) Difference in obesity phenotype between orexin-knockout mice and orexin neuron-deficient mice with same genetic background and environmental conditions. *Neurosci Lett* 380:239-242.
- Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* 437:556-559.
- Harrison C, Traynor JR (2003) The [35S]GTPgammaS binding assay: approaches and applications in pharmacology. *Life Sci* 74:489-508.
- Haynes AC, Chapman H, Taylor C, Moore GB, Cawthorne MA, Tadayyon M, Clapham JC, Arch JR (2002) Anorectic, thermogenic and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice. *Regul Pept* 104:153-159.
- Haynes AC, Jackson B, Chapman H, Tadayyon M, Johns A, Porter RA, Arch JR (2000) A selective orexin-1 receptor antagonist reduces food consumption in male and female rats. *Regul Pept* 96:45-51.
- Heckmann D, Kessler H (2007) Design and chemical synthesis of integrin ligands. *Methods Enzymol* 426:463-503.
- Heifetz A, Morris GB, Biggin PC, Barker O, Fryatt T, Bentley J, Hallett D, Manikowski D, Pal S, Reifegerste R, Slack M, Law R (2012) Study of human Orexin-1 and -2 G-protein-coupled receptors with novel and published antagonists by modeling, molecular dynamics simulations, and site-directed mutagenesis. *Biochemistry* 51:3178-3197.
- Helms JB (1995) Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles. *FEBS Lett* 369:84-88.
- Herring WJ, Snyder E, Budd K, Hutzelmann J, Snavelly D, Liu K, Lines C, Roth T, Michelson D (2012) Orexin receptor antagonism for treatment of insomnia: a randomized clinical trial of suvorexant. *Neurology* 79:2265-2274.
- Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA (2001) Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. *Neuroscience* 103:777-797.
- Hessler G, Zimmermann M, Matter H, Evers A, Naumann T, Lengauer T, Rarey M (2005) Multiple-ligand-based virtual screening: methods and applications of the MTree approach. *J Med Chem* 48:6575-6584.
- Heuss C, Gerber U (2000) G-protein-independent signaling by G-protein-coupled receptors. *Trends Neurosci* 23:469-475.
- Heuss C, Scanziani M, Gähwiler BH, Gerber U (1999) G-protein-independent signaling mediated by metabotropic glutamate receptors. *Nat Neurosci* 2:1070-1077.
- Hill SJ (2006) G-protein-coupled receptors: past, present and future. *Br J Pharmacol* 147 Suppl 1:S27-37.
- Ho YC, Lee HJ, Tung LW, Liao YY, Fu SY, Teng SF, Liao HT, Mackie K, Chiou LC (2011) Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to

- antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. *J Neurosci* 31:14600-14610.
- Hoeffer P, Dorffner G, Benes H, Penzel T, Danker-Hopfe H, Barbanj MJ, Pillar G, Saletu B, Polo O, Kunz D, Zeitlhofer J, Berg S, Partinen M, Bassetti CL, Hogl B, Ebrahim IO, Holsboer-Trachsler E, Bengtsson H, Peker Y, Hemmeter UM, Chiossi E, Hajak G, Dingemans J (2012) Orexin receptor antagonism, a new sleep-enabling paradigm: a proof-of-concept clinical trial. *Clin Pharmacol Ther* 91:975-985.
- Holmqvist T, Åkerman KE, Kukkonen JP (2002) Orexin signaling in recombinant neuron-like cells. *FEBS Lett* 526:11-14.
- Holmqvist T, Johansson L, Östman M, Ammoun S, Åkerman KE, Kukkonen JP (2005) OX1 orexin receptors couple to adenylyl cyclase regulation via multiple mechanisms. *J Biol Chem* 280:6570-6579.
- Huang HC, Klein PS (2004) The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biol* 5:234.
- Hughes JP, Rees S, Kalindjian SB, Philpott KL (2011) Principles of early drug discovery. *Br J Pharmacol* 162:1239-1249.
- Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I, Niki H (2002) Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? *Neurosci Res* 44:121-131.
- Insel PA, Snead A, Murray F, Zhang L, Yokouchi H, Katakia T, Kwon O, Dimucci D, Wilderman A (2012) GPCR expression in tissues and cells: are the optimal receptors being used as drug targets? *Br J Pharmacol* 165:1613-1616.
- Jääntti MH, Putula J, Somerharju P, Frohman MA, Kukkonen JP (2011) OX(1) orexin/hypocretin receptor activation of phospholipase D. *Br J Pharmacol* 165:1109-1123.
- Jääntti MH, Putula J, Turunen PM, Nasman J, Reijonen S, Lindqvist C, Kukkonen JP (2013) Autocrine Endocannabinoid Signaling through CB1 Receptors Potentiates OX1 Orexin Receptor Signaling. *Mol Pharmacol* 83:621-632.
- Jenkins GM, Frohman MA (2005) Phospholipase D: a lipid centric review. *Cell Mol Life Sci* 62:2305-2316.
- Ji TH, Grossmann M, Ji I (1998) G protein-coupled receptors. I. Diversity of receptor-ligand interactions. *J Biol Chem* 273:17299-17302.
- Johansson L, Ekholm ME, Kukkonen JP (2007) Regulation of OX(1) orexin/hypocretin receptor-coupling to phospholipase C by Ca(2+) influx. *Br J Pharmacol* 150:97-104.
- Johansson L, Ekholm ME, Kukkonen JP (2008) Multiple phospholipase activation by OX(1) orexin/hypocretin receptors. *Cell Mol Life Sci* 65:1948-1956.
- Johren O, Neidert SJ, Kummer M, Dendorfer A, Dominiak P (2001) Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. *Endocrinology* 142:3324-3331.
- Jupp B, Krivdic B, Krstew E, Lawrence AJ (2011) The orexin(1) receptor antagonist SB-334867 dissociates the motivational properties of alcohol and sucrose in rats. *Brain Res* 1391:54-59.
- Karnani MM, Apergis-Schoute J, Adamantidis A, Jensen LT, de Lecea L, Fugger L, Burdakov D (2011) Activation of central orexin/hypocretin neurons by dietary amino acids. *Neuron* 72:616-629.
- Karteris E, Chen J, Rande HS (2004) Expression of human prepro-orexin and signaling characteristics of orexin receptors in the male reproductive system. *J Clin Endocrinol Metab* 89:1957-1962.
- Kawabe J, Iwami G, Ebina T, Ohno S, Katada T, Ueda Y, Homcy CJ, Ishikawa Y (1994) Differential activation of adenylyl cyclase by protein kinase C isoenzymes. *J Biol Chem* 269:16554-16558.

- Kearns BG, McGee TP, Mayinger P, Gedvilaite A, Phillips SE, Kagiwada S, Bankaitis VA (1997) Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature* 387:101-105.
- Kenakin T (2010) Functional Selectivity and Biased Receptor Signaling. *J Pharmacol Exp Ther* 336:296-302.
- Kenakin T (2013) New concepts in pharmacological efficacy at 7TM receptors: IUPHAR review 2. *Br J Pharmacol* 168:554-575.
- Kenakin T, Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* 12:205-216.
- Kenakin TP (2012) Biased signalling and allosteric machines: new vistas and challenges for drug discovery. *Br J Pharmacol* 165:1659-1669.
- Khan SM, Sleno R, Gora S, Zylbergold P, Laverdure JP, Labbe JC, Miller GJ, Hebert TE (2013) The expanding roles of Gbetagamma subunits in G protein-coupled receptor signaling and drug action. *Pharmacol Rev* 65:545-577.
- Kirchgessner AL, Liu M (1999) Orexin synthesis and response in the gut. *Neuron* 24:941-951.
- Klein S, Reuveni H, Levitzki A (2000) Signal transduction by a nondissociable heterotrimeric yeast G protein. *Proc Natl Acad Sci U S A* 97:3219-3223.
- Kobilka BK (2007) G protein coupled receptor structure and activation. *Biochim Biophys Acta* 1768:794-807.
- Kozasa T, Hajicek N, Chow CR, Suzuki N (2011) Signalling mechanisms of RhoGTPase regulation by the heterotrimeric G proteins G12 and G13. *J Biochem* 150:357-369.
- Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* 103:21-80.
- Kukkonen JP (2004) Regulation of receptor-coupling to (multiple) G-proteins. A challenge for basic research and drug discovery. *Receptors Channels* 10:167-183.
- Kukkonen JP (2011) A menage a trois made in heaven: G-protein-coupled receptors, lipids and TRP channels. *Cell Calcium* 50:9-26.
- Kukkonen JP (2013a) Lipid signaling cascades of orexin/hypocretin receptors. *Biochimie*.
- Kukkonen JP (2013b) Physiology of the orexinergic/hypocretinergic system: a revisit in 2012. *Am J Physiol Cell Physiol* 304:C2-32.
- Kukkonen JP, Åkerman KEO (2001) Orexin receptors couple to Ca²⁺ channels different from store-operated Ca²⁺ channels. *Neuroreport* 12:2017-2020.
- Kukkonen JP, Holmqvist T, Ammoun S, Åkerman KE (2002) Functions of the orexinergic/hypocretinergic system. *Am J Physiol Cell Physiol* 283:C1567-C1591.
- Kukkonen JP, Leonard CS (2013) Orexin/hypocretin receptor signalling cascades. *Br J Pharmacol*.
- Lagerstrom MC, Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* 7:339-357.
- Lang M, Soll RM, Durrenberger F, Dautzenberg FM, Beck-Sickinger AG (2004) Structure-activity studies of orexin A and orexin B at the human orexin 1 and orexin 2 receptors led to orexin 2 receptor selective and orexin 1 receptor preferring ligands. *J Med Chem* 47:1153-1160.
- Lawrence AJ, Cowen MS, Yang HJ, Chen F, Oldfield B (2006) The orexin system regulates alcohol-seeking in rats. *Br J Pharmacol* 148:752-759.
- Lee J, Reddy MM, Kodadek T (2010) Discovery of an orexin receptor positive potentiator. *Chemical Science* 1:48-54.
- Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lanca AJ, O'Dowd BF, George SR (2004) Dopamine D1 and D2 receptor Co-activation generates a novel phospholipase C-mediated calcium signal. *J Biol Chem* 279:35671-35678.
- Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* 273:18677-18680.

- Lefkowitz RJ, Roth J, Pastan I (1970) Radioreceptor assay of adrenocorticotrophic hormone: new approach to assay of polypeptide hormones in plasma. *Science* 170:633-635.
- Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. *Science* 308:512-517.
- Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98:365-376.
- Liu AM, Ho MK, Wong CS, Chan JH, Pau AH, Wong YH (2003a) Galpha(16/z) chimeras efficiently link a wide range of G protein-coupled receptors to calcium mobilization. *J Biomol Screen* 8:39-49.
- Liu G, Duranteau L, Carel JC, Monroe J, Doyle DA, Shenker A (1999) Leydig-cell tumors caused by an activating mutation of the gene encoding the luteinizing hormone receptor. *N Engl J Med* 341:1731-1736.
- Liu J, Wang LN (2012) Ramelteon in the treatment of chronic insomnia: systematic review and meta-analysis. *Int J Clin Pract* 66:867-873.
- Liu JJ, Hartman DS, Bostwick JR (2003b) An immobilized metal ion affinity adsorption and scintillation proximity assay for receptor-stimulated phosphoinositide hydrolysis. *Anal Biochem* 318:91-99.
- Liu RJ, van den Pol AN, Aghajanian GK (2002) Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. *J Neurosci* 22:9453-9464.
- Lopez M, Senaris R, Gallego R, Garcia-Caballero T, Lago F, Seoane L, Casanueva F, Dieguez C (1999) Orexin receptors are expressed in the adrenal medulla of the rat. *Endocrinology* 140:5991-5994.
- Lund PE, Shariatmadari R, Uustare A, Detheux M, Parmentier M, Kukkonen JP, Åkerman KEO (2000) The orexin OX1 receptor activates a novel Ca²⁺ influx pathway necessary for coupling to phospholipase C. *J Biol Chem* 275:30806-30812.
- Magalhaes AC, Dunn H, Ferguson SS (2012) Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *Br J Pharmacol* 165:1717-1736.
- Magga J, Bart G, Oker-Blom C, Kukkonen JP, Åkerman KE, Näsman J (2006) Agonist potency differentiates G protein activation and Ca²⁺ signalling by the orexin receptor type 1. *Biochem Pharmacol* 71:827-836.
- Mahler SV, Smith RJ, Moorman DE, Sartor GC, Aston-Jones G (2012) Multiple roles for orexin/hypocretin in addiction. *Prog Brain Res* 198:79-121.
- Malherbe P, Borroni E, Gobbi L, Knust H, Nettekoven M, Pinard E, Roche O, Rogers-Evans M, Wettstein JG, Moreau JL (2009a) Biochemical and behavioural characterization of EMPA, a novel high-affinity, selective antagonist for the OX(2) receptor. *Br J Pharmacol* 156:1326-1341.
- Malherbe P, Borroni E, Pinard E, Wettstein JG, Knoflach F (2009b) Biochemical and electrophysiological characterization of almorexant, a dual orexin 1 receptor (OX1)/orexin 2 receptor (OX2) antagonist: comparison with selective OX1 and OX2 antagonists. *Mol Pharmacol* 76:618-631.
- Malherbe P, Roche O, Marcuz A, Kratzeisen C, Wettstein JG, Bissantz C (2010) Mapping the binding pocket of dual antagonist almorexant to human orexin 1 and orexin 2 receptors: Comparison with the selective OX1 (SB-674042) and OX2 (EMPA) antagonists. *Mol Pharmacol* 78:81-93.
- Mangmool S, Kurose H (2011) G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX). *Toxins (Basel)* 3:884-899.

- Mazzocchi G, Malendowicz LK, Gottardo L, Aragona F, Nussdorfer GG (2001) Orexin A stimulates cortisol secretion from human adrenocortical cells through activation of the adenylate cyclase-dependent signaling cascade. *J Clin Endocrinol Metab* 86:778-782.
- McAtee LC, Sutton SW, Rudolph DA, Li X, Aluisio LE, Phuong VK, Dvorak CA, Lovenberg TW, Carruthers NI, Jones TK (2004) Novel substituted 4-phenyl-[1,3]dioxanes: potent and selective orexin receptor 2 (OX(2)R) antagonists. *Bioorg Med Chem Lett* 14:4225-4229.
- McElhinny CJ, Jr., Lewin AH, Mascarella SW, Runyon S, Brieady L, Carroll FI (2012) Hydrolytic instability of the important orexin 1 receptor antagonist SB-334867: possible confounding effects on in vivo and in vitro studies. *Bioorg Med Chem Lett* 22:6661-6664.
- McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393:333-339.
- Mieda M, Willie JT, Hara J, Sinton CM, Sakurai T, Yanagisawa M (2004) Orexin peptides prevent cataplexy and improve wakefulness in an orexin neuron-ablated model of narcolepsy in mice. *Proc Natl Acad Sci U S A* 101:4649-4654.
- Mignot E (1998) Genetic and familial aspects of narcolepsy. *Neurology* 50:S16-22.
- Millar RP, Newton CL (2010) The year in G protein-coupled receptor research. *Mol Endocrinol* 24:261-274.
- Milligan G, Kostenis E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* 147 Suppl 1:S46-55.
- Milligan G, Marshall F, Rees S (1996) G16 as a universal G protein adapter: implications for agonist screening strategies. *Trends Pharmacol Sci* 17:235-237.
- Milligan G, Rees S (1999) Chimaeric G alpha proteins: their potential use in drug discovery. *Trends Pharmacol Sci* 20:118-124.
- Mitler MM, Hayduk R (2002) Benefits and risks of pharmacotherapy for narcolepsy. *Drug Saf* 25:791-809.
- Morishita M, Peppas NA (2006) Is the oral route possible for peptide and protein drug delivery? *Drug Discov Today* 11:905-910.
- Morrison SF, Madden CJ, Tupone D (2012) An orexinergic projection from perifornical hypothalamus to raphe pallidus increases rat brown adipose tissue thermogenesis. *Adipocyte* 1:116-120.
- Muroya S, Funahashi H, Yamanaka A, Kohno D, Uramura K, Nambu T, Shibahara M, Kuramochi M, Takigawa M, Yanagisawa M, Sakurai T, Shioda S, Yada T (2004) Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca²⁺ signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus. *Eur J Neurosci* 19:1524-1534.
- Murthy KS, Zhou H, Huang J, Pentylala SN (2004) Activation of PLC-delta1 by Gi/o-coupled receptor agonists. *Am J Physiol Cell Physiol* 287:C1679-1687.
- Nagayama S, Fukukawa C, Katagiri T, Okamoto T, Aoyama T, Oyaizu N, Imamura M, Toguchida J, Nakamura Y (2005) Therapeutic potential of antibodies against FZD 10, a cell-surface protein, for synovial sarcomas. *Oncogene* 24:6201-6212.
- Nakabayashi M, Suzuki T, Takahashi K, Totsune K, Muramatsu Y, Kaneko C, Date F, Takeyama J, Darnel AD, Moriya T, Sasano H (2003) Orexin-A expression in human peripheral tissues. *Mol Cell Endocrinol* 205:43-50.
- Nakamura T, Uramura K, Nambu T, Yada T, Goto K, Yanagisawa M, Sakurai T (2000) Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. *Brain Res* 873:181-187.
- Natochin M, Artemyev NO (1998) A single mutation Asp229 --> Ser confers upon Gs alpha the ability to interact with regulators of G protein signaling. *Biochemistry* 37:13776-13780.
- Neves SR, Ram PT, Iyengar R (2002) G protein pathways. *Science* 296:1636-1639.

- Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* 270:28495-28498.
- Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E (2000) Hypocretin (orexin) deficiency in human narcolepsy. *Lancet* 355:39-40.
- Nobles M, Benians A, Tinker A (2005) Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci U S A* 102:18706-18711.
- Offermanns S, Simon MI (1995) G alpha 15 and G alpha 16 couple a wide variety of receptors to phospholipase C. *J Biol Chem* 270:15175-15180.
- Offermanns S, Simon MI (1998) Genetic analysis of mammalian G-protein signalling. *Oncogene* 17:1375-1381.
- Olson KR, Eglen RM (2007) Beta galactosidase complementation: a cell-based luminescent assay platform for drug discovery. *Assay Drug Dev Technol* 5:137-144.
- Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745.
- Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* 365:649-651.
- Parnot C, Miserey-Lenkei S, Bardin S, Corvol P, Clauser E (2002) Lessons from constitutively active mutants of G protein-coupled receptors. *Trends Endocrinol Metab* 13:336-343.
- Peavy RD, Hubbard KB, Lau A, Fields RB, Xu K, Lee CJ, Lee TT, Gernert K, Murphy TJ, Hepler JR (2005) Differential effects of Gq alpha, G14 alpha, and G15 alpha on vascular smooth muscle cell survival and gene expression profiles. *Mol Pharmacol* 67:2102-2114.
- Perez-Leighton CE, Butterick-Peterson TA, Billington CJ, Kotz CM (2013) Role of orexin receptors in obesity: from cellular to behavioral evidence. *Int J Obes (Lond)* 37:167-174.
- Pertwee RG (2006) The pharmacology of cannabinoid receptors and their ligands: an overview. *Int J Obes (Lond)* 30 Suppl 1:S13-18.
- Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3:639-650.
- Pitha J, Milecki J, Czajkowska T, Kusiak JW (1983) beta-Adrenergic antagonists with multiple pharmacophores: persistent blockade of receptors. *J Med Chem* 26:7-11.
- Putula J, Kukkonen JP (2012) Mapping of the binding sites for the OX1 orexin receptor antagonist, SB-334867, using orexin/hypocretin receptor chimaeras. *Neurosci Lett* 506:111-115.
- Putula J, Turunen PM, Jantti MH, Ekholm ME, Kukkonen JP (2011a) Agonist ligand discrimination by the two orexin receptors depends on the expression system. *Neurosci Lett* 494:57-60.
- Putula J, Turunen PM, Johansson L, Nasman J, Ra R, Korhonen L, Kukkonen JP (2011b) Orexin/hypocretin receptor chimaeras reveal structural features important for orexin peptide distinction. *FEBS Lett* 585:1368-1374.
- Rajagopal K, Lefkowitz RJ, Rockman HA (2005) When 7 transmembrane receptors are not G protein-coupled receptors. *J Clin Invest* 115:2971-2974.
- Rebecchi MJ, Pentylala SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev* 80:1291-1335.
- Ritter SL, Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nat Rev Mol Cell Biol* 10:819-830.
- Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* 459:356-363.
- Rouet-Benzineb P, Rouyer-Fessard C, Jarry A, Avondo V, Pouzet C, Yanagisawa M, Laboisie C, Laburthe M, Voisin T (2004) Orexins acting at native OX(1) receptor in colon cancer and neuroblastoma cells or at recombinant OX(1) receptor suppress cell growth by inducing apoptosis. *J Biol Chem* 279:45875-45886.

- Rudolf R, Mongillo M, Rizzuto R, Pozzan T (2003) Looking forward to seeing calcium. *Nat Rev Mol Cell Biol* 4:579-586.
- Sadana R, Dessauer CW (2009) Physiological roles for G protein-regulated adenylyl cyclase isoforms: insights from knockout and overexpression studies. *Neurosignals* 17:5-22.
- Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-585.
- Sakurai T, Moriguchi T, Furuya K, Kajiwara N, Nakamura T, Yanagisawa M, Goto K (1999) Structure and function of human prepro-orexin gene. *J Biol Chem* 274:17771-17776.
- Scammell TE, Saper CB (2005) Orexin, drugs and motivated behaviors. *Nat Neurosci* 8:1286-1288.
- Scammell TE, Winrow CJ (2011) Orexin receptors: pharmacology and therapeutic opportunities. *Annu Rev Pharmacol Toxicol* 51:243-266.
- Scheer A, Fanelli F, Costa T, De Benedetti PG, Cotecchia S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *EMBO J* 15:3566-3578.
- Schneider G, Fechner U (2005) Computer-based de novo design of drug-like molecules. *Nat Rev Drug Discov* 4:649-663.
- Scholich K, Mullenix JB, Wittpoth C, Poppleton HM, Pierre SC, Lindorfer MA, Garrison JC, Patel TB (1999) Facilitation of signal onset and termination by adenylyl cyclase. *Science* 283:1328-1331.
- Seamon KB, Padgett W, Daly JW (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci U S A* 78:3363-3367.
- Sellayah D, Bharaj P, Sikder D (2011) Orexin is required for brown adipose tissue development, differentiation, and function. *Cell Metab* 14:478-490.
- Shimamura T, Shiroishi M, Weyand S, Tsujimoto H, Winter G, Katritch V, Abagyan R, Cherezov V, Liu W, Han GW, Kobayashi T, Stevens RC, Iwata S (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature* 475:65-70.
- Shirasaka T, Nakazato M, Matsukura S, Takasaki M, Kannan H (1999) Sympathetic and cardiovascular actions of orexins in conscious rats. *Am J Physiol* 277:R1780-1785.
- Shoblock JR, Welty N, Aluisio L, Fraser I, Motley ST, Morton K, Palmer J, Bonaventure P, Carruthers NI, Lovenberg TW, Boggs J, Galici R (2011) Selective blockade of the orexin-2 receptor attenuates ethanol self-administration, place preference, and reinstatement. *Psychopharmacology (Berl)* 215:191-203.
- Shoichet BK, Kobilka BK (2012) Structure-based drug screening for G-protein-coupled receptors. *Trends Pharmacol Sci* 33:268-272.
- Shukla AK, Xiao K, Lefkowitz RJ (2011) Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci* 36:457-469.
- Siehler S (2009) Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol* 158:41-49.
- Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. *Science* 252:802-808.
- Smart D, Jerman JC, Brough SJ, Rushton SL, Murdock PR, Jewitt F, Elshourbagy NA, Ellis CE, Middlemiss DN, Brown F (1999) Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br J Pharmacol* 128:1-3.
- Smart D, Sabido-David C, Brough SJ, Jewitt F, Johns A, Porter RA, Jerman JC (2001) SB-334867-A: the first selective orexin-1 receptor antagonist. *Br J Pharmacol* 132:1179-1182.
- Smith PM, Connolly BC, Ferguson AV (2002) Microinjection of orexin into the rat nucleus tractus solitarius causes increases in blood pressure. *Brain Res* 950:261-267.

- Soboloff J, Spassova M, Hewavitharana T, He LP, Luncsford P, Xu W, Venkatachalam K, van Rossum D, Patterson RL, Gill DL (2007) TRPC channels: integrators of multiple cellular signals. *Handb Exp Pharmacol* 575-591.
- Song CH, Xia JX, Ye JN, Chen XW, Zhang CQ, Gao EQ, Hu ZA (2005) Signaling pathways of hypocretin-1 actions on pyramidal neurons in the rat prefrontal cortex. *Neuroreport* 16:1529-1533.
- Spalding TA, Burstein ES (2001) Constitutively active muscarinic receptors. *Life Sci* 68:2511-2516.
- Spiegel K, Tasali E, Leproult R, Van Cauter E (2009) Effects of poor and short sleep on glucose metabolism and obesity risk. *Nat Rev Endocrinol* 5:253-261.
- Steegborn C, Litvin TN, Hess KC, Capper AB, Taussig R, Buck J, Levin LR, Wu H (2005) A novel mechanism for adenylyl cyclase inhibition from the crystal structure of its complex with catechol estrogen. *J Biol Chem* 280:31754-31759.
- Stoyanova, II, Rutten WL, le Feber J (2010) Orexin-A and orexin-B during the postnatal development of the rat brain. *Cell Mol Neurobiol* 30:81-89.
- Suh BC, Hille B (2005) Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. *Curr Opin Neurobiol* 15:370-378.
- Summers RJ (2012) Themed section: molecular pharmacology of GPCRs. *Br J Pharmacol* 165:1609-1612.
- Sun H, Kennedy WP, Wilbraham D, Lewis N, Calder N, Li X, Ma J, Yee KL, Ermlich S, Mangin E, Lines C, Rosen L, Chodakewitz J, Murphy GM (2013) Effects of suvorexant, an orexin receptor antagonist, on sleep parameters as measured by polysomnography in healthy men. *Sleep* 36:259-267.
- Taipale J, Chen JK, Cooper MK, Wang B, Mann RK, Milenkovic L, Scott MP, Beachy PA (2000) Effects of oncogenic mutations in Smoothed and Patched can be reversed by cyclopamine. *Nature* 406:1005-1009.
- Taussig R, Iniguez-Lluhi JA, Gilman AG (1993) Inhibition of adenylyl cyclase by Gi alpha. *Science* 261:218-221.
- Thannickal TC, Lai YY, Siegel JM (2007) Hypocretin (orexin) cell loss in Parkinson's disease. *Brain* 130:1586-1595.
- Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 27:469-474.
- Thomsen W, Frazer J, Unett D (2005) Functional assays for screening GPCR targets. *Curr Opin Biotechnol* 16:655-665.
- Tran DT, Bonaventure P, Hack M, Mirzadegan T, Dvorak C, Letavic M, Carruthers N, Lovenberg T, Sutton SW (2011) Chimeric, mutant orexin receptors show key interactions between orexin receptors, peptides and antagonists. *Eur J Pharmacol* 667:120-128.
- Tsujino N, Sakurai T (2013) Role of orexin in modulating arousal, feeding, and motivation. *Front Behav Neurosci* 7:28.
- Tsuneki H, Wada T, Sasaoka T (2012) Role of orexin in the central regulation of glucose and energy homeostasis. *Endocr J* 59:365-374.
- Turunen PM, Ekholm ME, Somerharju P, Kukkonen JP (2010) Arachidonic acid release mediated by OX1 orexin receptors. *Br J Pharmacol* 159:212-221.
- Turunen PM, Jantti MH, Kukkonen JP (2012) OX1 orexin/hypocretin receptor signaling through arachidonic acid and endocannabinoid release. *Mol Pharmacol* 82:156-167.
- Uramura K, Funahashi H, Muroya S, Shioda S, Takigawa M, Yada T (2001) Orexin-a activates phospholipase C- and protein kinase C-mediated Ca²⁺ signaling in dopamine neurons of the ventral tegmental area. *Neuroreport* 12:1885-1889.
- Van Cauter E, Knutson KL (2008) Sleep and the epidemic of obesity in children and adults. *Eur J Endocrinol* 159 Suppl 1:S59-66.

- van den Bout I, Divecha N (2009) PIP5K-driven PtdIns(4,5)P₂ synthesis: regulation and cellular functions. *J Cell Sci* 122:3837-3850.
- Voisin T, El Firar A, Fasseu M, Rouyer-Fessard C, Descatoire V, Walker F, Paradis V, Bedossa P, Henin D, Lehy T, Laburthe M (2011) Aberrant expression of OX1 receptors for orexins in colon cancers and liver metastases: an openable gate to apoptosis. *Cancer Res* 71:3341-3351.
- Voisin T, Firar AE, Avondo V, Laburthe M (2006) Orexin-induced apoptosis: the key role of the seven-transmembrane domain orexin type 2 receptor. *Endocrinology* 147:4977-4984.
- Ward RJ, Padiani JD, Milligan G (2011) Heteromultimerization of cannabinoid CB(1) receptor and orexin OX(1) receptor generates a unique complex in which both protomers are regulated by orexin A. *J Biol Chem* 286:37414-37428.
- Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* 100:10782-10787.
- Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J* 11:346-354.
- Wettschureck N, Moers A, Offermanns S (2004) Mouse models to study G-protein-mediated signaling. *Pharmacol Ther* 101:75-89.
- Wijker M, Wszolek ZK, Wolters EC, Rooimans MA, Pals G, Pfeiffer RF, Lynch T, Rodnitzky RL, Wilhelmssen KC, Arwert F (1996) Localization of the gene for rapidly progressive autosomal dominant parkinsonism and dementia with pallido-ponto-nigral degeneration to chromosome 17q21. *Hum Mol Genet* 5:151-154.
- Williams C (2004) cAMP detection methods in HTS: selecting the best from the rest. *Nat Rev Drug Discov* 3:125-135.
- Willie JT, Chemelli RM, Sinton CM, Tokita S, Williams SC, Kisanuki YY, Marcus JN, Lee C, Elmquist JK, Kohlmeier KA, Leonard CS, Richardson JA, Hammer RE, Yanagisawa M (2003) Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* 38:715-730.
- Wilson S, Bergsma DJ, Chambers JK, Muir AI, Fantom KG, Ellis C, Murdock PR, Herrity NC, Stadel JM (1998) Orphan G-protein-coupled receptors: the next generation of drug targets? *Br J Pharmacol* 125:1387-1392.
- Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330:1066-1071.
- Wu EH, Lo RK, Wong YH (2003) Regulation of STAT3 activity by G16-coupled receptors. *Biochem Biophys Res Commun* 303:920-925.
- Xu Y, Piston DW, Johnson CH (1999) A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. *Proc Natl Acad Sci U S A* 96:151-156.
- Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, Wang QP, Tominaga M, Goto K, Shioda S, Sakurai T (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem Biophys Res Commun* 290:1237-1245.
- Yanagisawa M (2010) Small-molecule agonists for type-2 orexin receptor. *US patent application no 2010/0150840 A1, Jun 17, 2010.*
- Yang B, Samson WK, Ferguson AV (2003) Excitatory effects of orexin-A on nucleus tractus solitarius neurons are mediated by phospholipase C and protein kinase C. *J Neurosci* 23:6215-6222.
- Yang SY (2010) Pharmacophore modeling and applications in drug discovery: challenges and recent advances. *Drug Discov Today* 15:444-450.

- Yu S, Castle A, Chen M, Lee R, Takeda K, Weinstein LS (2001) Increased insulin sensitivity in Gsalpha knockout mice. *J Biol Chem* 276:19994-19998.
- Zhai P, Yamamoto M, Galeotti J, Liu J, Masurekar M, Thaisz J, Irie K, Holle E, Yu X, Kupersmidt S, Roden DM, Wagner T, Yatani A, Vatner DE, Vatner SF, Sadoshima J (2005) Cardiac-specific overexpression of AT1 receptor mutant lacking G alpha q/G alpha i coupling causes hypertrophy and bradycardia in transgenic mice. *J Clin Invest* 115:3045-3056.
- Zhang R, Xie X (2012) Tools for GPCR drug discovery. *Acta Pharmacol Sin* 33:372-384.
- Zhong H, Neubig RR (2001) Regulator of G protein signaling proteins: novel multifunctional drug targets. *J Pharmacol Exp Ther* 297:837-845.
- Zhuang X, Belluscio L, Hen R (2000) G(olf)alpha mediates dopamine D1 receptor signaling. *J Neurosci* 20:RC91.