Orexin Receptors in Recombinant CHO Cells

Signaling to Short- and Long-Term Cell Responses

SYLVIA AMMOUN
Dissertation presented at Uppsala University to be publicly examined in B42, Biomedicinsk centrum (BMC), Uppsala, Thursday, September 22, 2005 at 09:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract


Recently discovered neuropeptides orexins (orexin-A and -B) act as endogenous ligands for G-protein-coupled receptors called OX1 and OX2 receptors. Our previous studies have established model systems for investigation of the pharmacology and signaling of these receptors in recombinant CHO cells. OX1 receptor-expressing CHO cells were mainly utilized in this thesis.

Orexin-A and -B activate both OX1 and OX2 receptors. However, orexin-B is less potent in activating OX2 receptors than orexin-A, whereas the peptides are equipotent on OX1 receptors. We have performed mutagenesis on orexin-A to investigate the basis for this selectivity. We show that OX2 receptor is generally less affected by the mutations and thus OX1 receptor appears to have less strict requirements for ligand binding, likely explaining the lack of difference in affinity/potency between orexin-A and orexin-B on OX2 receptor.

The other studies focus on orexin receptor signaling. OX1 receptors are shown to regulate adenylyl cyclase both in positive and negative manner, activate different MAP-kinases (ERK1/2 and p38) and induce cell death after long-lasting stimulation. Adenylyl cyclase regulation occurs likely through three different G-protein families, Gi, Gs and Gq. For ERK1/2, several downstream pathways, such as Ras, Src, PI3-kinase and protein kinase C (PKC) are implicated. OX1 receptor-mediated activation of ERK is suggested to be cytoprotective whereas p38 MAP-kinase induces programmed cell death.

Three particularly interesting findings were made. Firstly, novel PKC δ (delta) is suggested to regulate adenylyl cyclase, whereas conventional and atypical PKCs are involved in activation of ERK. Secondly, adenylyl cyclase and ERK activation is fully dependent on extracellular Ca++. Further experiments suggest that the previously discovered receptor-operated Ca++ influx is not affecting the downstream effectors of orexin receptors but that it instead enables orexin receptors to couple to several signal cascades. Thirdly, upon inhibition of caspases, classical mediators of programmed cell death, OX1 receptor-mediated cell death is not reversed, but instead the pathways to death are altered so de novo gene transcription is no longer required for cell death.

Keywords: orexins, cell signaling

Sylvia Ammoun, Department of Neuroscience, Box 593, Uppsala University, SE-75124 Uppsala, Sweden

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“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale”.

*Marie Curie-Sklodowska*

*To my family...*
List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals


II  OX$_1$ receptors couple to adenylyl cyclase regulation via multiple mechanisms. Tomas Holmqvist, Lisa Johanson, Marie Östman, Sylwia Ammoun, Karl E.O. Åkerman, Jyrki P. Kukkonen. *J. Biol. Chem.*, 280(8), 6570-6579, 2005

III  OX$_1$ orexin receptors activate extracellular signal-regulated kinase (ERK) in CHO cells via multiple mechanisms: The role of Ca$^{2+}$ influx in OX$_1$ receptor signaling. Sylwia Ammoun, Lisa Johansson, Marie Östman, Tomas Holmqvist, Alexander S. Danis, Laura Korhonen, Karl E.O. Åkerman, Jyrki P. Kukkonen. Accepted in *Molecular Endocrinology*, 2005


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase(s)</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis-inducing factor</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis stimulating kinase 1</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activation transcription factor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_i$</td>
<td>intracellular $\text{Ca}^{2+}$ concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase(s)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary-K</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>dn</td>
<td>dominant-negative</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein(s)</td>
</tr>
<tr>
<td>GCK</td>
<td>germinal center kinase</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide-exchange factor(s)</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein-gated inward rectifier $\text{K}^+$ channel</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth-factor-receptor-bound protein 2</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein-coupled receptor kinase(s)</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor(s)</td>
</tr>
<tr>
<td>HPK1</td>
<td>hematopoietic progenitor kinase 1</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase(s)</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase(s)</td>
</tr>
<tr>
<td>mdm2/hdm2</td>
<td>murine/human double minute 2</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP/ERK kinase</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase(s)</td>
</tr>
<tr>
<td>MKKK</td>
<td>MAPK kinase kinase(s)</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK phosphatase(s)</td>
</tr>
<tr>
<td>PAK1</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphatidylinositol 3-phosphate-dependent kinase-1</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP$_3$</td>
<td>phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B (=Akt)</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase(s)</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PYK2</td>
<td>proline-rich tyrosine kinase</td>
</tr>
<tr>
<td>Ras-GEF</td>
<td>Ras guanine nucleotide-exchange factor</td>
</tr>
<tr>
<td>Ras-GRP</td>
<td>Ras guanine nucleotide-release protein</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of GPCR signaling</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor-operated channel(s)</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase(s)</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase(s)</td>
</tr>
<tr>
<td>SERCA</td>
<td>endoplasmic/sarcoplasmic reticulum Ca$^{2+}$ ATPase(s)</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology and collagen protein</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2-containing inositol 5-phosphatase</td>
</tr>
<tr>
<td>SOC</td>
<td>store-operated channel(s)</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β-activated kinase 1</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated Ca$^{2+}$ channel(s)</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Orexinergic system

1.1.1 Distribution of orexins and orexin receptors.
Orexins, orexin-A and orexin-B, are recently discovered neurotransmitters/hormones (de Lecea et al., 1998; Sakurai et al., 1998). The neuronal orexinergic cell bodies are found in the lateral hypothalamus (reviewed in Kukkonen et al., 2002). The most important orexinergic projection areas are within the hypothalamus, thalamus, brain stem, spinal cord and cerebellum. Orexin receptors are found at the projection sites of orexinergic neurons. In the rat brain, the mRNA for OX1 and OX2 receptors is equally expressed and rather similar in distribution, even though some differences exist (Trivedi et al., 1998; Marcus et al., 2001). In the periphery, orexin receptors/receptor mRNA has mainly been found in the same organs as orexins. The cell types expressing orexin receptors include endocrine, muscle and nerve cells (reviewed in Kukkonen et al., 2002). Orexin receptors have been found in the gastrointestinal endocrine cells, endocrine pancreas, adrenal glands (cortex and medulla), testis, kidney, thyroid, lung, pituitary and pineal gland (Jöhren et al., 2001; Mikkelsen et al., 2001; Näslund et al., 2002; reviewed in Kukkonen et al., 2002). Orexin peptides have been detected in the gastrointestinal tract, pancreas, testis and pituitary (Jöhren et al., 2001; Näslund et al., 2002; reviewed in Kukkonen et al., 2002).

1.1.2. Systemic effects of orexins

1.1.2.1 Involvement of orexins in regulation of feeding
Orexins may participate in the short-term regulation of energy homeostasis by initiating feeding in response to a drop in glucose levels. Subsequent rise of glucose and leptin levels decrease orexin concentration which, in turn, would terminate feeding (reviewed in Willie et al., 2001). Intracerebroventricular injection of orexins induces feeding in rats (Sakurai et al., 1998) and the intraperitoneal administration of a selective OX1 receptor antagonist, 1-(2-methylbenzoxazol-6-yl)-3-[1,5] naphthyridin-4-yl urea hydrochloride (SB-334867-A) reduces food consumption in rats indicating the involvement of OX1 receptor (Haynes et al., 2000). It has also been proposed that the
effect of orexins on feeding behavior comes from the influence on metabolic rate by increase of arousal, rather than by up-regulation of food intake per se (reviewed in Sutcliffe & de Lecea, 2002). In the gastrointestinal tract, both neurons and some endocrine cells may express orexins. Orexinergic neurons in the gastrointestinal tract may affect intestinal secretion and uptake, endocrine secretion, sensory signaling and intestinal motility. Orexins also increase insulin release in the rat (reviewed in Kukkonen et al., 2002).

1.1.2.2 Involvement of orexins in sleep/wakefulness

Orexinergic neurons are present and project to different part of CNS involved in regulation of arousal state. Both OX₁ and OX₂ receptors are involved in this process. Injection of orexin-A into rat cerebral ventricles and the more specific CNS sites (locus coeruleus, basal forebrain, lateral preoptic area) in the rat or cat increases wakefulness and decreases sleep (reviewed in Kukkonen et al., 2002; Sutcliffe & de Lecea, 2002).

Histaminergic systems are also involved in the above processes. In the mouse, effects of orexin-A are blocked by both H₁ (histamine) receptor antagonists and H₂ receptor gene disruption (Huang et al., 2001; Yamanaka et al., 2001).

Reduced orexinergic signaling caused by either genetic disruption of the preproorexin gene, genetic destruction of the orexinergic cells or mutations in the OX₂ receptor gene lead to narcolepsy in animals (Chemelli et al., 1999; Lin et al., 1999; Gerashchenko et al., 2001; Hara et al., 2001; Hartwig et al., 2001; reviewed in Hungs & Mignot, 2001; Kukkonen et al., 2002; Sutcliffe & de Lecea, 2002; Chabas et al., 2003).

In the classical narcolepsy-model systems, genetically narcoleptic canines (Dobermans and Labradors), narcolepsy is caused by mutations in OX₂ receptor gene (canarc-1). Exon-skipping mutations resulted in a truncated receptor which localize incorrectly to the membrane, lack ligand binding proprieties and signal transduction function (reviewed in Hungs & Mignot, 2001; Mignot, 2004). Other mutations in OX₂ receptor have also been found. A single amino-acid substitution (E54 to K54) in the N-terminal region of the receptor created mutant with normal membrane location but lacking ability to bind the ligand (reviewed in Mignot, 2004).

In human narcolepsy, OX₁ and OX₂ receptor gene mutations are not detected. With probably one exception, human narcolepsy is a sporadic disease, suggested to be autoimmune in origin because of its tight association with HLA-DQB1*0602 (reviewed in Mignot, 2004). In most cases, orexins are not detectable in the cerebrospinal fluid of human narcoleptics, indirectly supporting the hypothesis that human narcolepsy is caused by death of orexinergic neurons (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000; Hartwig et al., 2001; Ripley et al., 2001; Mignot et al., 2002; reviewed in Hungs & Mignot, 2001; Chabas et al., 2003). Also, sporadically
narcoleptic canines seem to lack orexins in the cerebrospinal fluid (Ripley et al., 2001).

1.1.2.3 Orexins in autonomic/endocrine functions and regulation of stress response

Upon intracerebroventricular injection of orexins, the blood pressure, heart rate, intestinal motility, gastric acid secretion and sympathetic nerve activity increases (reviewed in Kukkonen et al., 2002). Intracerebroventricular administration of orexin-A increases plasma levels of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), corticosterone, vasopressin and epinephrine in the rat (reviewed in Kukkonen et al., 2002), suggesting that orexins could mediate stress responses.

1.1.3 Orexins and orexin receptors

1.1.3.1 Orexin peptides
Mammalian preproorexin, the precursor peptide, is composed of 130-131 amino acids (aa) (de Lecea et al., 1998; Sakurai et al., 1998). Cleavage of preproorexin and further modification leads to production of two peptides, orexin-A (33 aa) and orexin-B (28 aa). The peptides exhibit high sequence identity in the C-terminus (see Paper I, Fig. 1). There is also some sequence identity between orexin-B and secretin caused by an evolutionary relationship (de Lecea et al., 1998; Alvarez & Sutcliffe, 2002). Both peptides are C-terminally amidated, and orexin-A is pyroglutamyolated on its N-terminal (Sakurai et al., 1998). Structurally, both orexins consists of two α-helices connected by a kink, but only the structure of orexin-A is stabilized by disulphide bridges (Lee et al., 1999; Kim et al., 2004). Orexin-A is more stable than orexin-B in the physiological environment (Kastin & Akerstrom, 1999). This fact may explain the higher detectable concentration of orexin-A compared to orexin-B in the cerebrospinal fluid (Ripley et al., 2001; Yoshida et al., 2003). In the CNS, orexin-B levels are 2 to 5 times higher than orexin-A levels. Orexin-A also displays much higher lipid solubility than orexin-B, probably making orexin-A, in contrast to orexin-B, blood-brain-barrier-permeable (Kastin & Akerstrom, 1999). Despite this, it has been suggested that orexin-A in plasma originates from peripheral sources, and no brain penetration of intravenous orexin-A has been observed in the rat (reviewed in Kukkonen et al., 2002).

1.1.3.2 Orexin receptors
There are two receptors responding to orexin stimulation, OX₁ and OX₂ receptors. Orexin receptors belong to the class A G-protein-coupled receptors (GPCR). The OX₁ receptor is 425 aa and the OX₂ receptor 444 aa long in
humans, and there is a 64% sequence identity between them. There is a high
sequence identity (91-98%) between orexin receptors of different mammal-
ian species (human, pig, dog, rat and mouse) (reviewed in Kukkonen et al.,
2002). Orexin receptors show significant homology (28-35% identity) to
other peptide receptors including neuropeptide FF, neuropeptide Y (NPY),
thyrotropin-releasing hormone (TRH) and tachykinin NK₂ receptors
(reviewed in Kukkonen et al., 2002).

1.1.4 Cellular responses to orexins
Early studies demonstrated two cellular responses upon stimulation of orexin
receptors: i) Ca²⁺ elevations in Chinese hamster ovary-K1 (CHO) cells re-
combinantly expressing orexin receptors (Sakurai et al., 1998), and ii) in-
creased action potential frequency in hypothalamic neurons (de Lecea et al.,
1998).

1.1.4.1 Increased synaptic activity
Orexins cause an increase of either spontaneous or triggered action potential
frequency. Increased action potential frequency has been measured in the
hypothalamus, thalamus, hippocampus, brain stem, spinal cord and periph-
eral neurons (de Lecea et al., 1998; Grudt et al., 2002; Yang & Ferguson,
2003; Selbach et al., 2004; Ishibashi et al., 2005). Orexins probably exert
their effects both pre-and postsynaptically (reviewed in Beuckmann & Ya-
nagisawa, 2002; Kukkonen et al., 2002). It has also been found that orexin-A
causes a slow-onset long-term potentiation of synaptic transmission in the
hippocampus (Selbach et al., 2004).

1.1.4.2 Ca²⁺ elevation
In CHO cells recombinantly expressing OX₁ or OX₂ receptors, orexins cause
Ca²⁺ elevations (Sakurai et al., 1998; Smart et al., 1999; Lund et al., 2000;
Holmqvist et al., 2001; Okumura et al., 2001). This elevation in [Ca²⁺] (in-
tracellular [Ca²⁺]) was thought to proceed via activation of phospholipase Cβ
(PLCβ), production of inositol-1,4,5-trisphosphate (IP₃) and Ca²⁺ release
from internal stores. However, In CHO cells, removal of Ca²⁺ from extracel-
lular space significantly diminishes (8- to 100-fold) the potency of orexin-A
for the OX₁ receptor (Smart et al., 1999; Lund et al., 2000; Ammoun et al.,
2003). Further studies revealed that this effect is caused by activation of a
receptor-operated Ca²⁺ influx pathway as a primary response to orexin recep-
tor activation, which subsequently also apparently amplifies the PLC re-
sponse (Lund et al., 2000). However, Ca²⁺ alone is not sufficient to activate
PLC in these cells, suggesting that Ca²⁺ influx acts in concert with another
receptor-mediated mechanism (Lund et al., 2000). Further studies showed
that this influx pathway is different from the store-operated pathway
(Kukkonen & Akerman, 2001). Finally, the channel was electrophysiologi-
cally isolated (Larsson et al., 2005). These data also suggest that the channel activity may be mediated by some TRP (transient receptor potential) channels (Larsson et al., 2005), a recently cloned non-selective cation channel (super) family with wide expression but weakly characterized physiological functions and activation mechanisms (reviewed in Clapham et al., 2001). Elevation of \([\text{Ca}^{2+}]_i\) has also been observed in recombinant neuronal-like cells such as Neuro-2a and PC12 (Holmqvist et al., 2002) and also in neuronal cells from the hypothalamus (van den Pol et al., 1998), spinal cord (van den Pol, 1999; Van Den Pol et al., 2001) and cortex (Lambe & Aghajanian, 2003; reviewed in Kukkonen et al., 2002). In neurons, the \(\text{Ca}^{2+}\) rise has been suggested to occur via activation of voltage-gated \(\text{Ca}^{2+}\) channels, putatively mediated by protein kinase C (PKC) (van den Pol et al., 1998; Van Den Pol et al., 2001; Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2004). Activation of non-selective cation channels has also been observed in primary neurons, though their identity has yet to be determined (Lund et al., 2000; Hwang et al., 2001; Kukkonen & Akerman, 2001; Brown et al., 2002; Yang & Ferguson, 2003; Yang et al., 2003). However some studies indicate the involvement of transient receptor potential (TRP) channels (Larsson et al., 2005). In addition, orexin-responding neurons also express transient receptor potential (TRP) channels (Sergeeva et al., 2003).

1.1.4.3 cAMP

There are data showing orexin-mediated cAMP elevation in rat and human adrenal cortex, followed by the activation of protein kinase A and increased synthesis and release of glucocorticoids (Malendowicz et al., 1999; Mazzocchi et al., 2001). Surprisingly, data obtained with BIM hybridoma cells (thymidine auxotrophs (B3T) of rat nerve-like cells (B103) × IMR-32 human neuroblastoma) suggest that heterologously expressed OX2 but not OX1 receptors inhibit forskolin-stimulated cAMP accumulation in a pertussis toxin-sensitive manner (Zhu et al., 2003). This clearly does not hold true in CHO cells (Paper II). Additionally, in mouse mOX1, mOX2α, and mOX2β receptors stably expressed in HEK-293 cells did not display any cAMP response to orexin-A or -B stimulation (Chen & Randeva, 2004).

1.1.4.4 Pro-apoptotic effects of orexins

Orexins suppress serum-induced cell growth by induction of cell death. Activation of OX1 receptors endogenously expressed in HT29-D4 human colon adenocarcinoma and SK-N-MC human neuroblastoma cells and recombinantly in CHO-cells, induce apparently apoptotic cell death with cytochrome c release and caspase activation (Rouet-Benbineb et al., 2004) (see also Paper IV).
1.1.4.5 Involvement of orexins in the development

GPCR are involved in the regulation of cell proliferation (reviewed in Gutkind, 1998), death, differentiation (reviewed in Lowes et al., 2002), plasticity (Riedel, 1996) and motility (reviewed in Rozengurt, 1998). In the developing rat brain, orexin content as well as their receptors’ expression is quite variable. Up-regulation of the orexin receptor expression is observed during the fetal life followed by a postnatal decrease. In contrast, preproorexin level rises postnatally (van den Pol et al., 2001).

1.1.5 Pharmacology

When expressed in CHO cells, OX₁ receptor was shown to have a 10 times higher affinity for orexin-A than for orexin-B, whereas OX₂ receptor had the same affinity for both peptides. The same tendency was revealed even with Ca²⁺ measurements, where orexin-A had 10 times higher potency for OX₁ receptor than orexin-B (Sakurai et al., 1998; Smart et al., 1999; Okumura et al., 2001). Additional studies in CHO cells clarified the contribution of different residues of orexin peptide important for binding and activation of the receptors. The results indicated that N-terminal amino acids of both peptides (orexin-A and -B) are less important for receptor binding and activation when compared to the C-terminal amino acids. It was also observed that the truncation of the orexin-A to orexin-A₁₅₋₃₃ reduces its potency for both OX₁ and OX₂ receptors 20-to 60-fold and further truncation gradually abrogates Ca²⁺ response for OX₁ receptor (Darker et al., 2001). There are some specific antagonists for OX₁ receptors from GlaxoSmithKline, most notably SB-334867, which displays 100-fold higher affinity for OX₁ compared to OX₂ receptor. More recently, production of selective OX₂ receptor antagonists has been reported (McAtee et al., 2004).

1.2 Signaling of GPCR (G-protein-coupled receptor)

1.2.1 GPCR

GPCR contain a conserved structural motif consisting of seven α-helical membrane-spanning regions (reviewed in Gutkind, 2000). The N-terminus is located on the extracellular side and the C-terminus on the intracellular side. Highest sequence homology between GPCR is usually found within transmembrane parts of the receptors. High resolution X-ray crystallographic analysis of bovine rhodopsin revealed the presence of helix VIII, found in the intracellular C-terminus running parallel to the membrane (Palczewski et al., 2000). The activation of G-proteins by GPCR is mediated by the second-
and the third intracellular loops (i2 resp. i3) and even the proximal C-terminus (reviewed in Gether, 2000). A variety of kinases can phosphorylate GPCR, such as protein kinase A (PKA), protein kinase C (PKC) and GPCR kinases (GRK) (reviewed in Tobin, 1997). Most GPCR probably are glycoproteins with glycosylation sites located in the extracellular domains. The extracellular loops contain Cys residues, which may stabilize the conformation via disulphide bridges. Palmitoylation takes place on a Cys residue in the proximal C-terminus; this may be important for anchoring of the C-terminus in the membrane and generation of an i4 loop.

1.2.2 GTPase cycle

GPCR can exist in two principal conformations, active or inactive (reviewed in Gether, 2000; Kukkonen, 2004). Activation of GPCR leads to a conformational change, upon which the receptor can catalyze the release of GDP from the active site of a Ga-subunit of a heterotrimeric G-protein. The “Empty” Ga subunit binds GTP upon which Ga and the Gβγ-dimer are thought to dissociate from each other and from the receptor. Both Ga-subunit and Gβγ-dimer are able to initiate intracellular signaling pathways (reviewed in Schwindinger & Robishaw, 2001). The receptor released from the complex can activate other G-proteins, permitting amplification of the signal (Iiri et al., 1998). The signal transduction is terminated by the hydrolysis of GTP by the intrinsic GTPase activity of the Ga-subunit. This process is accelerated by GTPase-activating proteins (GAPs) such as regulators of G-protein signaling (RGS) (Wilkie, 2000). Signaling via Gβγ is inhibited by binding to phosducin or pleckstrin (reviewed in Schwindinger & Robishaw, 2001). Receptor activity is subject to negative feedback regulation by GRK, some of which are activated by Gβγ (reviewed in Schwindinger & Robishaw, 2001).

1.2.3 G-protein-independent GPCR signaling

GPCR signaling can also be mediated independently of G-proteins. GPCR are able to associate with a variety of different molecules other than G-proteins (reviewed in Hall et al., 1999). For instance, β-arrestin binds to phosphorylated GPCR leading to receptor internalization and sometimes to coupling of the receptor to Src (reviewed in Hall et al., 1999; Marinissen & Gutkind, 2001). G-protein-independent signals may also be mediated upon receptor interaction with PDZ-, SH2-, SH3-, WW- and EVH-domain-containing proteins (reviewed in Hall et al., 1999; Marinissen & Gutkind, 2001).
1.2.4 Gα subunits

Gα subunits are divided into four families: Gαs, Gαi/o, Gq, and G12/13 (reviewed in Gutkind, 2000; Marinissen & Gutkind, 2001; Kurose, 2003). Gα subunits of the Gq family activate PLCβ which results in IP₃ and diacylglycerol (DAG) production from hydrolyzed phosphatidylinositol-4,5-bisphosphate (PIP₂) (reviewed in Rhee, 2001). IP₃ and DAG lead to an increase in [Ca²⁺ᵢ] levels and the activation of protein kinase C (PKC) (reviewed in Gutkind, 2000). Gα subunits of the Gi family activate adenylyl cyclases (AC) whereas those of the Gi family have the opposite effects. Gα subunits of the G12 family, which includes Gα₁₂ and Gα₁₃, provide a link between GPCR and the activation of the small G-proteins of Rho family (reviewed in Gudermann et al., 2000). These proteins are involved in reorganisation of cytoskeleton (Majumdar et al., 1999), activation of c-Jun N-terminal kinase (JNK) and stimulation of Na⁺/H⁺-exchanger (reviewed in Voyno-Yasenetskaya, 1998; Avkiran & Haworth, 2003; Kurose, 2003) (see Table 1).

Table 1. G-protein α-subunits (Majumdar et al., 1999; Blomquist et al., 2000; reviewed in Voyno-Yasenetskaya, 1998; Gutkind, 2000; Marinissen & Gutkind, 2001; Avkiran & Haworth, 2003; Kurose, 2003)

<table>
<thead>
<tr>
<th>Family</th>
<th>Subunit</th>
<th>Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gₛ</td>
<td>αₛ, α₇₄</td>
<td>AC ↑, K⁻ channels ↑, Ca²⁺ channels ↑</td>
</tr>
<tr>
<td>Gᵢ</td>
<td>α₁₁, α₁₂, α₁₃, α₁₄, α₁₅, α₂, α₁₆, α₁₇, α₁₈, α₁₉, α₁₀, α₁₁, α₁₂, α₁₃</td>
<td>AC ↓, K⁻ channels ↑, Ca²⁺ channels ↓↑, cAMP and cGMP phosphodiesterases ↑</td>
</tr>
<tr>
<td>Gₖ</td>
<td>α₁₆, α₁₇, α₁₈, α₁₉, α₁₀, α₁₁, α₁₂, α₁₃, α₁₄, α₁₅/16</td>
<td>PLCβ↑, ion channels</td>
</tr>
<tr>
<td>G₁₂</td>
<td>α₁₁₂, α₁₁₃</td>
<td>guanine nucleotide exchange factors: p115 Rho-GEF /Lsc ↑, Lbc ↑, Lfc ↑, Tiam1↑, FGD1↑, p114-Rho-GEF ↑, PDZ-RhoGEF ↑; JNK ↑; Na⁺/H⁺-exchanger ↑</td>
</tr>
</tbody>
</table>
1.2.5 G\(\beta\gamma\)-mediated signaling

Independently of G\(\alpha\) subunits, G\(\beta\gamma\) subunits can also mediate signals to GPCR effectors (Figure 1). Though \(\beta\gamma\) subunits could in principle arise from activation of any G-protein species, most often they seem to originate from the G\(_i\) family (reviewed in Schwindinger & Robishaw, 2001).

![Signal Transduction Diagram](image_url)

**Figure 1.** G\(\beta\gamma\)-mediated signal transduction (Bayewitch et al., 1998; reviewed in Schwindinger & Robishaw, 2001)

1.3 Mitogen-activated protein kinase (MAPK) pathways

The stimulation of receptor tyrosine kinases (RTK) and GPCR can lead to activation of MAPK. These Ser/Thr kinases are stimulatory to intracellular signals controlling gene expression, cell proliferation and differentiation (reviewed in Lopez-Illasaca, 1998). There are five subfamilies of MAPK: ERK1/2 (ERK = extracellular signal-regulated kinase), ERK3/ERK4, ERK5, JNK (c-Jun N-terminal kinases) and p38 MAPK (reviewed in Widmann et al., 1999; Hagemann & Blank, 2001). Growth factors, phorbol esters and
hormones activate MAPK through a series of phosphorylation cascades. RTK and GPCR share common mediators in the cascades leading to activation of MAPK (reviewed in Widmann et al., 1999) (see Figure 2).

Figure 2. Mammalian MAPK signaling pathways (reviewed in Widmann et al., 1999; Gutkind, 2000; Kyriakis & Avruch, 2001; Marinissen & Gutkind, 2001; Zarrubin & Han, 2005). Three-component MAP-kinase module: MAP-kinase kinase (MKKK) is activated either by phosphorylation by MAP kinase kinase kinase (MKKK) or by interaction with Ras-, Rac1-, CDC42- and Rap-1-GTPases. Activated MKKK (serine/threonine kinase) phosphorylates MAP kinase kinase (MKK) (threonine/tyrosine kinase). MKK recognizes and phosphorylates a Thr-X-Tyr motif in the activation loop of MAP-kinase (serine/threonine kinase).
1.3.1 ERK

The GTP-bound Ras binds to a MAPKKK (Raf1) and translocates it to the plasma membrane (reviewed in Widmann et al., 1999). Membrane bound Raf1 is activated upon phosphorylation by membrane-bound kinases including c-Src and PKC. Further, MEK1 (MAPKK) is phosphorylated leading to activation, dimerization, and translocation of ERK1/2 (MAPK) to the nucleus (reviewed in Cobb & Goldsmith, 2000). Within the nucleus ERK1/2 regulate the activity of nuclear proteins including transcription factors such as ELK1, Ets1, Sap 1a, c-Myc, Tal and signal transducer and activator of transcription (STAT) (reviewed in Yordy & Muise-Helmericks, 2000). Nuclear ERK1/2, when dephosphorylated (by phosphatases), return to the cytoplasm and reassociate with MEK1 (MKK1). In addition, active ERK1/2 also phosphorylate several cytoplasmic proteins on Ser/Thr residues, such as p90 ribosomal S6 kinases (RSKs) (RSK1, RSK2 and RSK3) resulting in regulation of c-Fos, c-Jun and CREB (Roux et al., 2003; reviewed in Impey et al., 1999; Widmann et al., 1999; Chuderland & Seger, 2005) (Figure 2). Regulation of additional kinases by ERK1/2 can result in amplification of the signal downstream of ERK1/2. ERK1/2 is also able to phosphorylate the EGF receptor (EGFR), the RasGEF SOS, Raf1 and MEK1, which is believed to reduce their catalytic activity (negative feedback) (reviewed in Widmann et al., 1999; Pouyssegur et al., 2002; Chuderland & Seger, 2005). However, more recently other cytosolic targets have been found including cell adhesion molecules (CAMs), cytoskeletal proteins such as caldesmon (an actin filament-binding protein), ion channels and phospholipase A2 (cPLA2) (Bornfeldt et al., 1997; reviewed in Robinson & Cobb, 1997; Impey et al., 1999; Pouyssegur et al., 2002; Chuderland & Seger, 2005; Gerthoffer, 2005).

1.3.2 SAPK

p38 MAPK and JNK are sometimes referred to as stress-activated protein kinases (SAPK) as they are most classically activated under stressful conditions such as DNA damage (e.g. UV irradiation), osmotic shock, heat shock, lipopolysaccharide and protein synthesis inhibitors. Also cytokines (IL-1, IL-2, IL-7, TNF-α) and RTK, GPCR and death receptor activation (Watabe et al., 2004) can induce activation of these kinases (reviewed in Kyriakis & Avruch, 2001; Bulavin & Fornace, 2004; Zarubin & Han, 2005).

1.3.2.1 p38 MAPK

The mammalian p38 MAPK family consists at least of four different sub-types, p38α, -β, -γ and -δ, activated with different affinity by MKK3 and MKK6. Additionally, MKK4, an upstream JNK stimulator, can also partici-
pate in activation of p38α and p38δ, suggesting that the activation of p38 isoforms is specifically controlled through different coactivators and by various combinations of upstream regulators (reviewed in Zarubin & Han, 2005). Several MKKK have been implicated in MKK3 and MKK6 activation, including p21-activated kinases (PAK), TGF-β-activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1) and mixed lineage kinases (MLK1/3). Small Rho-family G-proteins, Cdc42 and Rac have been described as upstream regulators of these MKKK (reviewed in Widmann et al., 1999; Gutkind, 2000; Kyriakis & Avruch, 2001; Marinissen & Gutkind, 2001; Zarubin & Han, 2005) (Figure 2).

Several MAPK-activated protein (MAPKAP) kinases are downstream targets for p38 MAPK-mediated phosphorylation and activation. Other substrates of p38—either directly or via MAPKAP kinases—are 27-kDa heat-shock protein, transcription factors such as activating transcription factor 1, 2 and 6 (ATF-1/2/6), Elk1, cAMP-responsive element binding protein (CREB), C/EBP homologous protein (CHOP), myocyte enhancer factor (MEF2A and -C), p53 and Max. Elk1 acts as a convergence point for p38, ERK1/2 and JNK (reviewed in Widmann et al., 1999; Gutkind, 2000; Zarubin & Han, 2005). Max, in turn, heterodimerizes with c-Myc, an ERK substrate, which provides cross-talk between ERK and p38 pathways. cPLA2, Na+/H+ exchanger isoform-1 (NHE-1), tau and keratin 8 have also been reported as substrates for p38α. p38 MAPK activity can be downregulated by MAP kinase phosphatases (MKP). Several members of MKPs dephosphorylate p38α and p38β while p38δ and p38γ are resistant to all known MKP (reviewed in Zarubin & Han, 2005).

1.3.2.2 JNK

JNK family consists of three isoforms, JNK1, JNK2 and JNK 3. JNK1 and JNK2 are ubiquitously expressed while JNK3 is mainly expressed in neuronal and heart tissues. Similarly to other MAPK subfamilies (p38 and ERK) activation of JNK is mediated by a MAP kinase module, MKKK/MKK/MAPK protein phosphorylation cascade. To MKKK belong ASK1, MLK, TAK1 and TPL-2. JNK is directly phosphorylated and activated by dual-specificity protein kinases, MKK4 and MKK7 phosphorylating JNK at Thr183 and Tyr185. Downstream targets of JNK are transcription factors such as c-Jun, ATF2, Elk-1, p53, c-Myc and also cytosolic Bcl-2 family members (Bcl-2, Bcl-xl, Bim and BAD) (reviewed in Widmann et al., 1999; Gutkind, 2000; Liu & Lin, 2005) (Figure 2).
1.4 Ras

H-, K- and N-Ras are the archetypical small (monomeric) G-proteins (reviewed in Bos, 1998). Small G-proteins have the same propensity as heterotrimeric G-proteins by cycling between the inactive GDP-bound state and the active GTP-bound state. Ras proteins are located at the inner side of the plasma membrane and are activated by RasGEF (Ras GDP/GTP-exchange factors) (reviewed in Bos, 1998). Ras activation can integrate multiple signals via GEF sensitive to tyrosine phosphorylation, Ca²⁺ and DAG (diacylglycerol) and possibly even G-protein βγ-subunits (Gutkind, 2000). Ras-GTP complex binds and activates effector proteins (reviewed in Bos, 1998) such as members of Raf family (Raf1, A-Raf, B-Raf), class Ia phosphatidylinositol-3 kinases (PI3K), PLCɛ, members of the RalGEF family (RalGDS, RIf and RGl) and PKCζ (Lopez et al., 2001). The main pathway downstream from activated Ras is the MAPK (ERK) cascade via tyrosine kinase Raf. All Ras isoforms are posttranslationally modified and initial modifications at C-terminus, farnesylation and methylation, are common to all Ras proteins (reviewed in Hancock, 2003). Different posttranslational modification of Ras-isotypes targets different Ras-proteins to different plasma-membrane microdomains (reviewed in Hancock, 2003), which leads to the activation of distinct signal pathways. Ras isoforms show different cellular localization (Quilliam et al., 2002). They are activated by different stimuli (Prior & Hancock, 2001; Oliva et al., 2003) and have variable abilities to activate different effector pathways (Yan et al., 1998; Villalonga et al., 2001).

1.5 GPCR and activation of the ERK1/2 pathway

1.5.1 Receptor tyrosine kinases (RTK) and nonreceptor tyrosine kinases in GPCR signaling

GPCR- and RTK-mediated ERK activation converges in many cases. Both pathways involve tyrosine phosphorylation of adaptor protein Shc as well as Ras activation (reviewed in Pierce et al., 2001). GPCR, in addition to the phosphorylation of Shc, couples to activation of RTK, such as the EGFR (epidermal growth factor receptor), a process known as transactivation (reviewed in Pierce et al., 2001). Transactivation is triggered by different mechanisms such as GPCR-mediated activation of matrix metalloproteases with subsequent release of EGF (reviewed in Marinissen & Gutkind, 2001) or by Gβγ subunits-mediated activation of c-Src and Pyk2 (reviewed in Pierce et al., 2001). Recent data suggest involvement of β-arrestin (known to be involved in GPCR desensitization) in recruitment and activation of c-Src (reviewed in Marinissen & Gutkind, 2001). The β-arrestin-c-Src complexes
also contain ERK suggesting involvement of β-arrestins in regulating the assembly of ERK activation complexes on the GPCR (see Figure 3). Some GPCR, such as β₁ adrenoceptors, bind directly to c-Src (reviewed in Marinissen & Gutkind, 2001). Moreover it has been observed that Gαi and Gαs can directly interact with and activate c-Src via the catalytic domain of the kinase (reviewed in Pierce et al., 2001).

1.5.2 PI3K in GPCR-mediated MAPK pathway

PI3K plays a central role in cell signaling and can lead to cell proliferation, survival, motility, exocytosis and cytoskeletal rearrangements. Class I PI3Ks are activated upon receptor-stimulated translocation to the plasma membrane where they phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinisitol-3,4,5-trisphosphate (PIP₃). PIP₃ serves as a docking site for a variety of signaling proteins initiating cascades of downstream signals (reviewed in Vanhaesebroeck & Alessi, 2000). Signaling proteins with certain pleckstrin homology (PH) domains accumulate at sites of PI3K activation by directly binding to PIP₃. There are two types of class I PI3K, both of which are heterodimeric molecules composed of a p110 catalytic subunit and a regulatory subunit. The class IA enzymes contain a p110α, -β or -γ catalytic subunits and a SH2-domain-containing adapter subunit, p85α, p85β or p55γ. Binding of the SH2 domain of the p85/55 subunit to phosphotyrosine residue recruits the p110 catalytic subunit into the membrane. Class IB contains only one member, PI3Kγ, which is composed of p110γ catalytic subunit and p101 regulatory subunit. This enzyme can be translocated to the membrane by βγ subunits upon stimulation of GPCR (Suire et al., 2002). Moreover, the catalytic subunits of both PI3Kα and PI3Kγ can bind to Ras-GTP via their Ras-binding-domains (RBD) (Suire et al., 2002). PI3K-dependent signaling pathways are antagonized by tumor suppressor protein PTEN, which dephosphorylates PIP₃ at 3’-position (reviewed in Vanhaesebroeck & Alessi, 2000) and by SHIP, a 5’-phosphatase (reviewed in Huber et al., 1999).

1.5.2.1 PDK1

Phosphatidylinositol-3-phosphate-dependent kinase-1 (PDK1) is a 63 kDa Ser/Thr kinase ubiquitously expressed in human tissues. In vitro, its PH domain binds PIP₃. PDK1 is responsible for the PIP₃-dependent phosphorylation of the AGC family of protein kinases such as PKA, PKB/Akt (protein kinase B/Akt), PKC, PKG (protein kinase G), p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK) (reviewed in Vanhaesebroeck & Alessi, 2000; Biondi, 2004; Mora et al., 2004).
1.5.2.2 PKC

Protein kinases C (PKCs) belong to a family of Ser/Thr-specific protein kinases and are involved in the regulation of various cellular processes such as cell proliferation, cell plasticity, apoptosis and cell migration (reviewed in Mellor & Parker, 1998; Dempsey et al., 2000). PKC can be activated by calcium, phosphatidylserine (PS), diacylglycerol (DAG) and phosphorylation (reviewed in Battaini, 2001) (see Table 2).

Table 2. PKC isoforms (reviewed in Battaini, 2001; Dutil et al., 1998; Le Good et al., 1998 905; Steinberg, 2004)

<table>
<thead>
<tr>
<th>PKC subfamilies</th>
<th>activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>conventional (α, βI, βII, γ)</td>
<td>phosphatidylserine, DAG, calcium, PDK1</td>
</tr>
<tr>
<td>novel (δ, ε, η, θ)</td>
<td>phosphatidylserine, DAG, PDK1, Src</td>
</tr>
<tr>
<td>atypical (ζ/ι, ξ)</td>
<td>phosphatidylserine, PIP3, PDK1, Ras</td>
</tr>
</tbody>
</table>

Maturation and activity of PKCs is controlled by autophosphorylation and transphosphorylation (PDK). Additionally, a new mechanism for PKCδ activation has recently been reported involving tyrosine phosphorylation by Src. The tyrosine-phosphorylated PKCδ displays lipid-independent kinase activity explaining PKC-dependent phosphorylation of proteins in non-membrane compartments (reviewed in Ron & Kazanietz, 1999; Battaini, 2001; Steinberg, 2004). Recent studies demonstrate that all PKC subtypes and especially PKCδ are able to phosphorylate and activate RasGRP, a GEF for Ras (reviewed in Ron & Kazanietz, 1999; Brodie & Blumberg, 2003).

1.5.3 Gq/11-coupled receptor-mediated ERK activation

Gq/11 activation has been implicated in the control of the ERK cascade via PLCβ and PKC activation by both Ras-dependent and -independent mechanisms (reviewed in Luttrell, 2003). PKCα may activate c-Raf-1 by direct phosphorylation (Kolch et al., 1993) creating a Ras-independent pathway. It has also been demonstrated that stimulation of ERK by Gq/11-coupled a1b adrenoceptors and M1 muscarinic receptors in fibroblasts proceeds through a c-Raf-1- and PKC-dependent and Ras-independent manner. In many cases, however, Gq/11 activation causes ERK activation independently on PKC via RTK transactivation (Crespo et al., 1994; Daub et al., 1997; reviewed in Luttrell, 2003). Additionally, calcium may also contribute to Gq/11-mediated ERK activation, especially in neurons. Gq/11 activation causes PLCβ activation, inositol trisphosphate (IP3) production and release of intracellular calcium which triggers Pyk2 autophosphorylation, recruitment of non-receptor tyrosine kinase c-Src, tyrosine phosphorylation of Shc and Ras-dependent ERK activation (Dikic et al., 1996; Della Rocca et al., 1999; reviewed in Luttrell, 2003) (see Figure 3).
1.5.4 Gs-coupled receptor-mediated ERK activation

Gs-coupled receptors have been implicated in signaling pathways towards ERK inhibition by stimulation of adenylate cyclase and subsequent PKA-mediated phosphorylation and inhibition of c-Raf-1 (Wu et al., 1993; reviewed in Luttrell, 2003). However, in some cell types cAMP and PKA may instead trigger ERK activation, observed in neuronal and hematopoietic cells. In this case Gs activation leads to PKA-dependent phosphorylation of the Ras-family GTPase, Rap-1, and activation of the B-Raf isoform (Vossler et al., 1997; Grewal et al., 2000; Gutkind, 2000) involving non-receptor tyrosine kinase c-Src downstream of PKA (Schmitt & Stork, 2002). In addition, direct binding of cAMP to the Rap-1 guanine nucleotide exchange factor Epac may also contribute to cAMP-dependent stimulation of Rap-1 (de Rooij et al., 1998; reviewed in Luttrell, 2003) (Figure 3).

PKA-mediated phosphorylation of Gs-coupled receptors such as β2-adrenoceptors (Daaka et al., 1997) and prostacyclin receptors (Lawler et al., 2001) leads to a switch from Gs to Gi in receptor coupling. Thus, Gs-coupled receptors, upon phosphorylation by PKA, may activate ERK via Ras-dependent pathway mediated through Gβγ subunits derived from pertussis toxin-sensitive Gi proteins (reviewed in Lefkowitz et al., 2002).

1.5.5 Gi/o-coupled receptor-mediated ERK activation

Gi activates ERK cascade mainly via its Gβγ subunits leading to transactivation of receptor tyrosine kinase (RTK) (Figure 3). Additionally, inhibition of adenylate cyclase activity by Gaq subunits may positively regulate ERK activation through relief of the inhibitory effects of PKA on the c-Raf-1 isoform (reviewed in Luttrell, 2003). In contrast, the Gαo subunit may contribute to GPCR-mediated ERK activation in some cell types in both tyrosine kinase- and Ras-independent and PKC-dependent manner (reviewed in Luttrell, 2003). The expression of an active variant of Gaq in CHO cells leads to ERK activation via B-Raf, but not c-Raf-1 in which the Ras-independent mechanism required PKC and phosphatidylinositol 3-kinase activity (Antonelli et al., 2000; reviewed in Luttrell, 2003) (Figure 3).
1.5.6 Gβγ–ERK signaling

Both Gαi-coupled receptor (LPA [lysophosphatidic acid] receptors, α2A adrenoceptors, M2 muscarinic receptors) and Gq/11-coupled receptors (M1 muscarinic receptor) may activate ERK via Gβγ subunit using pathway requiring receptor tyrosine kinase (RTK) and Ras (Crespo et al., 1994; Hawes et al., 1995; van Biesen et al., 1995; reviewed in Luttrell, 2003). Gβγ subunits mediates ‘transactivation’ of RTK (EGFR) receptors through proteolysis of heparin-binding-epidermal growth factor (HB-EGF) by metalloproteases such as ADAM (a disintegrin-like and metalloprotease domain-containing protein) (reviewed in Carpenter, 2000; Gutkind, 2000; Gschwind et al., 2001). Further, RTK activation leads to recruitment of adaptor proteins (Shc and Grb2), activation of Ras and initiation of Raf–MEK–ERK cascade (see Figure 3). PI3K and non-receptor tyrosine kinase c-Src may act as mediators in this pathway (Hawes et al., 1995; Luttrell et al., 1997; Lopez-Ilasaca, 1998; Yart et al., 2002; reviewed in Gutkind, 2000; Luttrell, 2003) (Figure 3).

1.5.7 G-protein-independent signaling

GPCR can couple to ERK signaling independently of G-proteins, involving G-protein-coupled receptor kinases (GRK) and β-arrestins. β-arrestins (β-arrestin 1/2) act as a multifunctional adaptors and scaffold proteins recruiting different signaling molecules such as MEK, ERK, c-Raf-1 and Src (reviewed in Lefkowitz & Shenoy, 2005) (Figure 3).
Figure 3. GPCR-mediated signaling to ERK MAP kinase cascade via activation of various heterotrimeric G proteins, second messenger-dependent protein kinases (PKA and PKC), cross-talk between GPCR and EGF receptors and integrin heterodimers clustered in focal adhesions (Dikic et al., 1996; Della Rocca et al., 1999; Antonelli et al., 2000; reviewed in Gutkind, 2000; Luttrell, 2002; Luttrell, 2003; Lefkowitz & Shenoy, 2005).

1.6 Calcium signaling

Ca\(^{2+}\) is a universal intracellular messenger, which controls and regulates multiple cellular processes such as neurotransmission, muscle contraction, gene transcription and cell proliferation (reviewed in Berridge et al., 2000). Ca\(^{2+}\) exerts its regulatory functions upon elevation of its concentration within the cell cytoplasm. The mechanisms behind Ca\(^{2+}\) elevations include activation of different types of Ca\(^{2+}\) channels within the plasma membrane as well as Ca\(^{2+}\) release from the intracellular stores. Intracellular Ca\(^{2+}\) increase triggers processes to reduce [Ca\(^{2+}\)]\(i\), such as various Ca\(^{2+}\)-pumps within the plasma-and endoplasmic reticulum (ER) membranes (reviewed in Berridge et al., 2000; Bootman et al., 2001) as well the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (reviewed in Bootman et al., 2001). In addition, mito-
Chondria can also sequester Ca\textsuperscript{2+} (reviewed in Berridge et al., 2000). In resting conditions, Ca\textsuperscript{2+} level is below 100 nM, whereas in the course of activation intracellular Ca\textsuperscript{2+} levels reach more than 1 µM. Several types of Ca\textsuperscript{2+} channels within the plasma membrane supply cells with Ca\textsuperscript{2+} from the extracellular space. In excitable cells such as neurons, muscles and endocirunal cells, voltage-gated Ca\textsuperscript{2+} channels (VGCC) are activated upon depolarization. Another group of channels providing Ca\textsuperscript{2+} influx belongs to the superfamily of ligand-gated ion channels. There are also other Ca\textsuperscript{2+}-entry channels opening in response to different stimuli, like the receptor-operated channels (ROC), regulated by more or less direct receptor signals, and store-operated channels (SOC), which open in response to the ER Ca\textsuperscript{2+} store depletion (reviewed in Berridge et al., 2000). The best candidates for ROC and SOC are TRP (transient receptor potential) channels. Ca\textsuperscript{2+} release from ER is mostly mediated by IP\textsubscript{3} and ryanodine receptors (reviewed in Clapham et al., 2001).

1.7 PLC isozymes in GPCR signaling

PLC isozymes catalyze hydrolysis of PIP\textsubscript{2} resulting in formation of two intracellular messengers, DAG and IP\textsubscript{3}. These messengers then promote the activation of, e.g., PKC and release of Ca\textsuperscript{2+} from intracellular stores, respectively. PLC isozymes can be divided in subfamilies (Table 3). All the PLC isoforms require Ca\textsuperscript{2+} for their activity, but the stimulatory potential and sensitivity to Ca\textsuperscript{2+} varies widely as also does the sensitivity to other intracellular signals.

Table 3. PLC isotypes (reviewed in Cullen, 2001; Rhee, 2001; Ochocka & Pawelczyk, 2003; Wing et al., 2003; Liu & Wu, 2004; McCudden et al., 2005)

<table>
<thead>
<tr>
<th>GPCR signaling</th>
<th>PLC (\beta) (1-4)</th>
<th>PLC (\gamma) (1-2)</th>
<th>PLC (\delta) (1, 2, 4)</th>
<th>PLC(\varepsilon)</th>
<th>PLC(\zeta)</th>
<th>PLC(\eta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsuperscript{2+} RTK signaling</td>
<td>G(\alpha)\textsubscript{q}, G(\beta)\textsubscript{2}</td>
<td>direct interaction, Src?, PIP\textsubscript{3}, RTK trans-activation</td>
<td>G(\alpha) (transglutaminase II)</td>
<td>G(\beta)\textsubscript{2}/G(\alpha)\textsubscript{q} (Ras/Rap GEF via e.g. cAMP)</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} (strong) H-Ras Rap/Rho</td>
<td>Ca\textsuperscript{2+} (strong)</td>
<td>Ca\textsuperscript{2+} H-Ras Rap/Rho</td>
<td>Ca\textsuperscript{2+} H-Ras Rap/Rho</td>
<td>Ca\textsuperscript{2+} (strong)</td>
<td>Ca\textsuperscript{2+} (strong)</td>
<td>Ca\textsuperscript{2+} (strong)</td>
</tr>
</tbody>
</table>
1.8 cAMP

As a second messenger, cAMP is as central as Ca\textsuperscript{2+} involved in the regulation of gluconeogenesis, glycolysis, hormone synthesis, muscle contraction, learning processes, ion channels, cell differentiation, proliferation and apoptosis. The cAMP signaling is mainly linked to PKA activation. In addition, a PKA-independent pathway of cAMP signaling occurs via cAMP activation of Epac, a guanine nucleotide-exchange factor of the Ras-family G-protein Rap (de Rooij et al., 1998) or cAMP-sensitive ion channels such as cyclic nucleotide-gated (CNG) channels (reviewed in Kaupp & Seifert, 2002).

1.8.1 Regulation of adenylyl cyclase (AC) activity

The synthesis of cAMP is catalyzed by a family of enzymes known as adenylyl cyclases (AC). There are at least nine membrane-bound adenylyl cyclases in mammals (reviewed in Tesmer & Sprang, 1998) (see Table 4).

Table 4. Differential modulation of adenylyl cyclase (AC) activity (Tang & Gilman, 1991; Lustig et al., 1993; Taussig et al., 1994; Iwami et al., 1995; Zimmermann & Taussig, 1996; Harry et al., 1997; Lai et al., 1997; Bayewitch et al., 1998; Fagan & Romani, 2000; Lin et al., 2002; Cumbay & Watts, 2004; Cheung et al., 2005; reviewed in Cooper et al., 1995; Tesmer & Sprang, 1998).

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>G(_a_1)</td>
<td>AC1-9</td>
</tr>
<tr>
<td>G(_\alpha_1)</td>
<td>No</td>
</tr>
<tr>
<td>G(<em>\beta</em>\gamma)</td>
<td>AC2, AC4, AC7</td>
</tr>
<tr>
<td>Forskolin</td>
<td>AC1-8</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}/calmodulin</td>
<td>AC1, AC3, AC8</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>No</td>
</tr>
<tr>
<td>PKC</td>
<td>AC1 (weak ?), AC3 (weak)</td>
</tr>
<tr>
<td></td>
<td>AC2, AC5 (PKC-\alpha, -\zeta)</td>
</tr>
<tr>
<td></td>
<td>AC4 (weak), AC7</td>
</tr>
<tr>
<td>PKA</td>
<td>No</td>
</tr>
<tr>
<td>calcineurin</td>
<td>No</td>
</tr>
</tbody>
</table>
1.9 Cell Fate

1.9.1 Different pathways of cell death

Cell death has previously been classically divided into two main types: active (apoptotic) and passive (necrotic) cell death. However, the classical hallmarks of apoptosis are not fulfilled in many cases of execution of cell death programs, and thus the active forms of cells death are rather covered by the term programmed cell death (reviewed in Leist & Jaattela, 2001; Jaattela, 2004). For separation in different subforms (see Table 5).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Apoptosis</th>
<th>Apoptosis-like PCD</th>
<th>Necrosis-like PCD</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatin condensation</td>
<td>Sharp, compact</td>
<td>Less sharp</td>
<td>No/weak</td>
<td>No</td>
</tr>
<tr>
<td>&quot;DNA ladder&quot;</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
<td>No</td>
</tr>
<tr>
<td>Caspase activation</td>
<td>Yes, mandatory</td>
<td>Yes/No, not mandatory</td>
<td>Yes/No, not mandatory</td>
<td>No</td>
</tr>
<tr>
<td>Zeiosis</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Phosphatidylserine exposure</td>
<td>Early</td>
<td>Yes</td>
<td>?</td>
<td>No</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cytochrome C release</td>
<td>Yes</td>
<td>?</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Dependence on de novo gene transcription</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

1.9.2 Signal pathways in apoptosis

Apoptosis proceeds through two general pathways, the intrinsic and extrinsic. The intrinsic pathway is based on proapoptotic Bcl-2-related protein (Bax and Bak) activation in either transcriptional manner or by conformational change originating from cleavage or binding to BH3-only proteins (Bad, Bid, Bim, Bmf, Noxa, PUMA etc.). This is followed by mitochondrial leakage of cytochrome c, which forms a complex, named apoptosome, together with apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, a member of the caspase family of cysteine proteases. Next, activated caspase-9 is released from this complex to cleave and activate downstream caspases -3, -6 and -7. Also, apoptosis-inducing factor (AIF), endonuclease G (Endo G) and the DIABLO/Smac proteins are released from mitochondria. AIF and Endo G can induce DNA degradation in a caspase-independent manner. DIABLO/Smac blocks inhibitors of apoptosis proteins (IAP) and promotes caspase activation.
In contrast, the extrinsic pathway is dependent upon external stimuli and associated with the activation of cell surface death receptors (Fas, TNF receptors) and proceeds in caspase- and mitochondria-dependent or -independent manner. Caspases-8 and -10 participate in this process (Budihardjo et al., 1999; Jaattela, 2004; Pommier et al., 2004).

As also indicated in Table 5., these pathways, including caspases, the most classical executors of PCD, can be in varying degrees associated with other forms of PCD as well. Inhibition of caspases may uncover/enhance underlying caspase-independent death programs speaking in favor of the existence of backup for death pathways. Thus, the existence of overlapping death pathways initiated by a single stimulus may be a general phenomenon. The reported caspase-independent pathways utilize calpains and cathepsins as well as apoptosis-inducing factor (AIF) (reviewed in Leist & Jaattela, 2001). Caspases may thus determine the pattern of cell death rather than the decision to die (Jaattela, 2004).

1.9.3 Role of calcium in the control of cell fate

Ca²⁺ has also a central role in the control of cell plasticity, such as growth, proliferation, differentiation and programmed cell death. The choice between different pathways regulating cell fate is tightly associated with amplitude of Ca²⁺ signal as well as its spatiotemporal distribution (reviewed in Berridge et al., 2000). Various cell-surface receptors (GPCR and RTK) and ion channels can generate a multitude of different Ca²⁺ response patterns. Moreover, crosstalk between Ca²⁺ and other signaling pathways enhances variations in cellular responses (reviewed in Berridge et al., 2000).

1.9.3.1 Ca²⁺ and apoptosis

Involvement of [Ca²⁺], in cell death was first reported at early eighties when Ca²⁺ ionophores, molecules transporting Ca²⁺ across membrane, were found highly toxic to the cells (Wyllie et al., 1981; Wyllie et al., 1984). Thapsigargin, the endoplasmic/sarcoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor, which depletes intracellular Ca²⁺ stores and leads to constitutive activation of store-operated Ca²⁺ influx, is also toxic to the cells (reviewed in Kass & Orrenius, 1999; Niederau et al., 1999; Denmeade & Isaacs, 2005). The apoptotic cell death observed was suggested to be explained by mitochondrial Ca²⁺ overload, opening of the mitochondrial permeability transition pore and classical mitochondria driven apoptosis (Bustamante et al., 2004; reviewed in Orrenius et al., 2003; Rizzuto et al., 2003). Though this explanation may hold true in many circumstances, other mechanisms may also be involved, and the role of Ca²⁺ in physiological and pathological conditions may be more complex. For instance, it has been suggested that very high intracellular Ca²⁺ levels promote cell death through necrosis, whereas lower
concentrations induce apoptosis (Choi, 1995), and in some cases Ca\textsuperscript{2+} elevation may be antiapoptotic (reviewed in Berridge et al., 2000; Fahlman et al., 2002; Gray et al., 2005).

1.9.3.1 Targets of Ca\textsuperscript{2+} in cell death
In addition to apoptosis mediated by mitochondrial Ca\textsuperscript{2+} overload and leakage of cytochrome c, cytosolic Ca\textsuperscript{2+} elevation can trigger apoptosis via activation of protein kinases, phospholipases, proteases and endonucleases (reviewed in Rizzuto et al., 2003). Moreover, Ca\textsuperscript{2+} has been demonstrated to contribute to the control of gene expression for different proapoptotic factors belonging to Fas-dependent pathway (reviewed in Berridge et al., 2000). The PKC family plays an important modifying role in Ca\textsuperscript{2+}-mediated apoptosis, dependent upon both the PKC isoform and cell type (Dempsey et al., 2000). Due to variable signaling background in different cell types, a single PKC isoform can exert opposite effects, either accelerating or counteracting apoptosis (Liu et al., 2001; Liu et al., 2002). For instance, some data reports that conventional PKC\(\alpha\) exhibit antiapoptotic effects (Lee et al., 1996; Dempsey et al., 2000) while novel PKC\(\delta\) has been implicated in the mitochondria-dependent apoptotic cell death (Ghayur et al., 1996; Dempsey et al., 2000). Factors such as Ser/Thr-phosphatase calcineurin (protein phosphatase 2B) (Shibasaki & McKeon, 1995), cysteine proteases calpains (Nakagawa & Yuan, 2000) and caspases (caspase-3 and -12) can also mediate Ca\textsuperscript{2+} dependent apoptotic cell death (Yoneda et al., 2001; reviewed in Wang et al., 2005).

1.9.3.2 Anti-apoptotic Ca\textsuperscript{2+} signaling
It has been demonstrated that moderate Ca\textsuperscript{2+} rise together with Ras, ERK1/2, PKB/Akt and PLC signaling is implicated in neuronal survival, protecting cells from apoptotic cell death (Fahlman et al., 2002; Gray et al., 2005). In contrast, larger Ca\textsuperscript{2+} elevations have pro-apoptotic effects (Bickler & Fahlman, 2004). In cooperation with other signaling pathways such as MAPK and PI3K, \([\text{Ca}^{2+}]\), is one of the most important factors controlling cell proliferation, which depends on the temporal pattern of Ca\textsuperscript{2+} signal. For instance, gastrin-stimulated CHO cell proliferation correlates with prolonged, two-hour-long Ca\textsuperscript{2+} oscillations. In contrast, a short-lived Ca\textsuperscript{2+} response to M\textsubscript{3} muscarinic receptor stimulation failed to initiate cell proliferation (reviewed in Berridge et al., 2000).
1.9.4 p53 and cell fate

1.9.4.1 p53
p53 is a tumor suppressor that is normally present in low concentrations and in a repressed state, but stress signals such as DNA damage, hypoxia or oncogenic stress lead to its accumulation and de-repression. Functional p53 modulates various cellular functions such as DNA repair and synthesis, gene transcription, cell cycle arrest, cellular senescence and apoptosis (reviewed in Fridman & Lowe, 2003). The activity and function of p53 are controlled by various post-translational modifications, which influence its stability, subcellular localization, DNA-binding activity and binding to effector proteins (Sakaguchi et al., 1998). p53 mutations, occurring regularly in cancer, result in cell cycle checkpoint defects followed by cellular immortalization and proliferation of damaged cells (reviewed in Fridman & Lowe, 2003).

1.9.4.2 p53 de-repression, stabilization and functional activation
Under resting conditions, p53 protein is under strong negative control by mdm2/hdm2, which is an ubiquitin ligase which stimulates p53 degradation by 26S proteosome (Kubbutat et al., 1997). Stress signals such as UV or ionizing radiation and hypoxia suppress p53 degradation by Ser phosphorylation and diminished interaction with mdm2/hdm2 or by down-regulation of mdm2/hdm2 protein levels (Kishi et al., 2001). However, in the case of oncogenic stimuli, p53 stabilization requires expression of tumor suppressor protein p19Arf, which binds and neutralizes mdm2/hdm2 (Kaji et al., 2002). Ser phosphorylation may also affect p53 tetramerization or DNA binding ability (Fuchs et al., 1995; Huang et al., 1999; Sakaguchi et al., 1997). Moreover, a new natural isoform, ΔN-p53, lacking the first transactivation domain, has been detected. The transcriptional activity of this isoform is reduced and the ability to bind to mdm2/hdm2 totally abrogated. ΔN-p53 mutant may also oligomerize with full-length p53 and in that way negatively regulate its transcriptional activity by acting as a natural inhibitor of p53 (Courtois et al., 2002; Chan et al., 2004).

1.9.4.3 p53-mediated cell cycle arrest
The cell cycle is a very complex and highly organized process controlled by different proteins such as cyclins, cyclin-dependent kinases (CDK), and inhibitors of CDK (CKI) (Obaya & Sedivy, 2002). One of the most important effects of p53 is its ability to block cell cycle. p53 stimulates the expression of p21Waf1/Cip1 which directly inhibits CDK (Vogelstein et al., 2000; Taylor &
Stark, 2001) leading to the inhibition of G1 to S and G2 to M transitions in the cell cycle. Moreover, p53 may cause cell cycle arrest at G2 phase via transcriptional activation of other factors such as Reprimo, 14-3-3σ and GADD45. p53 inhibits the entry to M phase by repression of cyclin B1-dependent CDK (Vogelstein et al., 2000; Taylor & Stark, 2001; Guimaraes & Hainaut, 2002).

1.9.4.4 p53-mediated apoptosis

p53-mediated apoptosis proceeds primarily through the intrinsic apoptotic pathway where p53 acts as a transcription factor of various genes implicated in apoptosis (Bax, Puma, Noxa and Bid, caspase-6, Apaf-1) (Alarcon-Vargas & Ronai, 2002; Juin et al., 2002; Sax & El-Deiry, 2003; reviewed in Fridman & Lowe, 2003). In addition, p53 is able to regulate extrinsic apoptosis pathway in both transcriptional (Fas/CD95 and DR5 death receptors, Fas ligand) and nontranscriptional (Fas translocation) manner (Bennett et al., 1998; Sax et al., 2002; reviewed in Fridman & Lowe, 2003). The survival signaling pathway is also a target for p53-mediated regulation. p53 triggers transcriptional activation of genes of the lipid phosphatase PTEN which shuts-off antiapoptotic PI3K/Akt (reviewed in Fridman & Lowe, 2003). Further, p53 regulates apoptosis via transrepression of antiapoptotic genes such as survivin/IAP, Bcl-2 and MAP4 (Microtubule-associated protein 4) (reviewed in Fridman & Lowe, 2003; Slee et al., 2004). Additionally, p53 promotes apoptosis in a transcription-independent manner by either accumulation in the mitochondria (Mihara & Moll, 2003) or increase of ROS (reactive oxygen species) leading to subsequent cytochrome c release and caspase activation (reviewed in Fridman & Lowe, 2003) (see Table 6).

Table 6. p53-regulated genes and proteins. S, G1, G2 and M indicate the phases of cell cycle where the cell cycle arrest occurs.

<table>
<thead>
<tr>
<th>response</th>
<th>Genomic effects</th>
<th>Non-genomic effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>growth arrest</td>
<td>p21^Waf/Cip1 (G1,G2/M) GADD45 (G2/M) Reprimo (G2) 14-3-3σ (G2)</td>
<td>stimulated expression Inhibited expression</td>
</tr>
<tr>
<td>cell death</td>
<td>BH3-only proteins, BAX, caspase-6, Apaf-1, PTEN, CD95 (APO/Fas), DR5, Fas ligand</td>
<td></td>
</tr>
</tbody>
</table>
1.9.5 MAPK and cell fate

1.9.5.1 ERK1/2

p44/42 MAPK (ERK1/2) activity usually exhibits anti-apoptotic properties. In PC12 cells during NGF (nerve growth factor) withdrawal-induced apoptosis, activation of JNK and p38 MAPK and inhibition of ERK has been observed (Xia et al., 1995). Activation of ERK1/2 leads to inhibition of apoptosis induced by various stimuli such as hypoxia, growth factor withdrawal, H2O2 and chemotherapeutic agents (reviewed in Cross et al., 2000). Proliferative functions of ERK1/2 are mediated via gene expression. In NIH-3T3 cells expressing constitutively active MEK1-estrogen receptor fusion, the activity of which can be regulated by estrogen, MEK1 activity caused up-regulation of cyclin D1 and down-regulation of p27Kip1 expression with subsequent G1 progression (Greulich & Erikson, 1998). ERK1/2 also protects cells from apoptosis by postmitochondrial events at the level of caspses. For instance, B-Raf/MEK/ERK signaling increases the expression or activity of caspase inhibitors, inhibitors of apoptosis (IAP) (reviewed in Erhardt et al., 1999).

Regarding pre-mitochondrial events, ERK1/2-mediated phosphorylation of Bad has been observed (Scheid & Duronio, 1998). ERK1/2 activation may promote opposite cellular processes there final outcome is much dependent on the strength and duration of the response (reviewed in Agell et al., 2002). While transient activation followed by sustained lower ERK activity has been implicated in cell proliferation, sustained strong ERK activation can induce apoptosis or cell cycle arrest with senescence or differentiation (reviewed in Agell et al., 2002). Moreover, proapoptotic effects of ERK have also been observed in neurons cultured in K+-deprived media as well as pathogenesis of Parkinson's disease and during glutamate-induced oxidative synaptic stress (Cheung & Slack, 2004).

1.9.5.2 p38 MAPK and JNK

p38 MAPK and JNK are regularly activated in cells experiencing stress. Their function is to either arrest the proliferation of the putatively damaged cells allowing for cell repair or commit the cells to death (Xia et al., 1995; Davis, 2000; Cheung & Slack, 2004). There is a multitude of pathways engaged in these functions. For cell death, increased expression of proapoptotic proteins such as Bax and inhibited activity of survival factors such as ERK1/2 and PKB/Akt are worth mentioning (Berra et al., 1998; Lei et al., 2002; Park et al., 2002; Naderi et al., 2003; Aikin et al., 2004). On the other hand, activation of p38 MAPK and JNK has a dual role in promoting cell survival or demise, depending on e.g. other simultaneous signals (Park et al.,
Under certain conditions, these kinases can thus be involved in cell growth and differentiation (Juretic et al., 2001; Yosimichi et al., 2001).

1.9.5.3 p38 MAPK and ERK cross-talk

Activation of stress-activated kinases and a concomitant decrease in ERK activity have been observed in a number of cases when cells are undergoing programmed cell death (Berra et al., 1998; Singh et al., 1999). Berra et al. (Berra et al., 1998) report that the decreased basal activity of ERK and the activation of p38 MAPK but not JNK are sufficient to trigger caspase-dependent apoptosis. A simple transient activation of the stress kinase cascades may not always be sufficient to trigger apoptosis and a concomitant inactivation of survival signals may be required (Berra et al., 1998). In NGF-deprived PC12 cells and UV-irradiated mouse fibroblasts, inhibition of ERK activity correlates with the activation of JNK and p38 MAPK and induction of apoptosis (Xia et al., 1995; Berra et al., 1998). Inhibition of the atypical PKC leads to an increase in p38 MAPK activity and decrease in ERK activity followed by caspase-dependent apoptosis in mouse fibroblasts (Berra et al., 1997; Berra et al., 1998). Thus, to attain cell apoptosis, balance between activity of the pro-apoptotic (p38) and pro-survival (ERK) pathways should be shifted towards p38 (Canman & Kastan, 1996; Berra et al., 1998; Singh et al., 1999; Park et al., 2002; Park et al., 2003; Aikin et al., 2004; Ma et al., 2005).

1.9.5.4 Cross-talk between p38 MAPK and p53 in apoptosis

p53 is a known downstream target for activated p38 MAPK in pathways towards cell cycle arrest and cell death. The mechanisms behind this process are phosphorylation-dependent stabilization, functional activation (Bulavin et al., 1999; Huang et al., 1999; She et al., 2000; Kishi et al., 2001) and increase of de novo synthesis of p53 (Shimada et al., 2003). For example, UV-irradiation-induced p38 MAPK activation leads to phosphorylation of p53 at Ser33 and Ser46 with subsequent p53 stabilization and demise of human lung carcinoma cell line A549 (Bulavin et al., 1999). Ser15 and Ser389 have been suggested to be other p38 MAPK-dependent phosphorylation sites important for p53 activation (Keller et al., 1999; She et al., 2000). Other data disclose that mechanical stress-induced DNA damage also activates Rac-p38 MAPK signaling pathway followed by activation of p53 and apoptosis in vascular smooth muscle cells (Mayr et al., 2002). However, it is not always clear whether p38 directly or indirectly phosphorylates p53 as most of the suggested p38 MAPK-dependent phosphorylation sites do not contain p38 MAPK consensus motif (own findings).
1.9.5.4 Caspases in p38 MAPK-mediated cell death

p38 MAPK-mediated cell death, in most cases, involves caspase-dependent pathways (Schrantz et al., 2001; Jang et al., 2004; Rouet-Benzieb et al., 2004). However, caspase-independent mechanisms have also been observed, for example in signaling via ionotropic glutamate receptor (Cao et al., 2004) and upon treatment with Cd$^{2+}$ (Rockwell et al., 2004). In addition, it has also been observed that in some cases the same stimuli can induce both caspase-dependent and -independent pathways depending on the duration of the stimuli, as shown for ceramide induced apoptosis in A-431 human epithelial carcinoma cell line (Zhao et al., 2004). In that case, the short term stimuli leads to initiation of caspase-independent and p38-dependent apoptosis whereas longer incubation trigger both caspase-dependent and -independent pathways (Zhao et al., 2004).

1.9.7 GPCR and cell fate

It has now become known that a number of GPCR are involved in cell fate regulation. There are cases when activation of various GPCRs triggers either apoptotic cell death or survival signaling, though the mechanistic basis for these effects is often incompletely known.

1.9.7.1 GPCR and apoptosis

In a few cases GPCR have been implicated in cell death. The most significant cases are demonstrated in Table 7. As clearly seen, the pathways to cell death are very weakly characterized.
Table 7. Cell death/growth arrest mediated by GPCR. \textit{E} indicates endogenously and \textit{H} heterologously expressed receptors. References: (Sharma et al., 1996; Okazawa et al., 1998; Biard-Piechaczyk et al., 2000; Ferjoux et al., 2000; Saito et al., 2000; Adams & Brown, 2001; Teijeiro et al., 2002; Remondino et al., 2003)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>cell type</th>
<th>response</th>
<th>Signal pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSTR1-3 somatostatin receptors</td>
<td>• CHO (SSTR1) \textit{H} (SSTR2) \textit{H} (SSTR3) \textit{H} • human pancreatic adenocarcinoma (SST2R) \textit{E} • human pro-myelocytic HL-60 (SSTR2) \textit{E}</td>
<td>growth arrest apoptosis</td>
<td>(G_i/G_o) (SSTR1, SSTR2, SSTR3) Ras/ERK (SSTR1, SSTR2) tyrosine phosphatase SHP1 (SSTR2) tyrosine phosphatase SHP2 (SSTR1) p53 activation (SSTR3) BAX expression (SSTR3) p21\textsuperscript{Waf/Cip1} induction (SSTR1)</td>
</tr>
<tr>
<td>ET\textsubscript{B} endothelin receptor</td>
<td>• melanoma A375 \textit{H}</td>
<td>growth arrest apoptosis</td>
<td>(G_i/G_o) p53 activation and nuclear accumulation</td>
</tr>
<tr>
<td>AT1 angiotensin I receptor</td>
<td>• neonatal and adult myocytes \textit{E}</td>
<td>apoptosis</td>
<td>PKC(\delta) and -(\epsilon) ([Ca^{2+}]_i)↑</td>
</tr>
<tr>
<td>(\beta)-adrenergic receptor</td>
<td>• cardiac myocytes \textit{E} • rat ventricular myocytes (ARVMs) \textit{H}</td>
<td>apoptosis</td>
<td>• calcineurin-dependent Bad dephosphorylation, ([Ca^{2+}]_i)↑ • ROS, JNK, cytochrome c, caspases</td>
</tr>
<tr>
<td>CXCR4 chemokine receptors</td>
<td>• HEK293 \textit{H}</td>
<td>apoptosis</td>
<td>caspases p38 MAPK JNK</td>
</tr>
</tbody>
</table>

1.9.7.2 GPCR and cell proliferation

The GPCR may also exert mitogenic effects (Table 8), as demonstrated with neurotensin receptor, NTR1 (Maoret et al., 1999), protease-activated receptors 1/2 (PAR-1 and -2) (Darmoul et al., 2003), sphingosine 1-phosphate receptors 1 (S1P\(_1\), S1P\(_2\) and S1P\(_3\)) (Sato et al., 1999; An et al., 2000; Waeb er et al., 2004; Anelli et al., 2005; reviewed in Spiegel & Milstien, 2003; Radeff-Huang et al., 2004) and LPA\(_1\), LPA\(_2\) and LPA\(_3\) lysophosphatidic acid receptors (Sorensen et al., 2003; reviewed in Siess & Tigyi, 2004; Radeff-Huang et al., 2004). Chemokine receptors are also implicated in cell proliferation (Bajetto et al., 2001) even though some data indicate their participation in apoptosis (Biard-Piechaczyk et al., 2000).
Table 8. Cell growth stimulation mediated by GPCR. \( E \) indicates endogenously, \( Ec \) ectopically and \( H \) heterologously expressed receptors. References: (Maoret et al., 1999; Sato et al., 1999; An et al., 2000; Darmoul et al., 2003; Sorensen et al., 2003; Darmoul et al., 2004; Waeger et al., 2004; Anelli et al., 2005; reviewed in Bajetto et al., 2001; Bajetto et al., 2001; Spiegel & Milstien, 2003; Radeff-Huang et al., 2004; Siess & Tisgy, 2004)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell type</th>
<th>Signal pathway</th>
</tr>
</thead>
</table>
| CXCR4 chemokine receptor | • melanocytes \( E \)  
• rat type-1 astrocytes \( E \) | p38 MAPK  
\( G_\text{i}/G_\text{o} \), ERK1/2  
P13K, Pyk2, Akt |
| PAR-1 protease-activated receptor | • HT29 \( Ec \)  
• human colon cancer cells \( Ec \)  
• osteoblasts \( E \)  
• keratinocytes \( E \)  
• astrocytes \( E \) | ERK1/2  
[Ca\(^{2+} \)]\(_i\)↑  
[Ca\(^{2+} \)]\(_i\)↑  
[Ca\(^{2+} \)]\(_i\)↑  
[Ca\(^{2+} \)]\(_i\)↑ |
| PAR-2 protease activated receptor | • human colon cancer HT29 \( E \) | ERK1/2, EGFR-transactivation via Src, JNK, p38 MAPK |
| S1P\(_1\), S1P\(_2\) and S1P\(_3\) sphingosine 1-phosphate receptors | • Swiss 3T3 \( H \)  
• astrocytes \( E \)  
• endothelial cells \( E \)  
• vascular smooth muscle cells \( E \)  
• HTC4 hepatoma cells \( H \)  
• C6 glioma cell line \( H \)  
• breast cancer cell lines \( H \) | \( G_{12/13} \), Rho  
\( G_\text{q} \), Ras |
| LPA\(_1\), LPA\(_2\) and LPA\(_3\) lysophosphatidic acid receptors | • Rat1 cells \( H \)  
• human foreskin fibroblasts \( E \)  
• HeLa \( H \)  
• PC12 cells \( H \)  
• VSMC (vascular smooth muscle cells) \( E \)  
• astrocytes \( E \)  
• neuronal cells \( E \) | \( G_{12/13} \), Rho  
\( G_\text{q} \), Ras  
\( G_i \) |
| NTR1 neurotensin receptor | • human colon cancer HT29, SW480, SW620 \( Ec \)  
• human colon cancer cells \( Ec \)  
• prostate and pancreatic cancer cells \( Ec \) | PLC  
\( IP_3 \), [Ca\(^{2+} \)]↑ |

1.9.7.3 GPCR and cell survival

In contrast to pro-apoptotic properties, many GPCR have been implicated in protection against apoptosis induced by growth factor withdrawal, DNA damage and/or cell stress (see Table 9). Among these receptors are muscarinic receptors, which possess anti-apoptotic property in a number of neuronal and non-neuronal cell lines and primary cells (Leloup et al., 2000; De Sarno et al., 2003; Tobin & Budd, 2003). Moreover, lysosphosphatidic acid receptor 1 (LPA\(_1\)) and spingosine 1-phosphate receptor 1 (S1P\(_1\), S1P\(_2\), S1P\(_3\))
are also implicated in the protection from apoptosis (An et al., 2000; Grey et al., 2002; reviewed in Spiegel & Milstien, 2003; Radeff-Huang et al., 2004). However, some reports indicated that LPA receptors play a pro-apoptotic role (reviewed in Radeff-Huang et al., 2004).

Table 9. Cell survival mediated by GPCR. E indicates endogenous and H heterologously expressed receptors. References: (Leloup et al., 2000; De Sarno et al., 2003; Tobin & Budd, 2003; reviewed in Radeff-Huang et al., 2004).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell type</th>
<th>Signal pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1, M3, M5 muscarinic receptors</td>
<td>- CHO cells (M1, M3, M5) H</td>
<td>Bcl-2 expression ↑ (M1)</td>
</tr>
<tr>
<td></td>
<td>- COS-7 (M3) H</td>
<td>PI3K/Akt</td>
</tr>
<tr>
<td></td>
<td>- SH-SY5Y E</td>
<td>cytochrome c release ↓</td>
</tr>
<tr>
<td></td>
<td>- PC12 (M1) H</td>
<td>bcl-2 expression↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bax accumulation ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>caspase inhibition by ERK, PI3K</td>
</tr>
<tr>
<td>LPA1 lyso-phosphatidic acid receptor</td>
<td>- ovarian cancer cells</td>
<td>Gi, Rho, PI3K, Akt, ERK, eNOS</td>
</tr>
<tr>
<td></td>
<td>- intestinal epithelial cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- fibroblasts</td>
<td></td>
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<tr>
<td></td>
<td>- osteoblasts</td>
<td></td>
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<tr>
<td>S1P1, S1P2, S1P3 sphingosine 1-phosphate receptors</td>
<td>- melanocytes</td>
<td>Gi, Rho, Rac, PI3K, Akt, ERK, eNOS, p38 MAPK</td>
</tr>
<tr>
<td></td>
<td>- HTC4 hepatoma cells H</td>
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<td>- PC12 H</td>
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<td>- keratinocytes</td>
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<td></td>
<td>- CHO H</td>
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</table>
2. Aims of the study

As indicated in the Introduction, little is known about the cellular signaling of orexin receptors. The major aim of these studies was to map the different outputs of OX₁ receptors in order to gain knowledge that later would to be applied in endogenous systems. Recombinant CHO cells were chosen as a model system since they are stable, can be obtained in large quantities – in contrast to most orexin-responding native cells – and can be easily manipulated with molecular biological constructs. The outputs investigated were regulation of adenylyl cyclase (Paper II), ERK1/2 activity (Paper III) and cell death (Paper IV). Upon significant and, in part, unexpected discoveries, the studies were directed towards investigations of molecular mechanisms behind those different signaling pathways. We also investigated the molecular determinants of orexin peptide–orexin receptor interaction in order to i) find receptor subtype-selective peptides and ii) to find explanations to the reputed distinction of orexin-A and -B between OX₁ and OX₂ receptors (Paper I).
3. Methods

3.1 Cell culture

Chinese hamster ovary (CHO) (Papers I, II, III and IV) and Neuro-2a murine neuroblastoma (Paper III) express heterologously either the OX1 or the OX2 receptor. The cell lines were created by transfection of vectors containing cDNA for OX1 or OX2 receptors with liposome-forming reagents. Single-cell-based (CHO) or pan-clones (Neuro-2a) were isolated under selection pressure from Geneticin. The cell lines thus created were cultured in Ham's F-12 (CHO) or DMEM medium (Neuro-2a) supplemented with Geneticin, penicillin, streptomycin and fetal calf serum in a humidified incubator at 37 °C. For different assays, the cells were cultured in plastic culture flasks (bottom area 75-80 cm²), plastic culture dishes (Ø 32, 52 or 82 mm), 24- or 96-well plates or glass coverslips (Ø 25 mm).

For some experiments in Paper II, we have used rat primary striatal neurons. These were prepared form newborn (day 0-1) Sprague-Dawley rat pups (permission no. C 105/4, Tierps Tingsrätt, Tierp, Sweden, 2004). The isolated neuron-glial co-cultures were maintained in Minimum Essential Medium-Neurobasal medium mixture (50:50) supplemented with gentamicin, glucose, l-glutamine, insulin, B27, fetal calf serum and cytosine arabinoside (to inhibit glial over-growth) in the incubator as above. All the cells were cultured on polyethyleneimide-coated glass coverslips (Ø 13 mm).

3.2 Transient transfections

Most transient transfections of CHO cells using Lipofectamine were performed in order to introduce inhibitors of signal pathways. The signal pathways "attacked" were the following: Paper II: Gβγ (Gβγ scavengers); Paper III: Ras (dn-Ras), PI3K (PTEN, dn-p85α), Src (Csk, dn-Src), PKC (dn-PKCζ), cAMP (cAMP-phosphodiesterases); Paper IV: p53 (dn-p53). Secondly, transfections were used to introduce GFP- (green fluorescent protein; GFP- PLCδ1-PH in Paper III and GFP-PKCe in Papers II and III, p53-GFP in Paper IV and GST- (glutathione S-transferase; GST-ERK2) in Paper III. Thirdly, the cells were transfected with α2A- and β2-adrenoceptors to be used as a receptor-dependent positive control for Gβγ respectively Gs activation.
(Papers II and III), and with G\(\beta\gamma\) as a receptor-independent positive control for G\(\beta\gamma\) (Paper II). As transfection efficacy varied between 30 and 70\%, specific procedures were designed to correctly evaluate the inhibitor efficacy. In Ca\(^{2+}\) measurements (Papers II and III) and in evaluation of cell death, cotransfections of the "inhibitors" together with a GFP expressing plasmid allowed identification of the transfected cells. For Western blot analysis of ERK phosphorylation, GST-ERK (pEBG-ERK2), which migrates 25 kDa above the endogenous ERK, was introduced together with the "inhibitor" plasmids.

3.3 SDS-PAGE and immunoblotting

Western blotting was performed to detect specific protein expression levels (Papers II, III and IV) and levels of phosphorylated MAPK subfamilies (Papers III and IV). For detection of phospho-MAPK subfamilies, the cells were serum-starved for 24 h before the start of the experiment. The cells were lysed in the presence of phosphatase and protease inhibitors, the protein concentrations determined and the proteins separated by SDS-PAGE electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with specific antibodies against phosphorylated (active) ERK1/2, p38 MAPK and JNK (Papers II and III) and total ERK1/2, actin, p53 and PKC\(\delta\), -\(\epsilon\), -\(\eta\) and -\(\theta\) (Papers II, III and IV). Either actin (Papers II and IV) or total ERK (Paper III) was used as a loading control. The primary antibodies were detected using peroxidase conjugated donkey anti-rabbit or rabbit anti-mouse antibodies. Peroxidase enzymatic activity was visualized using a chemiluminescence assay and the exposed films were quantified densitometrically.

3.4 Immunocytochemistry

In order to detect activation of ERK1/2 in cultured primary striatal neurons, immunocytochemistry method was applied to diminish amount of cells needed for analysis compared to immunoblotting. Coverslips with cells were serum-starved for 24 h, stimulated and fixed/permeabilized in methanol. The cells were incubated with primary antibodies against phospho-ERK1/2 and the secondary Texas red-labeled antibody. After mounting on glass slides, the fluorescence was monitored by fluorescence microscopy.
3.5 PLC activity

PLC activity was determined by three different methods. In the first method, the cells were prelabeled with $[^3]$H-inositol and the tritiated inositol phosphates released upon receptor stimulation were isolated using anion-exchange chromatography and formic acid/NH$_4^+$-formate buffers of increasing ionic strength (Papers II and III).

The two other methods were based on translocation of GFP-fusion proteins (Papers II and III). Two different constructs were used: PKC$_{\varepsilon}$, which specifically binds to DAG and thus upon DAG generation (= PLC or PLD [phospholipase D] activity) translocates from the cytosol to the plasma membrane (Papers II and III), and the PH-domain of PLC$_{\varepsilon}$1, which specifically binds PIP$_2$ and IP$_3$ thus translocating from the plasma membrane to the cytosol upon PIP$_2$ breakdown (= PLC activation). These measurements were performed on cells cultured on glass coverslips mounted in a thermostated perfusion chamber (37 °C) of a confocal microscope (Bio-Rad Radiance 2100) or fluorescence imaging system (TILLvisION v. 4.01).

3.6 cAMP measurements

cAMP measurements were performed using intact or permeabilized cells in Papers II and III. For intact cells, the cells were preloaded with $[^3]$H-adenine which upon entering the cell is incorporated in ATP. Subsequently, $[^3]$H-ATP is converted into $[^3]$H-cAMP upon cell stimulation. For permeabilized cells, radiolabeled ATP was instead supplied directly as $[^3]$P-ATP. To avoid ATP depletion, an enzymatic regeneration system was included. In both types of experiments, phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (IBMX) was included in most of the experiments to allow larger cAMP accumulation. Radiolabeled cAMP was then isolated using sequential Dowex cation-exchange/alumina "affinity" chromatography.

3.7 Ca$^{2+}$ Measurements

All the Ca$^{2+}$ measurements were performed using fluorescent Ca$^{2+}$ indicators fura-2 and fluo-3. These indicators can be loaded in the cells as cell-permeant acetoxymethyl esters, which upon hydrolysis by the intracellular non-specific esterases release the free, Ca$^{2+}$-responding dyes. The advantage of fura-2 is that it allows ratiometric Ca$^{2+}$ measurements, which reduces non-Ca$^{2+}$-associated fluorescence artifacts. On the other hand, fluo-3 displays a much higher fluorescence increase upon Ca$^{2+}$ binding and has a lower affinity for Ca$^{2+}$, allowing more exact measurements of higher Ca$^{2+}$ levels. Ca$^{2+}$ levels were measured either with detached CHO cells in suspen-
sion (Papers I and III) with Fura-2 or Fluo-3 using Hitachi F-2000 and F-4000 fluorescence spectrophotometers or attached CHO cells on glass cover-slips (Papers II and III) with fura-2 using TILLvisION microscopic imaging system. Use of cells suspensions allows much faster generation of pharmacological data because of averaging of large numbers of cells and speed of the procedure, whereas imaging distinguishes responses in individual cells and allows much more flexible manipulation of the experiments because of the perfusion system. In all the cases, the cells were kept in constant temperature of 37 °C. The experimental medium was supplemented with probenecid, which inhibits the active extrusion of fura-2 and fluo-3 out of the cells.

3.8 Assessment of cell viability

Cell viability was mostly assessed using MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay (Paper IV). MTT stains viable mitochondria blue whereas the cells with non-functional mitochondria (= dead cells) remain non-stained. MTT staining was quantified either spectrophotometrically (absorbance at 690 nm) or by manual counting of stained and non-stained cells. Results of these two protocols were in good agreement and the data was thus mostly pooled.

Hoechst 33342 was used for staining of DNA (Paper IV). This was either to allow manual counting of condensed and non-condensed nuclei or as a counterstaining to allow identification of total numbers of cells in a fluorescence assay. Some experiments also included use of propidium iodide, which can only enter dead/dying cells (Paper IV). Total number of cells was obtained from Hoechst-staining.

3.9 Flow cytometric analysis of cell cycle

To assess the number of cells in specific phases of cell cycle, flow cytometry method was utilized (Paper IV). For this purpose, the cells were harvested, fixed in ethanol, and after RNase treatment, stained with propidium iodide. A minimum of 10000 cells was run from each sample and the intensity distribution of these analyzed using standard protocols of the FACSCalibur instrument.
3.10 Peptide synthesis
The truncated and alanine-scanned orexin-A peptides were synthesized using a peptide synthesizer (Paper I). The synthesized peptides were purified by reverse phase-HPLC and the quality controlled by reverse phase-HPLC and MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry.

3.11 Calculations and data analysis
Statistical significances were evaluated using Student's two-tailed $t$ test (paired or non-paired, depending on the data setup) or, in the case of more complex comparisons, ANOVA (analysis of variance) followed by Tukey's post-hoc test. Nonlinear curve fitting to determine the parameters of the peptide concentration-response curves was performed using SigmaPlot for Windows 4.01/4.1. F-test was used to evaluate the goodness of fit between cooperative or non-cooperative models (Paper I).
4. Results

4.1 Both OX₁ and OX₂ receptors require very similar determinants from orexin-A for activation (Paper I).

In this study we compared the abilities of orexin-A and orexin-B and mutated variants of orexin-A to trigger peak Ca²⁺ response in CHO cells expressing either human OX₁ or OX₂ receptors. The results of this study confirmed the previous results that there is a difference in the potency between orexin-A and orexin-B for OX₁ but not for OX₂ receptors with respect to this response. Thus the difference between both peptides is distinguished only by OX₁ receptors. When the extracellular Ca²⁺ was reduced, a right-shift in the EC₅₀ values of both orexin-A and -B peptides for both receptors was observed. The shift was more pronounced for orexin-A than for orexin-B. In agreement with our previous findings (Lund et al., 2000; Holmqvist et al., 2002) this shows that increase in intracellular Ca²⁺ level caused by orexins depends on the extracellular Ca²⁺ even for OX₂ receptor, and suggests that Ca²⁺ influx may be an important signal for both orexin receptor subtypes. The maximum response was not affected, but apparent cooperativity (n_Hill) for all the combinations of receptors and peptides was increased in the absence of extracellular Ca²⁺.

In this study we were mainly interested in investigation of requirements from orexin-A for interaction with the orexin receptors. Removal of the C-terminal part of the peptide, which, based on the high homology of orexin-A and orexin-B in this part can be thought to be of major importance for receptor interaction, lead to full abolishment of the response. Truncation of the N-terminal part of the orexin-A to orexin-A₁₄₋₃₃ (20 aa), which removes sulfhydryl bridges, reduced the potency for both OX₁ and OX₂ receptors. Interestingly, this truncation eliminated the differences between both receptors with respect to orexin-A. The maximum response was not affected. Further truncation of the N-terminal residues successively decreased peptides potency for both receptors. Truncation of the peptide to the length of 12 aa (orexin-A₂₂₋₃₃) totally abolished the response to 1 µM of the peptide for both OX₁ and OX₂ receptors, while the response to 10 µM of the peptide was retained. The response to 10 µM of the peptide for OX₁ receptor was obliterated upon
truncation to 10 aa (orexin-A24-33). OX2 receptor was more resistant to the truncation; the response was first completely abolished upon truncation to 6 aa (orexin-A28-33).

The next step in our investigation was to identify specific orexin-A residues critical for binding and activation of the receptors using alanine-scan. Instead of full-length orexin-A, orexin-A14-33 was chosen for this to avoid possible complex effect of removal of disulfide bridges. For the alanine-scan, aa residues 14-26 were successively replaced with alanine. Three areas with a significant decrease in the peptides potency were observed: amino acids 15-17, 20 and 25-26. The most dramatic reduction in the potency was observed with mutation of Leu20 to alanine where the potency for the OX1 receptor was reduced 25-fold and the potency for the OX2 receptor 11-fold.

A complete alanine-scan of the orexin-A15-33 with respect to OX1 receptor activation was published while we were performing our experiments (Darker et al., 2001) showing results mostly similar to ours. This study also showed that the receptor response is extremely sensitive to mutations in the outmost C-terminus (aa 26-33), as can be expected from the high homology in the C-termini of the native orexin peptides. Our results reveal that the similar areas/residues in orexin-A are important for both for OX1 and OX2 receptor activation. Alanine-scanned peptides activated the OX2 receptor with a potency very similar to that for the OX1 receptor. However OX2 receptor was less sensitive to those mutations.

These results suggest that both OX1 and OX2 receptors require very similar determinants from orexin-A to allow receptor binding and activation. However, OX2 receptors appear more resistant to truncation and mutation of orexin-A than OX1 receptors. Thus OX2 receptor may have less strict requirements for ligand binding, and minimum binding to OX2 receptor may be obtained with a lower number of interacting sites.

We also looked at the responses to orexin-A and -B and some selected alanine-scanned peptides with respect to Ca2+ responses in the absence of extracellular Ca2+. The potency of orexin-A was more reduced than that of orexin-B by removal of extracellular Ca2+. Both peptides showed increased $n_{Hill}$ (see below) Orexin-A14-33 showed similar shift as full-length orexin-A.

Most interestingly, some of the alanine-scanned peptides showed smaller shifts than orexin-A whereas some showed markedly larger shifts. This was interpreted as possible agonist-mediated signal-trafficking.

All peptides displayed slope factors above 1 and in addition many of the truncated and alanine-scanned peptides, through not orexin-A itself, displayed slope factors above 2.5, suggesting co-operative binding or signaling. In case of G-protein coupled receptors co-operativity could be caused by formation/presence of receptor di-/oligomers, but might also originate from a functional level. Experiments with orexin-A and -B in high and low extracellular Ca2+ indicate that the absence of amplification signals (low extracellu-
lar Ca\(^{2+}\)) led to even higher slope factors. Synergistic effect thus seems to reduce the apparent co-operativity.

4.2 The OX\(_1\) orexin receptor utilizes multiple mechanisms in adenylyl cyclase (AC) regulation (Paper II)

In this study we demonstrate a strong orexin-mediated elevation of cAMP in CHO cells expressing OX\(_1\) receptors. We therefore determined the basal regulatory properties of the AC isoforms expressed in CHO cells. The experiments were performed in the presence of the phosphodiesterase inhibitor IBMX in order to avoid any regulatory effects on cAMP phosphodiesterases. The cyclase was highly stimulated by G\(_s\) (cholera toxin, PGE\(_1\), \(\beta_2\)-adrenoceptors), while only weak stimulation was obtained via protein kinase C (phorbol ester TPA). Also calcium elevation (thapsigargin, ionomycin) and G\(\beta\gamma\) did not produce any significant AC stimulation. Thus, it appeared most likely that if OX\(_1\) receptors were to stimulate cAMP accumulation, this would be likely to occur via G\(_s\) and/or PKC. To simplify the situation, we treated the cells with cholera toxin at a concentration that produced apparently full saturation of AC with G\(_s\). Under these conditions, both orexin-A and the PKC stimulator TPA still produced a significant cAMP accumulation. This orexin-A response was fully inhibited by the PLC inhibitor U-73122 and the non-selective PKC inhibitor GF109203X, but not by the inhibitor of the conventional (Ca\(^{2+}\)-stimulated) PKC, Gö6976. In addition, the TPA response was fully inhibited by GF109203X, but not by Gö6976. However, TPA-stimulated cAMP accumulation was further potentiated by Ca\(^{2+}\) elevation (thapsigargin) and this potentiation was fully inhibited by Gö6976, confirming the selectivity of this inhibitor. Thus, these data suggests that in the absence of any possible G\(_s\) signal, orexin-A-induced AC activity is fully dependent on some novel PKC isoform.

In control cells, i.e. in absence of cholera toxin or \(\beta_2\)-adrenoceptor stimulus, PLC (U-73122) or PKC inhibition (GF109203X) only produced approximately 50% inhibition of the orexin-A-stimulated cAMP accumulation. The remaining part of the response was hypothesized to be mediated by activation of G\(_s\) by the OX\(_1\) receptors. In order to inhibit G\(_s\), we wanted to apply anti-G\(_s\) antibodies. In order to get these antibodies into the cells, the cells were permeabilized using digitonin. In the permeabilized cells, the response to orexin-A was inhibited by anti-G\(_s\) antibodies in an equal degree as that to \(\beta_2\)-adrenoceptor stimulation. In addition, there was no synergism between orexin-A and \(\beta_2\)-adrenoceptor stimulation. These data strongly suggest that in permeabilized CHO cells, orexin-A simulates AC solely via G\(_s\), and the PKC coupling is for some reason non-functional.
To determine which novel PKC isoform was responsible for the PKC-dependent part of orexin-A-induced cAMP accumulation, we investigated novel PKC isoform expression in CHO cells. In agreement with a previous study (Hill et al., 2003), we only found PKCδ and -ε in these cells. Inhibition of PKCδ with rottlerin inhibited orexin-A-stimulated cAMP activation in the same degree as U-73122 and GF109203X whereas inhibition of PKCε with the membrane-permeable peptide inhibitor KIE1–1 did not affect the orexin-A response. Thus, PKCδ seems to be the PKC isoform involved in this response.

In addition to the cAMP elevation, we could also observe a weak cAMP decrease at low orexin-A concentrations (1-10 nM). This response was fully reversed by pertussis toxin, indicating involvement of Gi/o proteins.

The potency of orexins to stimulate cAMP accumulation was low in intact cells (EC₅₀ [orexin-A]=300 nM), EC₅₀ [orexin-B]=500 nM) and the "usual" potency difference was essentially absent. In the cholera toxin-treated cells, the potencies of orexin-A and -B were highly enhanced (EC₅₀<9 nM resp. 100 nM), and the potency difference (≅10-fold) was restored. These potencies are very close to the potencies for Ca²⁺ elevation and inositol phosphate production. Transient expression of β₂-adrenoceptors and activation of these receptors produced a similar (or even stronger) shift in the potency of orexin-A as cholera toxin confirming that this is mediated by an effect of Gs on AC. In permeabilized cells, the potency of orexin-A was equal to the potency in intact cells in the absence of Gs stimulus.

Altogether, the results suggest that OX₁ receptors couple to AC stimulation through high-efficacy PKCδ-coupling possibly via Gs ⇒ PLCβ as well as low-efficacy Gs coupling. The inability of the phorbol ester TPA to strongly stimulate AC activity in the absence of simultaneous Gs activation suggests that Gs stimulation is required even for OX₁-mediated PKC activation to stimulate AC, and that the low efficacy of Gs coupling of OX₁ receptors thus determines the overall potency of the response. In addition, OX₁ receptors are able to couple to Gi/o proteins at high efficacy.

4.3 Stimulation of the OX₁ orexin receptor leads to extracellular Ca²⁺ influx-dependent activation of ERK in CHO cells (Paper III)

In this study we demonstrate that orexins (orexin-A and -B) are able to cause ERK1/2 phosphorylation via both the OX₁ and OX₂ receptor in a time-and concentration-dependent manner. In the studies we focused on the mechanism of ERK phosphorylation for OX₁ receptors. The relative potencies of both peptides for the OX₁ receptor (orexin-A was 14-fold more potent than orexin-B) correlate with a previous studies where Ca²⁺ responses and receptor
binding have been measured (Sakurai et al., 1998) (Paper I). However, in comparison with the potencies for Ca\(^{2+}\) elevation, the potencies of orexins for ERK1/2 activation were 10 times lower (Paper I) yet they were markedly higher than for cAMP elevation (Paper II). We could also observe a similar ERK activation in OX\(_1\) receptor-expressing Neuro-2a cells and cultured rat striatal neurons. However, detailed studies were performed in OX\(_1\) receptor-expressing CHO cells. The response was rapid (full response was obtained within 3 min) and relatively long-lasting (not fully reversing during 3 h).

4.3.1 Involvement of classical signal pathways to ERK

The OX\(_1\) receptor-induced ERK phosphorylation was fully inhibited by an inhibitor of MEK1 activation, U0126, and dominant-negative MEK1. Also rather expectedly, co-expression of dominant-negative mutants of the classical Ras proteins (H-, K- and N-Ras) almost fully abrogated the response. Involvement of PLC ⇒ PKC pathway was assessed using the PLC inhibitor U-73122 and the PKC inhibitors GF109203X (conventional and novel PKC) and Gö6976 (conventional PKC). All these compounds inhibited the response by ≈50-60%, as also did long-term (24 h) TPA treatment, which is thought to downregulate PKC. The results upon treatment with Gö6976 suggests that this response is mediated via PLC-dependent activation of conventional PKC. In addition, dominant-negative constructs of atypical PKC\(\zeta\) produced inhibition by ≈50-60%. Pathways mediated by PIP\(_3\) were targeted by PI3K inhibitors wortmannin and LY294002, dominant-negative p85\(\alpha\) and the PIP\(_3\) 3-phosphatase PTEN. All these constructs produced inhibition of an equal degree, ≈60%. PKC and PI3K, suggested to lie in the same signal cascade as GF109203X, did not produce any further inhibition when applied together with wortmannin or dominant-negative p85\(\alpha\). Src, an archetypical non-receptor tyrosine kinase, was targeted using Src inhibitor SU6656 and over-expression of dominant-negative Src and the physiological Src-inhibitor, Csk. Csk and dominant-negative Src produced an inhibition of ≈60%; however, SU6696 only inhibited the response by 30%.

Thus, most "usual" players in the activation of ERK cascades seem to be involved in the OX\(_1\) receptor-induced ERK phosphorylation. The results, however, do not show the internal relationships of these proteins. In contrast, cAMP elevation (Paper II), does not appear to be involved in ERK phosphorylation induced by OX\(_1\) receptors, although CHO cells have previously been suggested to display Epac- and Rap-dependent ERK activation in response to cAMP. Most convincingly, 100 nM orexin used in most experiments does not result in any measurable cAMP elevation in the absence of the phosphodiesterase inhibitor IBMX. We could also observe that exogenously applied 8-Br-cAMP and forskolin were very weak in stimulating ERK phosphorylation and exogenous expression of cAMP phosphodiesterases only weakly inhibited orexin-A-induced ERK phosphorylation.
4.3.2 Involvement of Ca$^{2+}$ signaling in the ERK response

From previous studies we know that activation of OX$_1$ receptors causes Ca$^{2+}$ influx as the primary response (Lund et al., 2000). Rather surprisingly, removal of extracellular Ca$^{2+}$ fully reversed the orexin-A-induced ERK phosphorylations in OX$_1$ receptors-expressing CHO and Neuro-2a cells and in striatal neurons. Further experiments in CHO cells showed that the ERK response could also be blocked by Ni$^{2+}$, which inhibits the receptor- and store-operated Ca$^{2+}$ influx pathways but not by 2-APB, that only blocks the store-operated pathway. Thapsigargin, an inhibitor of ER Ca$^{2+}$ pumps (SERCA pumps), which also activates the store-operated Ca$^{2+}$ influx, caused ERK phosphorylation that was inhibited by removal of extracellular Ca$^{2+}$ and both by Ni$^{2+}$ and 2-APB. Ca$^{2+}$ elevation-induced ERK response (thapsigargin, ionomycin) was very transient and much weaker than that induced by OX$_1$ receptor stimulation.

Thus, Ca$^{2+}$ influx occurs either via the receptor-operated pathway alone or via this pathway together with the store-operated pathway. Some compounds have recently been identified as more selective blockers of the receptor-operated pathway (Larsson et al., 2005). However, these compounds produced apparently non-specific effects of the ERK cascade and the data was not conclusive. Instead, we utilized the fact that thapsigargin somehow inactivates the receptor-operated pathway. Thapsigargin, however, also activates the store-operated pathway. After application of thapsigargin, orexin-A still induced ERK phosphorylation to an equal degree as in the absence of thapsigargin. This suggests that Ca$^{2+}$ influx via either pathway, receptor-operated or store-operated, is enough to support OX$_1$ receptor-mediated ERK phosphorylation.

There are two reasonable explanations concerning the dependency of ERK activation on Ca$^{2+}$ influx: 1) Ca$^{2+}$ itself affects ERK phosphorylation via PKC and other Ca$^{2+}$ sensitive effectors like PLC and RasGEFs, likely together with other receptor-mediated signals thus generating much stronger and more long-lasting ERK activation; 2) Ca$^{2+}$ elevations are important for the receptor to couple to intracellular signaling pathways. The first and the second possibilities are difficult to separate, because we cannot activate only the orexin receptor-operated Ca$^{2+}$ channels without activating the receptor, and even if we could, we could not then investigate the synergistic effects of this influx and other receptor-mediated signals. Instead, we decided to look into other effectors of orexin receptors in CHO cells, AC and PLC. Activation of AC was also seen to require Ca$^{2+}$ influx, yet there obviously was no Ca$^{2+}$-sensitive AC expressed in CHO cells (as also shown in Paper II). We had previously shown for PLC that its activation at low orexin-A concentrations requires Ca$^{2+}$ influx and although it is modestly activated by Ca$^{2+}$, the response to orexin receptor stimulus is several-fold higher (Lund et al., 2000). Thus, for both AC and PLC response we can suggest that the effect of...
Ca$^{2+}$ lies upstream of the effectors themselves (AC, PLC), which in these particular cases would likely mean at the receptor. We thus suggest that there is a local, submembrane Ca$^{2+}$ elevation which allows coupling of the activated receptor to cascades leading to downstream effectors like MEK1, AC and PLC. A molecular mechanism for this effect cannot be suggested yet.

4.4 The OX$_1$ orexin receptor induces programmed cell death through p38 MAPK in both classical caspase-dependent and caspase-independent manner bypassing p53 activation (Paper IV)

We observed that exposure to orexin-A triggered death of CHO cells expressing either OX$_1$ or OX$_2$ receptors. During our studies, another group reported that OX$_1$ receptor-expressing CHO cells undergo apoptotic cell death upon exposure to either orexin-A or -B (Rouet-Benzineb et al., 2004). However, in contrast to this study, cell demise was induced in our CHO cells only in the absence of fetal calf serum. We, once again, focused on the OX$_1$ receptors when investigating the mechanisms in more detail. Cell demise was time- ($t_{1/2}$=36 h) and concentration-dependent (EC$_{50}$ [orexin-A]=20 nM), following classical program of cell death (apoptosis), i.e. showing caspase-dependent chromatin condensation as well as dependency on protein synthesis and gene transcription. OX$_1$ receptor activation caused activation of ERK and p38 MAPK pathways. p38 MAPK activation was strong and still significant after 48 h, while ERK activation was reversed prior to the 24 h time point. Inhibition of p38$_{\alpha}$ and -$\beta$ with SB203580 completely reversed the orexin-A-induced cell death, whereas inhibition of MEK1 activation (and thus ERK activation) with U0126 sensitized the cells to orexin-A-induced cell death. In contrast, orexin-A-mediated cell death was not affected by the JNK inhibitor SP600125, PKC inhibitor GF109203X, PI3K inhibitor wortmannin or buffering of [Ca$^{2+}$], with BAPTA acetoxyethyl ester. Thapsigargin, the ER Ca$^{2+}$ pump (SERCA pump) inhibitor that causes persistent elevation in [Ca$^{2+}$], also induced apparently apoptotic death of CHO cells. In contrast to orexin-A, this was not inhibited by SB203580 but in part reversed by BAPTA acetoxyethyl ester. Thus, orexin-A-induced programmed cell death seems independent of Ca$^{2+}$ elevation but dependent on p38 MAPK, whereas the case for thapsigargin is the opposite.

Treatment of the CHO cells with the pan-caspase inhibitor z-VAD-fmk fully reversed chromatin condensation induced by OX$_1$ receptor activation, however, the cell death was not inhibited. The cell death was still dependent on p38 MAPK and *de novo* protein synthesis, but it did not require gene transcription. Thus, it appears that caspase inhibition leads to engagement of an alternative cell death pathway from p38 MAPK.
p53 has been suggested as one central downstream effector for p38 MAPK in cell cycle arrest and cell death. This interaction could theoretically occur via p53 stabilization (inhibition of interaction with mdm2/hdm2), increased transcription of p53 gene or via functional enhancement of p53 such as an increase of DNA binding. We could observe a slight but non-significant increase of p53 level. However, expression of several dominant-negative p53 constructs did not inhibit the cell death. Also, no transcriptional activity of p53 could be observed when p53-dependent reporter plasmid (p53-GFP) was used. We thus conclude that p38 MAPK most likely mediates effects via targets other than p53.
5. Discussion

5.1 Orexin receptor–peptide recognition

The results of the Paper I are mostly in agreement with the previous studies (Darker et al., 2001; Okumura et al., 2001). Thus, similar areas in orexin-A are recognized by OX₁ and OX₂ receptors. Therefore, approaches to development of subtype-selective ligands for orexin receptor subtypes can not be derived from this study. In contrast to orexin-A, orexin-B has been shown to display selectivity for OX₂ receptor at the level of receptor binding and Ca²⁺ elevation (e.g. Sakurai et al., 1998). This is also seen in Paper I. Since the C-termini of orexin-A and -B are very similar, this selectivity should lie in the N-terminus of orexin-B, which is clearly different from that of orexin-A. We can confirm the low potency of orexin-B also for other OX₁ receptors responses such as cAMP elevation in the cholera toxin-primed CHO cells (Paper II) and ERK phosphorylation in CHO cells (Paper III). However, in contrast to these data and other studies with recombinant expressed orexin receptors, there is essentially no difference in the ability of orexin-A and -B to elevate cAMP in control CHO cells, a response likely to be determined by receptor coupling to Gs. Similarly, the death of OX₁ receptor-expressing SK-N-MC cells does not show any pharmacological selection between orexin-A and -B (Rouet-Benzineb et al., 2004). Thus, it appears that orexin-A and -B can show signal-trafficking (Kukkonen, 2005) i.e. induction/stabilization of different active states of OX₁ receptors, which can then differentially interact with G-proteins. Thus, orexin-A and -B could show differential signaling in physiological situations. Therefore, the difference in potency between orexin-A and -B cannot be used as a proof for involvement of OX₁ receptors.
5.2 Primary coupling of OX₁ receptors

The results of the studies, especially those of Paper II, suggest that OX₁ receptors can functionally couple to at least three G-protein families, namely G₁₁₀, G₆ and G₉₁₁. This is in accordance with previous studies with OX₂ receptors utilizing GTP-azidoanilide labeling of activated G-proteins (Karteris et al., 2001; Randeva et al., 2001; Karteris et al., 2005). In addition, intuitive argumentation based on GTPγS labeling and the readout signals in the CNS suggest that both pertussis toxin-sensitive and -insensitive G-proteins could be involved in orexin receptor signaling. Altogether, a much larger repertoire of intracellular signals than that suggested by the studies so far may be regulated by orexin receptors, even in the CNS.

The results of Paper III suggest that Ca²⁺ may regulate OX₁ receptor signaling in a novel manner. In essence, removal of extracellular Ca²⁺ fully blocks OX₁ receptor signaling to AC and ERK and shifts the activation of PLC to 10-100-fold higher orexin concentrations. The data from AC measurements in particular suggest that Ca²⁺ must act somewhere upstream of AC. Since previous results suggest that extracellular Ca²⁺ does not affect orexin binding, we assume that the site of action of Ca²⁺ would be between the receptor and the AC. In the case of AC, as shown in Paper II, this would have to affect the receptor–G-protein-interaction or G-protein function. Similar is suggested for PLC and ERK regulation, though some direct effects of Ca²⁺ on these pathways cannot be excluded. If Ca²⁺ is to act at the receptor–G-protein-interface, this would require ability of either the receptor or G-protein to be regulated by Ca²⁺ or that some additional macromolecule would be the target for Ca²⁺. We speculate that, the last possibility appears most likely. Additionally, it would appear most likely to us that this macromolecule is blocking the normal signaling in low intracellular Ca²⁺ but that the elevated intracellular Ca²⁺ – via the receptor-operated influx – would translocate it away from the receptor and allow the normal signaling to G-proteins. However, at the moment this is mere speculation, the value of which has to be evaluated in future studies.

5.3 PKC

One interesting finding in these studies is the involvement of different PKC isoforms in the different responses investigated. Intuitively, one would believe that orexin receptors would signal through at least the Ca²⁺-sensitive conventional PKC. We were surprised to find that this was not the case for the AC regulation; instead, novel PKCδ was found to be responsible for the PKC-dependent signaling (Paper II). In contrast, conventional and atypical PKC, but not novel PKC, appeared to be involved in the ERK signaling. As both conventional and novel PKC show strong membrane localization upon
OX₁ receptor activation and as this most likely occurs in rather non-membrane compartment-specific manner (Ca²⁺ and/or diacylglycerol generation) it is unlikely that the specificity of signaling would depend upon signal complexes by the receptor. Rather, it appears that different PKC classes have different substrate specificities (reviewed in Poole et al., 2004; Farhadi et al., 2005) which has also been recognized in studies with peptide libraries (see e.g. http://scansite.mit.edu/).

5.4 Cell plasticity

Regulation of cell plasticity is altogether an interesting though weakly characterized area of GPCR signaling. There are ex vivo and cell line studies suggesting that plastic changes may also occur upon orexin receptor activation. These responses include enhancement of hippocampal LTP (Selbach et al., 2004) and induction of cell death (Rouet-Benzineb et al., 2004). In addition, adrenal cortical cells seem to be affected by orexins in a short-term manner similar to ACTH (Malendowicz et al., 1999; Mazzocchi et al., 2001). Surprisingly, no developmental abnormalities have been reported in orexin knock-out mice.

In Papers III and IV we show that ERK and p38 MAPK/SAPK are activated by OX₁ receptors and suggest that ERK can counteract the p38-induced cell death. A central question that arises is whether the activation of MAP/SAPK kinase pathways is important in the physiological signaling of the orexin receptor. Some recent data from adrenal cortex indicate that this is the case (Spinazzi et al., 2005). Our preliminary data with cultured striatal neurons (Paper III) and some personal communications suggest that ERK activation would occur upon orexin receptor activation in the CNS as well. Considering the likely extrasynaptic release sites of orexins, signaling lasting long enough for ERK activation is likely to occur in the CNS. Cell death response, despite the rapid activation of p38 MAPK, requires much longer time to occur (Paper IV). Would orexins be around for times long enough and concentrations large enough for cell death to take place in vivo? On average, human cerebrospinal fluid contains 300-400 pg/ml orexin-A (Banno et al., 2001; Scammell et al., 2001; Gledhill et al., 2004). This concentration corresponds to approximately 0.1 nM, which is well below the concentrations required for cell death. However, there is likely to be a large concentration gradient between the sites where orexins are released and the cerebrospinal fluid and orexins might thus be available in the concentrations that induce cell death in Paper IV. Yet, it does not seem likely that orexins would induce cell death in the CNS. The lack of this response could be related to temporal profile of orexin release and the different responses in the neuronal than in CHO cells. It would be interesting to monitor p38 MAPK activation upon orexin receptor signaling in neuronal cells, as
it might have specific functions. It has previously been suggested that cell death cascades, such as caspase activation, have cell compartment-specific roles in the CNS in e.g. synaptic plasticity (Oo et al., 2002).

5.5 Physiological relevance of the current results

These studies have mainly been performed with recombinant OX₁ receptors in CHO cells and it is thus relevant to ask whether the engagement of some of the signal pathways would simply be caused by receptor overexpression. Receptor overexpression might indeed cause receptor promiscuity; however, it is also acknowledged that altogether higher receptor expression levels might be required for the same responses in recombinant systems than in native cells (Kukkonen, 2005). We believe that the responses measured in this study are relevant for the physiological signaling of orexin receptors due to the fact that the signal pathways involved are also present in other tissues. ERK activation is suggested to occur in the CNS neurons and in the adrenal cortex, p38 activation in the adrenal cortex and AC activation in the hypothalamus and adrenal cortex. Cell death in tumor cell lines expressing native OX₁ receptors and activation of G<sub>iα</sub>, G<sub>α</sub> and G<sub>q11</sub> proteins in the hypothalamus and adrenal cortex has also been observed (Karteris et al., 2001; Randeve et al., 2000; Karteris et al., 2005; Rouet-Benzineb et al., 2004). The current studies have confirmed these effects with more direct measurements and deepened the insight in the signal pathways between the orexin receptor activation and cellular responses; many of the approaches utilized would have been impossible in native cells. The results of Paper III suggest that orexin receptors require Ca²⁺ influx in order to effectively signal to other pathways. Further studies should be performed to validate this view in native target cells of orexins. This suggestion is of much interest also in the perspective that orexin receptors, both in CHO cells and in the CNS, have often been shown to effectively connect to non-selective cation channels (Sergeeva et al., 2003; Larsson et al., 2005).

The results of the studies also suggest a novel mode of signaling for GPCR altogether. The apparent Ca²⁺-dependence of orexin receptor signaling (Paper III) suggests a novel paradigm for regulation of proximal GPCR signaling. Secondly, we show, for the first time for GPCR, caspase-dependent and -independent cell death (Paper IV). The finding of the involvement of p38 MAPK/SAPK in the GPCR-dependent cell death (Paper IV) is also unique. These results thus point out new directions in the investigations of GPCR signaling.
6. Conclusions

- Both OX₁ and OX₂ receptors require very similar determinants and areas/residues from orexin-A to allow binding to and activation of the receptor. OX₂ receptor appears to be more resistant to both truncation and mutation of orexin-A than OX₁ receptor, probably explaining the equal potency of orexin-A and orexin-B for OX₂ receptor. The results strongly suggest that OX₁ receptor does not necessarily display any selectivity between orexin-A and -B.

- OX₁ receptor-mediated signaling demonstrates both inhibitory and stimulatory effects towards AC activation: high-potency inhibitory and low-potency stimulatory effects. High-efficacy inhibitory effect likely represents OX₁ receptor coupling to Goᵢ. The low-potency stimulatory effect is characterized by two components: a low-efficacy Goᵢ₅ coupling and high-efficacy PKCθ coupling (likely via Gq → PLC).

- OX₁ receptor activation causes strong, rapid and long-lasting activation of ERK. Logical in the light of the multiple primary couplings of orexin receptors, several pathways, such as conventional and atypical PKC, PI3K, Src and Ras, are involved in the cascades.

- Most centrally, Ca²⁺ influx is mandatory for the OX₁ receptor mediated ERK phosphorylation. Ca²⁺ supply via receptor-operated channels can be replaced by influx via store-operated channels. Ca²⁺ is suggested to act locally allowing OX₁ receptors to couple to intracellular signal cascades.

- A long-term activation of orexin receptors mediates signals towards caspase-dependent and -independent cell death independently of Ca²⁺ elevations. p38MAPK is central in these responses.
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