Orexin/Hypocretin Signaling

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Abstract Orexin/hypocretin peptide (orexin-A and orexin-B) signaling is believed to take place via the two G-protein-coupled receptors (GPCRs), named OX_1 and OX_2 orexin receptors, as described in the previous chapters. Signaling of orexin peptides has been investigated in diverse endogenously orexin receptor-expressing cells – mainly neurons but also other types of cells – and in recombinant cells expressing the receptors in a heterologous manner. Findings in the different systems are partially convergent but also indicate cellular background-specific signaling. The general picture suggests an inherently high degree of diversity in orexin receptor signaling.

In the current chapter, I present orexin signaling on the cellular and molecular levels. Discussion of the connection to (potential) physiological orexin responses is only brief since these are in focus of other chapters in this book. The same goes for the post-synaptic signaling mechanisms, which are dealt with in Burdakov: Postsynaptic actions of orexin. The current chapter is organized according to the tissue type, starting from the central nervous system. Finally, receptor signaling pathways are discussed across tissues, cell types, and even species.

Keywords Adenylyl cyclase • Calcium • Diacylglycerol lipase • Endocannabinoid • G_i • G_q • G_s • Heterotrimeric G-protein • Hypocretin • K^+ channel • Na^+/Ca^{2+}-exchanger • Non-selective cation channel • Orexin • OX_1 receptor • OX_2 receptor • Phospholipase A_2 • Phospholipase C • Phospholipase D • Protein kinase C

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Abbreviations

2-AG	2-Arachidonoyl glycerol
σ_1	Sigma 1 receptor
κOR	κ Opioid receptor
AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
BRET and FRET	Bioluminescence and fluorescence/Förster resonance energy transfer, respectively
CHO	Chinese hamster ovary (cells)
CNS	Central nervous system
CRF and CRF_1	Corticotropin-releasing factor and corticotropin-releasing factor receptor 1, respectively
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GEF	Guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
Gαβγ, Gα and Gβγ	Heterotrimeric G-protein heterotrimeric complex, α-subunit, and the βγ-subunit complex, respectively
IP_3	Inositol-1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast

mTORC1	Mammalian target of rapamycin complex 1
NCX	Na^+/Ca^{2+}-exchanger
NSCC	Non-selective cation channel
$p70^{S6K}$	p70 ribosomal S6 kinase
$p90^{RSK}$	p90 ribosomal S6 kinase
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA, PKB, and PKC	Protein kinase A, B, and C, respectively
PLA_2, PLC, and PLD	Phospholipase A_2, C, and D, respectively
PP5	Protein phosphatase 5
TRP	Transient receptor potential (channel)
VTA	Ventral tegmental area

1 Introduction to the Signaling of G-Protein-Coupled Receptors

As their name implies, GPCRs classically signal via G-proteins, specifically heterotrimeric G-proteins composed of α-, β-, and γ-subunits. In the resting state, the G-protein subunits remain in complex (also called Gαβγ) and GDP is bound to the α-subunit. The activated receptor interacts with the G-protein catalyzing the release of GDP and binding of GTP (which is dominant inside the cell), upon which the G-protein structure changes (reviewed in [1]). In most cases this is thought to lead to dissociation of the α-subunit (Gα) from the β- and γ-subunits (Gβγ), but there also are suggestions of structural reorganization without dissociation (reviewed in [2]). Nevertheless, both Gα and Gβγ should be able to interact with downstream effectors. Upon hydrolysis of GTP to GDP, the G-protein can return to the resting state.

Heterotrimeric G-proteins are divided into four families based on Gα: G_i, G_s, G_q, and $G_{12/13}$. Gα's of each family mediate characteristic responses (Fig. 1). These are, in simplified terms, for $G\alpha_i$ and $G\alpha_s$ inhibition and stimulation, respectively, of adenylyl cyclase (AC). ACs produce cAMP, which is a classical second messenger with protein kinase A (PKA) as the most ubiquitous target. $G\alpha_q$-family members stimulate phospholipase Cβ (PLCβ). PLC hydrolyzes inositol phospholipids producing diacylglycerol (DAG) and inositol phosphates. In case of hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), the latter is inositol-1,4,5-trisphosphate (IP_3). Both DAG and IP_3 are second messengers, with the most well-known roles in the activation of protein kinase C (PKC) and Ca^{2+} release from the endoplasmic reticulum (ER), respectively. PLC also has a role in regulating membrane levels of inositol phospholipids, which, especially PIP_2, have been shown to interact and regulate the activity of membrane proteins such as ion channels (reviewed in [3–5]). $G\alpha_{12/13}$ have been shown to directly activate protein phosphatase 5 and some guanine nucleotide exchange factors (GEFs) for Rho

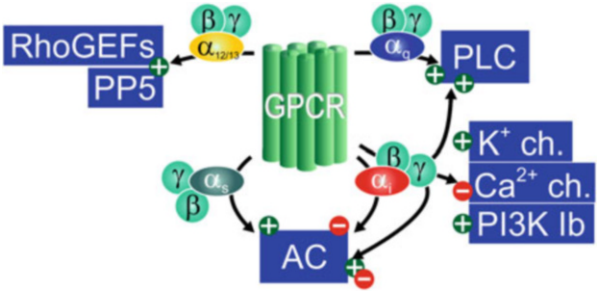

Fig. 1 Heterotrimeric G-protein signaling pathways. Only the most central pathways are shown; the pathways also end at the G-protein target. $G\alpha_s$ is a stimulant of all membrane-bound AC isoforms, while some G_i family $G\alpha$'s inhibit some AC isoforms. Some AC isoforms are also stimulated or inhibited by $G\beta\gamma$, and additional regulation comes from PKC and Ca^2, e.g. downstream of the PLC cascade. AC produces cAMP, which acts as a second messenger via PKA, some ion channels and EPAC, a GEF for the monomeric G-protein Rap. $G\beta\gamma$ signaling is usually supposed to originate from G_i-family proteins. $G\beta\gamma$ can, in addition to AC, inhibit some voltage-gated Ca^{2+} channels and stimulate class 1b PI3K, some voltage-gated K^+ channels, some PLC isoforms and some GEFs. $G\alpha_q$ stimulates all PLCβ species, which lead to phosphoinositide hydrolysis (a message in itself) and generation of the second messengers DAG and IP_3. DAG activates, for instance, PKC while IP_3 induces Ca^{2+} release from the endoplasmic reticulum. $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to directly interact with protein phosphatase 5 (PP5) and certain GEFs for Rho (e.g., lsc/p115 RhoGEF and LARG), but RhoGEFs may also be regulated by $G\alpha_q$ and $G\beta\gamma$

(Fig. 1). Most well-known targets for $G\beta\gamma$ signaling are some PLC isoforms and, in the brain, K^+ and Ca^{2+} channels (Fig. 1). Gβ and Gγ appear rather promiscuous in coupling to different $G\alpha$'s. However, $G\beta\gamma$ signaling is often assumed to originate from the G_i-family, in part based on the use of the G_i-family-inactivating toxin, Pertussis toxin. $G\beta\gamma$ has lower potency for many effectors than $G\alpha$'s; as G_i-family proteins are assumed to be present at higher levels than the other heterotrimeric G-proteins, they should also be better able to supply the higher levels of $G\beta\gamma$ required for activity. For reviews on heterotrimeric G-protein signaling, see [6–9].

GPCRs have also been shown to interact with other proteins. A ubiquitous signaling partner is β-arrestin (reviewed in [10]). Also a number of other proteins interact with specific receptors; in some cases, these have been shown relevant for the signaling, trafficking, etc. while their role in other cases is unclear (reviewed in [11, 12]).

Different receptors are able to preferentially couple to specific G-proteins. For some receptors this selectivity can be very high while other receptors – like orexin receptors – may be strongly promiscuous. The determinants of selectivity are not known; there are, for instance, no obvious general receptor features behind this. Also, for some promiscuous receptors the preferential G-protein coupling has been shown to be different in different tissues, possibly determined by tissue-specific receptor-interacting proteins (reviewed in [12]). Promiscuous signaling, already at the level of receptor coupling to primary signal transducers (G-proteins or other), is one way of explaining the differential effects of receptor activation in different tissues, but also downstream signal transducers and such can affect this. Oligomerization of GPCRs may affect the signal transduction or other receptor properties (Sect. 5). Yet a complicating factor is so-called *biased agonism* [13]. This implies

that each chemically different endo- or exogenous receptor activator produces a complex with the receptor with a potentially unique conformation and ability to interact with signal transducers. Thus, one ligand may preferentially stimulate activation of G_q and β-arrestin, while another might direct the signaling towards G_s. This complicates the analysis of native (physiological) signaling, if there is more than one endogenous ligand and also constitutes a challenge in drug discovery. This is a highly relevant issue for orexin receptors as well, as these have been shown to connect to three families of heterotrimeric G-protein – G_q, G_i, and G_s – and other proteins like β-arrestin as well ([14, 15]; reviewed in [16]), and the coupling also seems to be different in different tissues, as discussed below.

2 Orexin Signaling in Native Neurons

2.1 Impact on Electrical Activity

In central nervous system (CNS) neurons, usually investigated in rat or mouse slice preparations, orexins are excitatory. Both pre- and post-synaptic mechanisms have been suggested to be operational. Dozens of highly relevant papers have been published, and we apologize for not being able to cite these here; the readers are referred to other chapters of this book (especially Burdakov: Postsynaptic actions of orexin) and our own recent reviews [16, 17] for full citations and detailed analyses.

Orexin receptor activation is associated with post-synaptic depolarization in a number of neurons (reviewed in [16, 17]). Three different mechanisms have been suggested for this depolarization. In some cases, depolarization is associated with an increase in input resistance, indicative of K^+ channel inhibition (see, e.g., [18–20]; reviewed in [16, 17]). This has been verified by I–V curve analysis. The identity of the channels has not been directly determined, but as these seem to be open at rest, the two-pore-domain K^+ (K_{2P}) channels or possibly inward rectifier (K_{ir}) channels are the most likely channel types regulated by orexin receptors. Both interact with inositol phospholipids (e.g. PIP_2), and hydrolysis of such upon PLC activity would inhibit the channels (reviewed in [21, 22]). For K_{2P} channels, phosphorylation by PKA and PKC and direct interaction with $G\alpha_q$ are inhibitory factors (reviewed in [6, 21]). K_{ir} channels can also be inhibited by phosphorylation and direct interaction with $G\alpha_q$ (reviewed in [6, 22]). There is evidence that orexin receptor activation indeed can inhibit both K_{ir} and K_{2P} channels ([23–25]; reviewed in [16]). The G_q – PLC(−PKC) cascade is an especially likely signal pathway for orexin receptors (see Sects. 2.2, 3, and 4), but the mechanisms have not been fully investigated for orexin responses in CNS neurons.

Another common finding upon orexin receptor-mediated depolarization is activation of non-selective cation fluxes. Usually, this is ascribed to activation of non-selective cation channels (NSCCs) (see, e.g., [19, 26–29]), but sometimes the Na^+/Ca^{2+} exchanger (NCX) has been suggested as an alternative explanation (see, e.g., [30–32]). However, we have analyzed this for another recent review [17], and most often the methods employed do not allow distinction of NCX from NSCC. It

should also be pointed out that it is unclear whether NCX is likely to produce currents high enough to allow significant depolarization of most neurons, but, on the other hand, even a minor Na^+ influx may be enough to increase the excitability, especially if K^+ channels are inhibited at the same time (below). Nevertheless, there are some findings which clearly exclude one or the other and thus strongly point in the direction of either NCX or NSCCs (reviewed in [17]). A "normal" mode for NCX implies exchange of intracellular Ca^{2+} for extracellular Na^+, which produces a depolarizing current due to the 1:3 exchange ratio. NCX can also act in "reverse" mode, exchanging intracellular Na^+ for extracellular Ca^{2+} and hyperpolarizing the cell. The direction of the exchange is thus dependent on the extra- and intracellular concentrations of Na^+ and Ca^{2+} and the membrane potential. The normal mode of action would likely require an elevated intracellular Ca^{2+} concentration. This has not been investigated in orexin studies, but one likely source for this could be the G_q – PLC cascade-dependent inositol-1,4,5-trisphosphate (IP_3) production and Ca^{2+} release from the ER (see Sects. 3 and 4). In addition, NCX activity is regulated by phosphorylation by, e.g., PKC and by PIP_2 (reviewed in [33]), offering additional links to orexin receptor signaling. See further discussion on the collaboration of NCX and NSCCs in recombinant cells under Sect. 4.3.

Most cloned NSCCs are predicted to structurally resemble Shaker-type K^+ channels with six transmembrane helices per subunit and four subunits per channel. The most likely channels among NSCCs to be regulated by orexin receptors (and other GPCRs) belong to the transient receptor potential (TRP) channel family (reviewed in [34, 35]). However, this is speculation since the channel identities have not been assessed for orexin receptors in the CNS, except for one study, where Na^+ influx is suggested to be TRPC channel-mediated [36]. Also the receptor mechanisms involved in the regulation of NSCCs are unclear. We have speculated that the activation of the channels could take place via lipids, regulated via the "classical" GPCR-mediated regulation of phospholipases such as PLC but also phospholipase A_2 or D (PLA_2 and PLD, respectively) (reviewed in [37]), as is known for TRP channels (reviewed in [34]). Such signals could relate to the reduction or increase in PIP_2 or release of messengers such as DAG or arachidonic acid, which are known to affect TRP channel activity (reviewed in [34]). There are indications of engagement of TRP channels in orexin signaling in recombinant cell models (Sect. 4), supporting the idea of at least a principal ability of orexin receptors to couple to TRP channels; however, firm evidence from native neurons is lacking.

In some cases, orexins have been found to affect synaptic events, i.e. miniature synaptic potentials or currents. Especially interesting are the findings of increased frequency of events in the presence of action potential block, as this suggests a direct presynaptic effect of orexins ([38–49]; reviewed in [16, 17]). However, one also needs to keep in mind that such an effect could also be obtained upon engagement of new (silent) post-synaptic receptors/synapses. Further studies are required to resolve this.

Also other types of currents have been shown to be affected by orexin signaling, e.g. producing enhancement or inhibition of other depolarizing input (reviewed in

[17]). This is in agreement with the spectrum of signaling orexin receptors show (see, e.g., Sect. 4.1), which is likely to affect many ion channels. Orexin signaling has also been shown to be involved in plasticity, i.e. long-term potentiation in hippocampal circuitry [50–52], but the cellular targets and molecular mechanisms are thus far unclear.

In many sites, several mechanisms of depolarization have been seen to be utilized by orexin receptors in parallel, e.g. K^+ channel inhibition and NSCC/NCX activation co-occur ([19, 53–63]; reviewed in [17]). This is logical if we are to assume that basic orexin receptor signaling and in some degree also the principal channel/transporter expression profiles are rather similar in different neurons. However, as indicated above, details of the channel identities and signaling are not known. In addition, pre- and post-synaptic mechanisms may co-occur in the same site, as on the orexinergic neurons themselves [44, 49, 64, 65], neocortex [39, 66], nucleus tractus solitarius [48, 61, 62], dorsal motor nucleus of vagus [19, 42, 67], and POMC-ergic neurons of arcuate nucleus [38, 68]; however, it should be noted that it is in most cases unclear whether the pre- and post-synaptic actions of orexins take place in the same synapses.

The studies of molecular details of signaling in primary neurons are often hampered by methodological obstacles. Acutely isolated neurons are not an easy preparation (limitations of patch-clamp technique such as space-clamp problems and the different problems in whole-cell and perforated patch recordings, poor pharmacological tools, difficulties in applying molecular biological tools). In some neurons, the depolarizing inputs from orexins have been assessed using inhibitors of, e.g., PLC and protein kinases, but the targets are not clear. It also should be noted that PLC inhibitors often show both false negative and positive responses, and not all protein kinase or ion channel inhibitors are as selective as optimistically assumed by the users. Some novel pharmacological tools are now available for such receptor and channel studies, and these should be applied in orexin research. The tools are discussed under Sect. 6.4.

2.2 Other Responses in Neurons

Ca^{2+} is a second messenger often associated with orexin responses (Sects. 2.1 and 4). Few neuronal preparations have been investigated for Ca^{2+} elevations, but when done, this has been observed (e.g., a recent report of [69]; reviewed [17]). The source of Ca^{2+} elevations could be intracellular release or influx. The influx has been either shown or suggested in several cases for orexin, but there are no direct data demonstrating orexin-mediated Ca^{2+} release in native neurons – some indirect indications though (reviewed [17]). The pathway for Ca^{2+} influx is usually not clear, but it may be a result of activation of voltage-gated Ca^{2+} channels secondary to depolarization, direct influx via NSCCs or even NCX-mediated Ca^{2+} influx secondary to NSCC-mediated Na^+ influx. Among voltage-gated Ca^{2+} channels, L-type channels have been implicated in orexin responses in several sites by Ca^{2+}

or electrophysiological measurements. While Ca^{2+}-measurements do not separate depolarization-mediated and directly enhanced flux as such, patch-clamp measurements have often identified a PLC – PKC-dependent enhancement of depolarization-induced L-type channel current [70–72]. Ca^{2+} and PKC have been shown to be central for the activation of AMP-activated protein kinase in rat arcuate nucleus neurons in culture, partially via the L-type channels [72]. Ca^{2+} elevation has also been indirectly assessed using the intracellular Ca^{2+} chelator BAPTA, but one must remember that the heavy intracellular Ca^{2+}-buffering, required to effectively block Ca^{2+}-dependent orexin signaling, may also affect other (constitutive) processes. Reduction of extracellular Ca^{2+} concentration should block Ca^{2+} influx, but this also seems to reduce orexin binding as we have recently shown [73], and thus this may not be a suitable method to assess the role of Ca^{2+} influx in orexin responses. Ca^{2+} elevation is indirectly supported by the concept of orexin signaling involving NCX, since the normal (depolarizing) mode of action of this transporter requires intracellularly elevated Ca^{2+}.

Orexin receptor coupling to G-proteins has been investigated in a few studies. GTPγS binding in slice preparations suggests orexin receptor regulation of G_i-family G-proteins [74, 75], while direct measurements in the hypothalamus show coupling to G_i, G_s, and G_q family proteins [76]. Also potential responses downstream of the latter two, cAMP elevation and IP_3 generation, respectively, have been observed in the hypothalamus [76].

The PLC pathway is involved in orexin-mediated endocannabinoid generation [77, 78]. PLC produces DAG which can be hydrolyzed in the sn-1 position by DAG lipase, producing sn-2-monoacylglycerol. In cases where the sn-2 fatty acid is arachidonic acid, the resulting monoacylglycerol is 2-arachidonoyl glycerol (2-AG), which is a potent endocannabinoid, i.e. agonist at CB_1 and CB_2 cannabinoid receptors. Orexin signaling has been shown to rely on CB_1 receptor activation downstream of orexin receptors and DAG lipase in rat dorsal raphé nucleus and periaqueductal gray matter, suggesting the cascade orexin receptor → G_q → DAG (PLC) → 2-AG (DAG lipase) → CB_1 receptor. Endocannabinoid action elicits retrograde homo- or heterosynaptic presynaptic inhibition, which, depending on the neurons affected, produces either inhibition or disinhibition of the post-synaptic output (reviewed in [79]).

Also other CNS cells than neurons, such as astrocytes and microglia, have been suggested to express orexin responses, but the findings are thus far rather preliminary.

2.3 Conclusions

Orexin receptor signaling is well mapped at the level of the potential impact on neuronal electrical activity, but the molecular identities of the target channels/transporters as well as the signal cascades between the activated receptors and the channels/transporters are only weakly known, and the few observations to date of

the second messenger generation or kinase activation in neurons are not well connected to the "final" outputs. This is typical for GPCR signaling, and is partially caused by poor tools for such investigations, but there clearly also is a lack of consequent approaches. As discussed under Sect. 6.4, novel tools should assist in the studies.

3 Orexin Signaling Features in Other Native Cell Types and Cell Lines

Different peripheral cell types and tumor cell lines express orexin receptors and show functional responses to orexins. The physiological significance of this is rather unknown, since the utilization of orexins as transmitters in the periphery has not been demonstrated, and in most sites where the receptors are expressed, prepro-orexin protein expression has not been demonstrated – though often not even investigated. Studies utilizing these cells and cell lines are, under any circumstance, useful in revealing orexin receptor signaling mechanisms and may also pave the way for discoveries of the physiological role of orexin signaling in these tissues.

3.1 Endocrine Cells

Several types of endocrine cells display responses to orexins. The most thoroughly investigated ones are adrenal cortical (i.e., zona fasciculata and reticularis) cells, which have been studied in native preparations of both man and rat ([76, 80–82]; reviewed in [17]). In different studies, both OX_1 and OX_2 receptors are found. Depending on the species and developmental stage, orexin receptor activation leads to activation of G-proteins of the families G_i, G_s, and G_q, and activation of both AC and PLC is seen. Further downstream, a strong stimulation of glucocorticoid release is observed. The release is similar in magnitude to that induced by adrenocorticotropic hormone (ACTH) and apparently utilizes the same classical cascade AC → cAMP → PKA. However, it is not known, whether the orexin response is simply a result of $G\alpha_s$-mediated AC stimulation or whether also G_i and G_q proteins contribute to this; different AC isoforms can be stimulated by also $G\beta\gamma$, Ca^{2+}, and PKC (reviewed in [83]). Steroidogenesis has been further investigated in the human tumor cell line H295R, which expresses both OX_1 and OX_2 receptors. Orexin stimulation leads to activation of multiple kinase pathways, i.e. PKC and the mitogen-activated protein kinases (MAPKs) ERK1/2 and p38, and also Ca^{2+} elevation [84]. Orexin stimulation induces an increased steroidogenesis but also a generally more mature phenotype [84, 85], and thus it is difficult to judge whether the signaling cascades identified relate to the former or the latter (discussed in [17]). In adrenal medulla, as investigated in both native rat chromaffin cells and human

tumor cells, orexins stimulate catecholamine release [86, 87]. As investigated in the latter, orexin signaling activates PLC, and the catecholamine release appears to be mediated by the PKC cascade [87], which is a classical enhancer of catecholamine release (via voltage-gated Ca^{2+} channels?). Prepro-orexin mRNA and protein have been found in human adrenal gland [82, 88].

Orexin responses in the different endocrine cell types of the pituitary gland have been investigated in many studies but few of these offer insights in the cellular and molecular mechanisms. Two studies suggest involvement of PKC in the response [89, 90].

Orexin responses in the endocrine pancreas have been investigated in explants, isolated cells, and tumor cell lines. In InR1-G9 Chinese hamster glucagon-producing tumor cells, orexin signaling induces activating phosphorylation of phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB a.k.a. Akt), indicating activation of phosphoinostide-3-kinase (PI3K) [91]. GPCRs can activate class I PI3K either via Gβγ (Fig. 1) or via the Ras cascade (reviewed in [92]).

Orexin receptors are also expressed in the male reproductive tract of human and rat. In undefined testicular cells, their activation stimulates PLC [93]. Increased testosterone production as well as altered gene transcription is also seen [94, 95], but whether these responses are connected to the PLC signaling is not known. Expression of prepro-orexin mRNA and protein has been detected in the male reproductive tract [93, 94, 96–98], thus suggesting a possibility of paracrine orexin signaling.

3.2 Other Cell Types

The gastrointestinal tract was early on found to be a target of orexin responses and possibly also a site for orexin production (prepro-orexin mRNA detected) [99]. Both OX_1 and OX_2 receptor mRNA is expressed in neurons and ileal smooth muscle cells, and orexins seem to display direct actions on these cell types. The responses are complex and do not allow a clear-cut analysis; for instance, many different players are involved in the orexin-induced ileal contraction [100, 101].

Acutely patient-derived human colon carcinoma cells (and their metastases) express OX_1 receptors while the native colon epithelial cells do not [102, 103]. AR42J rat pancreatic acinar carcinoma cells express OX_2 receptors, and are also induced to undergo programmed cell death upon orexin challenge [104]. Thus induction of cell death is a possible response for both orexin receptors. See Sect. 4.1 for the cell death mechanisms.

Mesenchymal stem cells give rise to a number of different cell types, including brown and white adipocytes. Differentiation of mouse brown adipose tissue has been shown to require placentally produced orexins likely acting via OX_1 receptors [105, 106]. Orexin signaling was suggested to proceed in part via p38 MAPK and in part by induction of bone morphogenic protein 7 and its receptor, BMPR1A, which

generates a potential autocrine loop [105]. No other details of the signal cascades are known. White adipocytes and white adipocyte precursor cell lines also express orexin receptors. Whether orexins induce differentiation of adipocytes is questionable. Upon orexin challenge, human subcutaneous adipocytes, rat (undefined) adipocytes, and differentiated 3T3-L1 adipocytes show apparent maturation as indicated by expression of peroxisome proliferator-activated receptor γ2 (PPARγ2), hormone-sensitive lipase and lipoprotein lipase, and increased glucose uptake and triglyceride synthesis, while the human omental adipocytes rather show the opposite [107, 108]. 3T3-L1 preadipocytes are not differentiated by orexin-A, which is suggested to be due to the lack of PI3K – PKB activation [109]. Intracellular signals detected are activation of the MAPK pathways ERK1/2 and p38, while the PI3K – PKB cascade seems to be activated in differentiated 3T3-L1 cells [108]. cAMP is one of the major stimulants of differentiation of both brown and white adipocytes, and it is surprising orexin signaling via cAMP has not been assessed in any of these studies. Adipocyte studies also show an unexpected finding: while orexin-A stimulates the proliferation of 3T3-L1 preadipocytes, orexin-B does the opposite [109, 110].

Utilizing human, mouse, and rat models (vascular endothelial cells, and in and ex vivo), it was shown that orexin-A, at the high nanomolar range, induces heme oxygenase 1 expression and apparent activity as well as angiogenesis [111].

4 Orexin Signaling Features in Recombinant Orexin Receptor-Expressing Cells

Native orexin receptor-expressing cells are difficult to isolate, do not survive long in culture, and are present in rather small amounts – this is most obvious for the native neurons. Thus, researchers have resorted to recombinant cell lines, as in much other research as well. These cells have the additional advantage of expressing only a single orexin receptor subtype, which allows analysis of the receptor subtype differences. However, the cell background is also very different from the native cells (especially CNS neurons) and the results may thus not be directly physiologically applicable. Receptors from species other than human have barely been investigated in recombinant cells.

4.1 CHO Cells

Chinese hamster ovary K1 cells (CHO-K1; usually cited just as CHO) and HEK293 human embryonic kidney cells are the two most used cell lines in orexin receptor studies to date. The exact cell type is unclear for both cells, but they appear epithelial. Both are widely used cells for heterologous expression, especially of

receptors. Orexin signaling in CHO cells involves many second messenger pathways. The PLC pathway releases DAG and inositol phosphates including IP_3, which releases Ca^{2+} from the ER and leads to secondary (capacitative) Ca^{2+} influx (see, e.g., [112, 113]). The PLC pathway also gives rise to the endocannabinoid 2-AG (Sect. 2.2); recombinant CHO-K1 cells (and similarly recombinant HEK293 and neuro-2a cells; Sects. 4.2 and 4.3) are indeed the only cells where 2-AG production has been directly shown [114, 115], while the findings in native neurons (Sect. 2.2) are solely based on the use of pharmacological inhibitors. Breakdown of 2-AG gives rise to free arachidonic acid, but the latter is also produced upon action of PLA_2 (likely specifically $cPLA_2$) [114, 115]. PLD (primarily PLD1) is also activated upon orexin receptor stimulation [114, 116]. The G-protein family behind the entire Ca^{2+} response (see below), and PLA_2, PLC, and PLD activation was recently shown to be G_q, based on the use of the depsipeptide inhibitor of $G_{q/11/14}$, UBO-QIC a.k.a. FR900359 [117]. G_i signaling is seen in the inhibition and G_s in the stimulation of AC, respectively ([114, 118], a); however, it is possible that also the G_q family proteins play a part in the AC inhibition [117]. These responses are seen for both OX_1 and OX_2 receptors. The studies may not be directly used to assess the efficiency of coupling, but OX_2 receptors show weaker signaling to PLD and G_s. OX_1 signaling has been assessed for a wider set of responses than OX_2 responses. These include activation of PKC, MAPKs ERK1 and -2, p38 (α/β isoform), and c-jun N-terminal kinase (JNK) [119, 120]. PKC is likely activated downstream of PLC – DAG, though there also are conflicting findings [116], and even PI3K and Src are indirectly associated with the ERK cascade, though not directly measured [119]. Ras activation seems to be necessary for ERK activity [119].

One characteristic finding in OX_1 signaling is Ca^{2+} influx. There is an apparent receptor-operated Ca^{2+} influx that occurs at low orexin concentration and that does not require IP_3 [113, 121]. This influx is also seen as a non-selective cation conductance in patch-clamp [122]. Some inhibitor studies have been conducted but these are not conclusive for the channel type, and the patch-clamp studies are technically demanding as the conductance is not easily seen. The influx is partially inhibited by dominant-negative TRPC1 and TRPC3 channels, but these have not been tested in combination. In the lack of an effective and selective inhibitor, Ca^{2+} influx has in many studies been inhibited by reduction of extracellular Ca^{2+} concentration or depolarization, which reduces the driving force for Ca^{2+} entry. These procedures but especially the former, produce a significant inhibition of many downstream orexin responses [73, 113, 115, 116, 119, 123, 124], suggesting that intracellular Ca^{2+} elevation is somehow required for them. However, we have recently shown that extracellular Ca^{2+} is also required for orexin-A binding [73], and thus some earlier conclusions are not valid. Nevertheless, depolarization also produces significant inhibition of some orexin responses, and thus the issue requires further studies. It should be noted that activation of a non-selective cation conductance is also seen in native neurons (Sect. 2.1) and in other recombinant cells (Sects. 4.2 and 4.3), and thus we assume that this reflects the same general coupling ability (though not necessarily a single mechanism; Sect. 6.1) for orexin receptors.

We have recently seen that the $cPLA_2$ cascade, also activated by orexin receptors at high potency, is required for this influx, offering a clue to its regulation [115]. Unfortunately, we have not been able to continue these studies yet.

Another interesting feature of orexin signaling in CHO cells is the range of orexin concentrations it involves. The most high-potency responses are AC inhibition, Ca^{2+}/cation influx and $cPLA_2$ activation in the subnanomolar or low nanomolar range. These are followed by apparently two different PLC activities, PLD and DAG lipase in the low – mid-nanomolar range, and then finally G_s in the high nanomolar range (reviewed for OX_1 in [17]; see [114] for OX_2). As mentioned earlier, we do not know the physiological orexin concentrations, but one might assume that peptides usually are present at rather low levels. Thus, a response requiring rather high orexin concentration, such as G_s activation in CHO cells, would not be physiologically likely, unless mediated by rather direct synaptic contact. However, the apparent G_s-coupling takes place at high potency in other cells, such as hypothalamic neurons (Sect. 2.2) and adrenal cortex (Sect. 3.1). Thus we may assume that different cell backgrounds may direct orexin signaling to different outputs (Sect. 6.2). One such background factor is expression of other receptors capable of dimerizing/oligomerizing with orexin receptors (Sect. 5).

Programmed cell death is also seen in CHO-K1 cells (only investigated for OX_1). The cell death shows the hallmarks of apoptosis by requirements of gene transcription and protein synthesis and nuclear condensation [120]. However, upon inhibition of the caspases, the profile of the cell death changes, no longer requiring gene transcription. p38 MAPK is driving the cell death, but the activation mechanism for this kinase is not known in this context. In contrast, in the suspension-adapted CHO cell clone, CHO-S, orexin receptor-mediated signaling in this response has been suggested to involve activation of the tyrosine kinase Src, which phosphorylates the OX_1 receptor at Tyr83 within *protein immunoreceptor tyrosine-based switch motif* (ITSM), which then can attract protein phosphatase SHP-2 [125]. The cascades between the receptor and Src and from SHP-2 to cell death are thus far unclear. OX_1 receptor was previously suggested to also contain another similarly signaling motif, ITIM, but this finding may have alternative explanations [16]. The findings of the orexin-induced programmed cell death in CHO-K1 and CHO-S cells are also different in the perspective that the former is inhibited by serum while the latter is not. Nevertheless, the responses should be examined across the cell types, to judge, for instance, whether the conclusions are distorted by compromised selectivity of some of the inhibitors used. Interestingly, it is not known whether neurons are susceptible to this cell death. If not, then the question arises, why not, and whether this response might be contextually activated even in neurons.

4.2 HEK293 Cells

Both human orexin receptor subtypes are able to elevate intracellular Ca^{2+} and activate PLC in HEK293 cells [97, 126, 127], but the role of Ca^{2+} influx has not been assessed in such detail as for CHO-K1 cells. An oscillatory Ca^{2+} response is obtained at low orexin-A concentrations, and this response is nearly fully blocked by the non-selective serine hydrolase and protease inhibitor, MAFP [128], linking this to the findings with both MAFP and a more selective $cPLA_2$ inhibitor in CHO-K1 cells (Sect. 4.1) [115, 124]. It is not known whether only the PLC – DAG lipase cascade is operational in these cells or also the PLA_2 cascade [115, 128]. Several dominant-negative TRPC channel constructs, i.e. of TRPC1, and -3 and -4, reduce the Ca^{2+} response magnitude at low orexin-A concentrations (1 nM).

Both orexin receptor subtypes activate ERK1 and -2, and OX_2 receptor stimulation has additionally been shown to activate p38 MAPK (not investigated for OX_1) [15, 129]. Also cAMP elevation has been measured in OX_2 cells [129]. The MAPK cascades appear to be regulated by several signal cascades, similar to CHO-K1 cells [119]; even if one disregards the results obtained with dominant-negative G-proteins, which are uncertain, the use of other inhibitors demonstrates cooperation of the cascades [129]. OX_1 receptors also couple to β-arrestin in HEK293 cells, and this contributes to the ERK1/2 activation [14, 15]. Upon expression of GIRK1 and -2 (G-protein-regulated K_{IR}) channels together with orexin receptors, orexin receptor activation inhibits the channels; however, the potency of orexin-A is in the high nanomolar range [24].

One aspect distinguishes the studies with CHO-K1 and HEK293 cells: while all the studies in Sect. 4.1 for CHO-K1 cells have been performed with the same clones of OX_1 and OX_2 cells, different clones (some stable and some even transient) have been used for HEK293 cells. It is thus possible that even background clonal differences affect the results.

4.3 Neuron-Like Recombinant Models

A few other cell lines and transient expression models have been utilized in orexin studies. We generated PC12 rat pheochromocytoma and neuro-2a mouse neuroblastoma cells as neuronal models for orexin investigations. Unfortunately, these cells have not been used much since the original characterization of Ca^{2+} elevation and PLC activation in both cell types [130]. Ca^{2+} elevation in OX_1-expressing neuro-2a cells seems to involve IP_3-independent Ca^{2+} influx similar to CHO-K1 cells [121]. We recently also observed 2-AG generation in OX_1-expressing neuro-2a cells [115].

Another neuronal cell model, utilized in a single study, are the BIM hybridoma cells [131]. This study is often cited as reference for the ability of OX_2 receptors but not OX_1 receptors to couple to G_i proteins. Unfortunately the authors miss the

classical finding with receptors coupling to AC in multiple ways; usually nothing can be seen under basal conditions. Thus, a most likely explanation is that the positive and negative signaling from the receptors take each other out under basal conditions but can be seen when each pathway is selectively inhibited; this is evident with, e.g., CHO-K1 cells [114].

IMR-32 neuroblastoma cells, differentiated with 5-Br-2′-deoxyuridine and transiently transduced with baculoviral vectors harboring the human OX_1 receptor under cytomegalovirus promoter, have been utilized in two studies of orexin-A-induced cation fluxes by Åkerman & co-workers [132, 133]. It was found that orexin-A triggers a Ca^{2+} influx that is partially sensitive to extracellular Na^+: in most cells Ca^{2+} influx was either strongly inhibited by removal of extracellular Na^+ or not affected by it. In the former case, the studies indicate that Ca^{2+} influx is secondary to Na^+ entry (i.e., Ca^{2+} taken in by the reverse mode of NCX) while in the latter case it is caused by a direct channel-mediated Ca^{2+} influx. Expression of dominant-negative TRP channel subunits and RNA interference suggest that the apparent Na^+ influx in the former case is in most part mediated by TRPC3 channels. In contrast, the channels mediating the direct Ca^{2+} influx remain unknown, but their pharmacology is clearly different from the other channels. In OX_1-expressing CHO-K1 or HEK293 cells, where partial sensitivity to dominant-negative TRPC channels has been noted for the orexin-A-induced Ca^{2+} influx (Sects. 4.1 and 4.2), the role of Na^+ has not been examined [122].

Hypothalamic N41 & N42 cells, i.e. SV40 large T antigen-immortalized mouse embryonic hypothalamic neurons, stably transduced with OX_1 and OX_2 receptors (species unclear), respectively, have been reported in a recent study [134]. The cells were used to demonstrate orexin-mediated activation of mammalian target of rapamycin complex 1 (mTORC1) as indicated by, for instance, rapamycin-sensitive activation of p70 ribosomal S6 kinase ($p70^{S6K}$). The same was seen in HEK293 cells expressing OX_1 and OX_2 receptors. In N41 and -42 cells, orexin-A and -B stimulus activated the p90 ribosomal S6 kinase ($p90^{RSK}$) and ERK1/2 but not PKB, but neither $p90^{RSK}$ nor ERKs were involved in mTORC1 activation. In contrast, Ca^{2+} elevation appeared sufficient to activate mTORC1 as this could also be induced by thapsigargin or ionomycin and was, for orexin, abolished by intracellular Ca^{2+} chelation with BAPTA. Ca^{2+} was determined to elicit its effect via the lysosomal pathway of vacuolar H^+-translocating ATPase (v-ATPase) – Ragulator – Rag. Ragulator is a GEF for the monomeric G-protein Rag. How Ca^{2+} works in this cascade is unclear.

SV40 large T antigen has also been used to immortalize rat olfactory epithelium neurons to create the Odora cell line, which has been used for orexin studies upon transient expression of human orexin receptors [135]. Weak IP_3 and Ca^{2+} elevations were observed, while the cAMP elevation was more robust, though this was only tested at 100 nM orexin-A/B. ERK1 and -2 phosphorylation was also increased at this concentration.

4.4 Other Recombinant Cells

Some other cell types have also been used in orexin studies, but these have mainly offered supporting information. Mouse embryonic fibroblasts (MEF) expressing OX_1 receptors have been used in two studies. Laburthe & co-workers have used the wild-type and $G\alpha_{q/11}$ knock-out cells to demonstrate that orexin-promoted cell death requires $G_{q/11}$ signaling [136]. MEF cells were also used for RNA interference studies in [134] (Sect. 4.3).

4.5 Species Variants Other Than Human

Canine OX_2 receptors induce elevation of intracellular Ca^{2+} [137], and murine OX_1 and the two OX_2 splice variants IP_3 release in HEK293 cells [138, 139].

5 Orexin Receptor Oligomerization

GPCRs are assumed to exist in complexes (reviewed in [140, 141]). Though this is often called dimerization, the actual number of units is usually unknown, but often either dimers or tetramers are assumed. The most often utilized resonance energy transfer-based techniques, FRET and BRET, usually assess dimerization, and neither show nor exclude higher order complexes. The findings imply that the receptors can make both homomeric and heteromeric complexes; the latter may have radically different properties as compared to homomeric ones as concerns trafficking, pharmacology and signaling. It should be noted that complex formation is not easily investigated and, even if a principal ability to form complexes is present, its physiological significance is seldom clear.

Orexin receptors have been shown to be able to form homomeric complexes as well as heteromers between the subtypes when expressed in recombinant systems [139, 142, 143]. This is a rather trivial finding for GPCRs, and the significance of this is unknown as also is the proportion of complexes in relation to receptor monomers (see below). Another complex formation can occur between either orexin receptor subtype and CB_1 cannabinoid receptors [142, 144]. The interaction between OX_1 and CB_1 has been investigated in detail in HEK293 and CHO-K1 cells, and the studies suggest clear effects on trafficking and signaling. The problem, however, is that since orexin signaling also generates 2-AG [115], there is also a functional interaction, and current studies have not separated these levels of interaction. We assume that the 2-AG-mediated interaction is more important [145], especially in the physiological context, since OX_1 and CB_1 receptors have not been demonstrated to be expressed in the same cells. However, it is possible that

this could occur, and may be discovered once orexin receptor antibodies evolve and allow reliable analysis.

OX_1 receptors and κ-opioid receptors (κOR) have also been shown to display both direct molecular and functional interactions. In contrast to the $OX_1 - CB_1$ pairing, the functional interaction was suggested to occur through purely intracellular signaling pathways, i.e. OX_1 receptor interaction enhanced JNK-mediated phosphorylation of κOR in CHO-K1 cells, inhibiting κOR signaling to AC and enhancing its signaling via β-arrestin and p38 MAPK, apparently via G_q [146]. In another study, OX_1 and κOR were shown to heteromerize. The heteromerization reduced the coupling of OX_1 and κOR to G_q and G_i, respectively, while it instigated coupling of the receptor heteromers to G_s and thus to cAMP elevation in HEK293 cells [147]. OX_1 and κ-opioid receptor mRNA is expressed in the same cells in the hippocampus, thus suggesting a possible native interaction [147].

Another recent study demonstrated interaction of either orexin receptor subtype with GPR103 utilizing BRET and FRET techniques in HEK293 cells [148]. ERK1/2 phosphorylation in receptor co-expressing cells was stimulated by orexins as well as the GRP103 agonist, QRFP, and the effect of each peptide was blocked by orexin receptor antagonists. This indicates a very high degree of complex formation, while the FRET results are somewhat in disagreement with this. Expression of GPR103 correlates with the expression of the orexin receptor subtypes in the hippocampus, and the authors speculate that the receptor interaction could be relevant in Alzheimer's disease.

The most comprehensive study involves a receptor complex between OX_1, corticotropin-releasing factor receptor 1 (CRF_1) and the non-GPCR sigma 1 receptor (σ_1) [149]. The $OX_1 - CRF_1$ complex was shown in HEK293 by FRET and a functional response; the response to an agonist of each receptor was antagonized by an antagonist against either the same receptor or the other partner (cross-antagonism). Activation of each receptor was, in itself, antagonistic to the action of the other receptor with respect to β-arrestin recruitment and phosphorylation of PKB and ERK1/2 (negative crosstalk). The dimerization/oligomerization was blocked by cell-permeable peptides composed of either the OX_1 receptor transmembrane segment 1 or 5, which also abrogated the cross-antagonism and negative crosstalk. In rat ventral tegmental area (VTA) neurons, the dimerization/oligomerization was shown by cross-antagonism and negative crosstalk with respect to ERK1/2 phosphorylation. Dopamine release was stimulated by orexin-A, and this was inhibited by antagonists of both OX_1 and CRF_1 receptors and by CRF itself. However, whether the orexin-A response was enhanced by the disruption of the receptor complex by the cell-permeable peptides (above) is questionable. The complex could also harbor the σ_1 receptor, as was shown by the authors in HEK293 cells, and activation of σ_1 receptors with cocaine disrupted this. Application of cocaine on VTA neurons freed OX_1 and CRF_1 receptors from the cross-antagonism and negative crosstalk, suggesting a native $OX_1 - CRF_1 - \sigma_1$ interaction. Finally, the selectivity of the cocaine action for σ_1 receptor was verified and also the impact on VTA dopamine release, which both CRF and orexin-A were able to stimulate in the presence of cocaine (on top of the cocaine effect).

It is interesting to note that OX_1 receptor complex formation with either κOR, CRF_1 or GPR103 appears very efficient. For other complexes reported, i.e. those among orexin receptor subtypes or with CB_1 receptors, this is not known. The FRET and BRET methods mainly utilized do not easily give the efficiency of the complex formation, since resonance energy transfer efficiency is dependent on the orientation and distance of the donor and acceptor, which is also shown in orexin receptor studies [142]. While some studies indicate that receptor activation may affect the energy transfer [139, 142, 143, 147], this cannot be interpreted to suggest difference in dimerization/oligomerization, since any conformational change may also either increase or decrease the energy transfer efficiency. However, studies on GPCRs other than orexin receptors by, e.g., fluorescence correlation spectroscopy suggest that dimerization/oligomerization can be a dynamic process (reviewed in [150]).

6 Summary and Conclusions

6.1 Convergence of the Findings?

The first conclusion that arises is that orexin receptors have certain signaling properties that come to expression independent of the background, suggesting that these represent some strong intrinsic receptor properties. One such coupling is to PLC, which projects, in one hand, to DAG and therewith either to activation of PKC (or other targets) or breakdown of DAG to 2-AG, an endocannabinoid messenger. The other product of PLC activity is IP_3 and IP_3-dependent Ca^{2+} release, the role of which, however, is unclear for orexins in CNS neurons. The third role of PLC relates to the regulation of membrane phosphoinositide levels, which are known to affect membrane protein properties, especially ion channels but also, for instance, NCX. In this perspective it is interesting to note that phosphatidic acid, produced by, e.g., PLD activity, is able to stimulate phosphatidylinositol-4-phosphate 5-kinase, elevating PIP_2 levels (reviewed in [151]); we have observed activation of PLD1 in CHO-K1 cells expressing either OX_1 or OX_2 receptors (Sect. 4.1). PLD has also been suggested to be involved in orexin signaling in the brain [152], but the implications are thus far unclear. It is logical to assume that orexin receptors would couple to G_q proteins, which trigger the activation of PLCβ. Indeed, studies in native neurons and adrenal cortical cells indicate that orexin receptors are able to couple to G_q proteins (Sects. 2.2 and 3.1), and in CHO-K1 cells these seem to mediate most of the responses too (Sect. 4.1). In CHO-K1 cells expressing human OX_1 receptors, there seem to be two different PLC activities activated upon stimulation of these receptors [112], and we have also obtained indications that protein kinase Cδ is activated independent of PLC [116]. Thus, though it is logical to assume that the – for GPCRs classical – $G\alpha_q$ – PLCβ pathway is of high significance for orexin receptor signaling, there may be

additional features of this signaling. We also have to admit that the involvement of this signaling has seldom been assessed in the brain, not even at the level of PKC (below).

Another general feature is the ability to activate non-selective cation fluxes. As reviewed for neurons (Sect. 2.1), there is an ongoing discussion of whether these depolarizing inward currents are mediated by NSCCs or the reverse mode of NCX, as most of the studies do not allow distinction. Nevertheless, it feels to the author of this review that NSCCs are the more likely candidate for the response in most cases. If so, what are the types of channels and how are they regulated by orexin receptors? The clearest identification of the channel originates from recombinant IMR-32 neuroblastoma cells, in which TRPC3 is suggested to lie behind the Na^+-dependent OX_1 response (Sect. 4.3) but also findings in CHO-K1 (Sect. 4.1) and HEK293 (Sect. 4.2) cells and native neurons (Sect. 2.1) indicate TRPC subfamily channels. The common theme for regulation of many TRP channels are lipid mediators, and thus, for instance the PLC pathway could offer activating signals by releasing DAG or reducing phosphatidylinositol species (reviewed in [34]) – remarkably, the PLC pathway also regulates NCX (Sect. 3.1). However, alike the channel identities, the regulatory mechanisms for the orexin-activated channel are unknown. The only direct pieces of evidence as to the regulatory mechanisms for the channels come from recombinant CHO-K1 cells, in which the $cPLA_2$ cascade is suggested to be essential for the activation (Sect. 4.1), and from rat thalamic neurons, in which Ca^{2+} influx, mediated by the T-type voltage-gated Ca^{2+} channels, has been suggested to drive the channel activation [36]. Clearly we have different channel types and mechanisms in different cells (see also Sect. 4.3). Studies in CHO-K1 cells show activation of multiple lipid signaling pathways by the receptors, and thus we might not find it surprising if somewhat different channel types were regulated by orexin receptors depending on the cell background. This also makes sense in the perspective of the multiple TRP channel genes (>20) and their regulatory mechanisms (reviewed in [34, 35]).

6.2 Divergence of the Findings?

Apparently divergent signaling is found in different tissues, especially in peripheral tissues as compared to the findings in CNS neurons. The obvious findings in peripheral cells include strong elevation of cAMP in the adrenal gland (Sect. 3.1), and activation of the PI3K – PKC cascade in InR1-G9 glucagonoma cells (Sect. 3.1) and 3T3-L1 adipocytes (Sect. 3.2) and indications of the same in some other cells. As discussed above, the physiological significance of orexin receptors in tissues outside the CNS is unclear. Are we then to assume that these responses may be irrelevant? G_s-coupling and cAMP elevation is, however, also seen in hypothalamic membranes (Sect. 2.2). In other sites this has not been investigated. It should also be noted that while the neuronal responses usually have only been assessed at high orexin concentrations (≥100 nM), the peripheral responses usually have been

analyzed by concentration–response relationships and shown to display high potency. Thus many of these responses are significantly activated at low nanomolar orexins while most of the CNS neuron responses have been proven only at 100–1,000-fold higher orexin concentrations. Transmitter levels fall rapidly upon increasing distance (especially relevant for extrasynaptic signaling), so would this not rather question the physiological relevance of the orexin responses measured in CNS neurons? I guess we do not need go that far, but as long as we do not know the physiological orexin levels in different *loci* of the body (Sect. 6.2), we need to be more careful in our analyses and conclusions. For instance, we may need to test the concentration–response relationships for the responses we are measuring, and we should also look for potential other responses at different orexin concentrations; GPCR signaling is not only subject to an increasing amplification upon increased agonist concentration, but the signaling profile may change more radically according to the ligand concentration and time (see, e.g., [153–158]). Nevertheless, the studies in neurons and in recombinant cells are largely in agreement, and they are also in agreement with the more systemic orexin responses, which supports the notion that the responses measured both in neurons and other cell types are physiologically relevant.

Nevertheless it is clear that orexin signaling is also different in different cell types. For instance, G_s-coupling is apparently of high potency in hypothalamus and adrenal cortex (Sects. 2.2 and 3.1), while the potency is very low in CHO-K1 cells (Sect. 4.1) – quite the opposite to what would be expected from the assumed *overexpression artifacts* of recombinant cells. G_s is a stimulant of all AC isoforms. The expression of different AC isoforms in different tissues is unlikely to cause the apparent discrepancy, but we may assume that indeed the G-protein-coupling of orexin receptors is likely to be different in different tissues. Such has been observed for other GPCRs, but the mechanisms are unclear. One possibility is interaction with other proteins, maybe receptor oligomerization (Sect. 5).

The role of OX_1 and OX_2 receptors in neuronal responses was analyzed in our recent review [17]. In the analysis, we cannot see an obvious difference, but the material is too small to allow a firm conclusion. It should be noted that it is not easy to separate between the responses since many sites express both receptor subtypes and neither the subtype-selective agonists nor antagonists are quite as selective as hoped for. The recombinant cells have not been consequently analyzed but some studies with CHO-K1 cells suggest possible different coupling of OX_1 and OX_2 receptors (Sect. 4.1); however, this material also is too limited to allow conclusions.

6.3 A Matter of Receptor Subtypes and Ligands

The vast majority of the studies on the neuronal effects of orexins, from intact animals to isolated neurons, have been done with rodents (most often rat) while the native cell lines are of variable origin and the recombinant studies usually assess human receptors. Can we assume that these findings stand on a common ground?

This is always a relevant question and there are previous cases with other targets that would suggest either way. Orexin receptor sequences are very similar across species orthologs. This would suggest that findings at the pure receptor signaling and pharmacological level are likely to be rather similar. Another relevant general comparison may the different drug development campaigns that usually utilize animal models as first background. History offers examples of successes but also of failures due to species differences, but many successes – as concerns the physiological part – are encouraging even for the orexin field. For orexin receptors, there are species-specific identified (and predicted) splice variants, which may affect both the receptor expression and signaling [138, 159]. Based on the evidence accessible, we can assume that the findings can be extrapolated across species, although some converse findings are also likely to surface sooner or later. One significant physiological difference is already known: while incapacitation of the OX_2 receptor induces a strong narcoleptic phenotype in dogs, OX_2 knockout in mice gives a clearly milder phenotype [160–162]. However, this is more likely to relate to the receptor subtype expression profiles and not to the signaling properties of the receptors *per se*.

There are two native ligands for orexin receptors, orexin-A and orexin-B. These are utilized in most of the studies, but sometimes the synthetic peptide variant, Ala^{11}-D-Leu^{15}-orexin-B, has also been used. The latter peptide was originally reported as a highly OX_2-selective receptor activator [163], and has been used as such in a number of studies. The potency difference between orexin-A and orexin-B – the original study reports that orexin-A is tenfold more potent on OX_1 receptors while both peptides are equipotent on OX_2 for Ca^{2+} (and binding) in HEK293 cells [97] – has also been utilized as an indicator of involvement of a specific orexin receptor subtype in a given response. However, there is a built-in problem in this. For many GPCRs, it has been shown that chemically different ligands may induce/stabilize distinct active receptor conformations, which may interact with the effectors with different affinity profiles, and thus lead to preferentially different response profiles (Sect. 1). This is termed *biased agonism* [13]. For GPCRs, this can effect interaction with G-proteins as well as with other signal transducers. Furthermore, receptor structure itself and signaling can be affected by interaction with other proteins. Thus the use of the so-called subtype-selective agonists with any receptors known to have several potentially physiologically important signal pathways is discouraged. Orexin receptors are such receptors; indeed, we have demonstrated this for the human variants. On one hand, orexin receptor signaling to apparently the same response, Ca^{2+} elevation, shows different relative potencies in two recombinant cell lines [127]. On the other, when the potencies of orexin-A and orexin-B are compared in CHO-OX_1 cells for different responses, the difference is not constant, and orexin-B induces some of the responses nearly as potently as orexin-A while the difference is 20-fold for other responses [164]. Thus, the use of any agonist-based pharmacology to separate orexin receptor subtypes is discouraged. An additional reason for this is that Ala^{11}-D-Leu^{15}-orexin-B shows much poorer selectivity even in recombinant cells than originally described [127].

The orexin-A sequence is identical in different mammalian species, and also the post-translational processing is assumed to be identical. However, orexin-B is not identical. Pig and dog express orexin-B, which differs by one amino acid from the human variant (Pro2 vs. Ser2, respectively), and mouse and rat one that differs by this and also another one (Asn18 vs. Ser18, respectively). Many studies in rat or mouse do not report whether the rodent variant of orexin-B was used. Based on mutagenesis and modeling studies, amino acid 2 is unlikely to have a major impact on orexin-B binding while amino acid 18 is suggested to enter the putative binding cleft of the receptor [165], and could thus contribute to binding. However, it is not conserved among orexin-A and orexin-B unlike many amino acids in the same region, and thus it may be of less importance. In fact, rodent orexin-B has been tested on human orexin receptors: Ca^{2+} response to the rodent peptide was not significantly different as compared to human orexin-B for either receptor subtype [166]. Although rodent receptors have not been exposed to human orexin-B, the rodent receptors are either completely (OX_2) or almost completely (OX_1) identical to the human orthologs for the transmembrane regions likely composing the binding cleft, and this together with the published data suggest that there is no major difference between the human and the rodent orexin-B peptides. However, there is no guarantee that this does not impact on the potency of the peptide to activate the receptor signaling, and thus constitutes a further reason not to utilize agonist-based pharmacology for orexin receptor subtype determination.

A fairer chance to obtain reliable information on the receptor subtype involvement in a response comes from the use of antagonists. Plenty of antagonists have been reported (please see Hoyer: OX_2 receptor antagonists as sleep aids of this book), and even though only very few are commercially available, luckily some subtype-selective ones are. Even with the antagonists one has to use common sense. The selectivities of the ligands even between orexin receptor subtypes are far from absolute, and thus the antagonist concentration needs to be adjusted to the agonist concentration to, on the one hand, get any effect at all, and on the other, to retain selectivity. One also needs to consider the access and stability issues, especially when working with whole animals; for instance, the much used OX_1-selective antagonist, SB-334867, has been reported to suffer from inherently low stability [167]. For a more complicated physiological response, it is unclear whether the response can be pin-pointed to a single orexin receptor subtype, as the measured output may be a result of more than one orexinergic pathway either in series or in parallel.

6.4 Where Are We Heading?

We can see that much has been done to resolve orexin signaling mechanisms, but also much remains to be done. It is understandable from the perspective of physiological interest and the methodological shortcomings that the major focus has been on the cell physiological details of orexin signaling. However, with the knowledge we now

have – the signaling of other GPCRs in the CNS and the details of the signal cascade of orexin receptors as known from different cell types – and the tools we can access and which are actively developed – e.g., different TRP channel inhibitors – we should go for higher molecular detail. However, many signal pathways lack efficient and selective inhibitors and, as indicated above, work with especially native cells involves many obstacles related to the properties of these cells. Therefore it is not surprising that many questions regarding orexin signaling in the (at least currently) most interesting CNS neurons are open. There also is, however, a lack of consequent approaches. One such problem consists of pharmacological and molecular biological tools and poor use of these. For instance, many kinase inhibitors are not very selective at all, and thus a finding should rather be repeated with another one against the same assumed target but with a different chemical structure and off-target profile. The most non-selective inhibitors should altogether give way for more novel and selective ones. Little may be concluded from the PLC inhibitor U73122, which may affect other targets or be completely incapable of inhibiting PLC, or D609, the target of which is unknown. Use of the most non-selective inhibitors needs to be substantiated by a sensible tracking of the assumed pathway. Also general common sense needs to be used: for instance, when does a result, which indicates a nearly full abolishment of a response upon inhibition of almost any pathway make sense and when not? The other side of the coin is that the findings in cell lines, native or recombinant, are slowly translated to investigations of native cells (neurons). There are indications of, for instance, cAMP, PI3K, $cPLA_2$, and PLD signaling of orexins (Sects. 3.1, 3.2, 4.1), but little has been done to assess these in neurons, although there are decent inhibitors available. More selective inhibitors of the TRP channels are emerging, and a recently characterized inhibitor of G_q, UBO-QIC [168, 169] is already commercially available, and even tested for recombinant orexin receptors [117]. My hope thus is that the molecular mechanisms of orexin signaling in neurons are gradually revealed. In addition to its importance for understanding of orexin physiology, this would be highly valuable for understanding the actions of the entire superfamily of GPCRs in neurons.

The first orexin receptor antagonist, suvorexant, is on the market in the USA for insomnia (see Hoyer: OX_2 receptor antagonists as sleep aids). From a research point of view this finally gives us a glimpse into the role of orexins in human physiology, and thus the validity of the results mainly extrapolated from other species. Suvorexant is a rather non-subtype-selective antagonist, but pharmaceutical companies are also developing OX_2-selective ones for the same indication (Hoyer: OX_2 receptor antagonists as sleep aids) and, if reaching the market, these should also give equally valuable information. As noted above, there are significant species differences in orexin physiology, and thus we may expect other similar findings. Methods to reliably assess orexin levels (brain tissue, cerebrospinal fluid, plasma) should also be developed [170]. This would allow us to answer the question of whether the peripheral orexin receptors ever "see" orexins, and to adjust the experimentally used orexin concentrations to a sensible physiological level for the tissue under investigation. The ligand concentration affects the signaling, as shown in recombinant CHO-K1 cells, and thus we might see a different response profile even in neurons.

Determination of the crystal structures of antagonist-bound OX_1 and OX_2 receptors (Rosenbaum: OX receptor crystal structures; [171, 172]) gives a fresh start for the orexin receptor ligand discovery. It may not be too much to hope that also an orexin peptide-bound receptor could be crystallized in the hands of the utmost experts. This would give us much needed information on the active state of the receptor and help tremendously in agonist ligand discovery. Needless to say, such ligands would be much needed as drugs but also as tools in orexin signaling research.

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