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## Physiology of the orexinergic/hypocretinergic system: a revisit in 2012

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**Kukkonen JP.** Physiology of the orexinergic/hypocretinergic system: a revisit in 2012. *Am J Physiol Cell Physiol* 304: C2–C32, 2013. First published October 3, 2012; doi:10.1152/ajpcell.00227.2012.—The neuropeptides orexins and their G protein-coupled receptors, OX<sub>1</sub> and OX<sub>2</sub>, were discovered in 1998, and since then, their role has been investigated in many functions mediated by the central nervous system, including sleep and wakefulness, appetite/metabolism, stress response, reward/addiction, and analgesia. Orexins also have peripheral actions of less clear physiological significance still. Cellular responses to the orexin receptor activity are highly diverse. The receptors couple to at least three families of heterotrimeric G proteins and other proteins that ultimately regulate entities such as phospholipases and kinases, which impact on neuronal excitation, synaptic plasticity, and cell death. This article is a 10-year update of my previous review on the physiology of the orexinergic/hypocretinergic system. I seek to provide a comprehensive update of orexin physiology that spans from the molecular players in orexin receptor signaling to the systemic responses yet emphasizing the cellular physiological aspects of this system.

orexin receptor; OX<sub>1</sub> receptor; OX<sub>2</sub> receptor; neuropeptide; G protein-coupled receptor

IN 2002, I SUMMARIZED the initial knowledge regarding orexin receptors in a review in *American Journal of Physiology-Cell Physiology* (201). Orexins had made a kick-start with some ~290 articles published during that initial period. Work during the subsequent 10 years has expanded and refined early ideas through new knowledge, development, and application of new techniques and critical thinking. Even so, answers to many key questions, for instance, the physiological role of orexins outside the central nervous system (CNS) and existence of orexinergic neuron subpopulations, have been elusive. Many reviews have appeared over the past 10 years but most have focused on specific aspects of orexin physiology. My aim here is to emphasize cellular mechanisms of orexin receptor signaling but also to present the full spectrum of orexin physiology at the tissue and organismal level.

## Glossary

2-AG	2-arachidonoylglycerol
AC	adenylyl cyclase
ACTH	adrenocorticotrophic hormone
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
anandamide	<i>N</i> -arachidonylethanolamine
BAT	brown adipose tissue
BIM	a neuroblastoma-hybridoma cell line
CB <sub>1</sub> and CB <sub>2</sub>	CB <sub>1</sub> and CB <sub>2</sub> cannabinoid receptors, respectively

CHO	Chinese hamster ovary-K1 (cells)
CMV	cytomegalovirus
CNS	central nervous system
cPLA <sub>2</sub>	cytosolic (Ca <sup>2+</sup> -sensitive) PLA <sub>2</sub>
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DKO	double-orexin receptor knock-out (knockout of both OX <sub>1</sub> and OX <sub>2</sub> receptors)
EPSC and IPSC	excitatory and inhibitory post-synaptic current, respectively
ERK	extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GIRK channels	G protein-coupled inward rectifier K <sup>+</sup> channels
GPCR	G protein-coupled receptor
HLA	human leukocyte antigen
HEK-293	human embryonic kidney cells
icv	intracerebroventricular
IHC	immunohistochemistry
IP <sub>3</sub>	inositol-1,4,5-trisphosphate
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
iv	intravenous

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Kir channels	inward rectifier K <sup>+</sup> channels
MAPK	mitogen-activated protein kinase
MCH	melanin-concentrating hormone
MEK1	MAPK/ERK kinase 1
MHC	major histocompatibility complex
NCX	Na <sup>+</sup> /Ca <sup>2+</sup> exchanger
neuro-2a	a mouse neuroblastoma cell line
NMDA	<i>N</i> -methyl-D-aspartic acid
NMR	nuclear magnetic resonance
NSCC	nonselective cation channel
OX <sub>1</sub> and OX <sub>2</sub>	OX <sub>1</sub> and OX <sub>2</sub> orexin receptors, respectively
OX <sub>1</sub> - and OX <sub>2</sub> -KO	OX <sub>1</sub> - and OX <sub>2</sub> -knockout, respectively
PC12	a rat pheochromocytoma cell line
PCR	polymerase chain reaction
PI3K	phosphoinositide-3-kinase
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP <sub>2</sub>	phosphatidylinositol-4,5-bisphosphate
PKA, PKB, PKC, and PKD	protein kinase A, B, C, and D
PLA <sub>2</sub> , PLC, and PLD	phospholipase A <sub>2</sub> , C, and D, respectively
PPAR $\gamma$	peroxisome proliferator-activated receptor- $\gamma$
PPO	preproorexin
PPO-KO	PPO-knockout
PPO-pro-ataxin-3	knockin with ataxin-3 under the proximal PPO promoter
PPO-pro-channelrhodopsin-2	channelrhodopsin-2 under the proximal PPO promoter (in lentivirus)
PPO-pro-halorhodopsin	knockin with halorhodopsin under the proximal PPO promoter
PTx	pertussis toxin
RT-PCR	reverse-transcriptase PCR
SAPK	stress-activated protein kinase
SHP-2	a protein tyrosine phosphatase
TM	transmembrane helix (of orexin receptor)
TTC-GFP	knockin with GFP-fused to the COOH-terminal tetanus toxin fragment under the proximal PPO promoter
TRPC	transient receptor potential channel of the canonical subfamily
TTx	tetrodotoxin

VGCC	voltage-gated Ca <sup>2+</sup> channels
VLPO wt	ventrolateral preoptic nucleus wild-type

#### HISTORICAL PERSPECTIVE

The discovery of orexin peptides and receptors was reported in two independent publications in 1998. de Lecea and Sutcliffe with colleagues described two putative peptide transmitters, encoded by the same propeptide (78). They named the peptides hypocretins (“hypo” for hypothalamus, “cretin” for the homology of hypocretin-2 with some incretin peptides). The peptides were expressed in synaptic vesicles of hypothalamic neurons, and hypocretin-2 was shown to be strongly neuroexcitatory in neuronal cultures. Shortly thereafter, Sakurai and Yanagisawa used a different approach, deorphanization of a putative G protein-coupled receptor (GPCR), HFGAN72, and identified two peptide transmitters that activated the receptor, the common precursor peptide and its gene, and, finally, a second receptor based on a sequence homology search (307). Furthermore, they investigated the peptide-receptor pharmacology, mapped the peptide and receptor mRNA expression in the CNS, and linked the peptides to the regulation of appetite by showing stimulation of feeding upon intracerebroventricular infusion and increased peptide mRNA expression in the hypothalamus upon fasting. The peptides were termed orexins for their orexinergic function and the receptors OX<sub>1</sub> and OX<sub>2</sub> receptors. It was soon discovered that hypocretin-1 and orexin-A as well as hypocretin-2 and orexin-B were the same peptides. Both sets of names are still in use.

The next major breakthrough in orexin research occurred a year later. The group of Mignot isolated two *canarc* gene mutations responsible for hereditary canine narcolepsy; both mutations were in the OX<sub>2</sub> receptor gene, causing frameshift and premature stop (218). Later, both mutants were shown to produce a receptor blockade at intracellular sites (159). Yanagisawa's group then showed that knockout of the precursor peptide, preproorexin (PPO), causes narcoleptic phenotype in mice (61). In 2000, a report found orexin-A to be at very low or undetectable levels in the cerebrospinal fluid (CSF) of human narcoleptics with cataplexy (270).

These findings began an avalanche of studies with mapping of the orexinergic pathways, regulation of different physiological functions by orexins, cellular signaling of orexins, and pharmacology, which thus far has resulted in nearly 3,000 articles.

#### OVERVIEW OF OREXINS AND OREXIN RECEPTORS

##### *Orexin Peptides*

Native orexin peptides consist of the receptor agonists, orexin-A (33 amino acids) and orexin-B (28 amino acids) (aka hypocretin-1 and hypocretin-2) (Fig. 1), and their precursor, preproorexin (PPO, aka preprohypocretin). The PPO gene encodes one copy of each peptide; the gene structure has apparently been generated with an intraexon duplication followed by diversification (8). The PPO gene may have arisen by circular permutation of an ancestral secretin gene in a procordeate species [teleost fish are the lowest animal subclass that express orexins and also show functional responses (8, 99, 182,

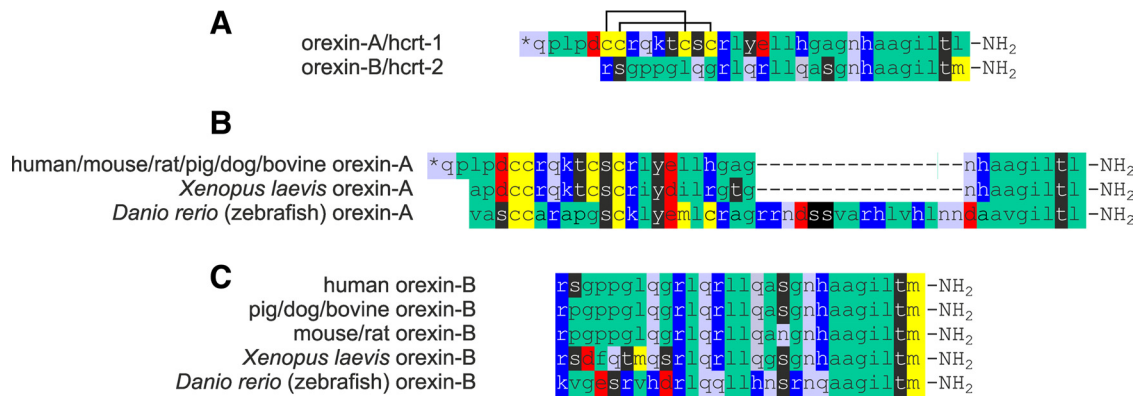


Fig. 1. Orexin peptide sequences. Note that the *Xenopus* and zebrafish sequences are predicted. The peptides are COOH-terminally amidated (although this is not verified for all species variants). The NH<sub>2</sub>-terminal glutamine of mammalian orexin-A is cyclized to pyroglutamate (marked \*q) and two disulfide bridges, as indicated in A, are present (not verified for nonmammalian variants). Amino acids are marked in different color code based on selected side-chain properties: pure CH-containing (aliphatic and aromatic) in green, OH-containing in black with white lettering, S-containing in yellow, amide-containing in mauve, other N-containing in blue with white lettering, and COOH-containing in red.

408)], though the relationship to secretin has recently been questioned (345). Sequences of orexin peptides are rather conserved in mammalian species while the sequences diverge in lower vertebrates (Fig. 1). Other neuropeptides have not been shown to bind to orexin receptors (150, 307, 329).

Both mature peptides are amidated in their COOH terminus; orexin-A, in addition, possesses two disulfide bridges and an NH<sub>2</sub>-terminal glutamine cyclized to pyroglutamate (307) (Fig. 1). The enzymes taking part in the processing of PPO are not known although predictions can be made. Several solution NMR structures, suggesting partly helical structure for both peptides, are available (188, 209, 344).

Orexin-A appears to be the more stable and more lipophilic of the two mature peptides (183) and can be detected in the CSF. In mice, orexin-A was found to be able to cross the blood-brain barrier in both directions (183). In contrast, Bingham et al. (35) found essentially no brain penetration of intravenous orexin-A in mice or rats. Poor penetration of intravenous orexin-A into the brain was also noted with orexin replacement therapy in a dog with sporadic narcolepsy (109). Some studies suggest that orexin-B levels are higher than those of orexin-A in several brain regions (256, 257) as well as selective enhancement of orexin-B levels in some conditions (55, 56). However, orexin peptide determinations may be uncertain because of possibly low specificity of orexin antibodies (see *Antibodies*).

### Orexin Receptors

Orexin receptors (Fig. 2) are class A GPCRs and comprise a subgroup of peptide receptors with low homology (~30%) to other GPCRs (201). Homology between the species orthologs is rather high and there are no known pharmacological differences.

Orexin receptors have not been crystallized, and thus their structures can only be surmised on the basis of computer model predictions. There are numerous GPCR structures that can be used as templates for homology modeling; these include (rhod)opsin,  $\beta_1$  and  $\beta_2$  adrenoceptors, M<sub>2</sub> muscarinic cholinergic receptors, and  $\kappa$  and  $\mu$  opioid, A<sub>2A</sub> adenosine, D<sub>3</sub> dopamine, and CXCR4 chemokine receptors, certain of which have been solved in different conformations [(66, 128, 238, 390, 391);

reviewed in (70)]. Homology modeling and molecular dynamics simulations have been performed for orexin receptors (135, 237, 356). OX<sub>1</sub> and OX<sub>2</sub> were modeled, the ligands were “docked,” and the amino acids predicted to be important for binding were replaced. These are useful approaches, but receptor mutagenesis can lead to an incorrect conclusion because a receptor’s structural integrity may be compromised in such mutants. Domain exchange mutagenesis between OX<sub>1</sub> and OX<sub>2</sub> receptors, an approach that may be less perturbing of receptor structure, has also been done (286, 356). However, this approach is also not free from effects on receptor structure, as indicated by poor expression of some constructs (286, 287). These studies identify the region around TM3 and TM4 as most important for high-affinity OX<sub>1</sub> receptor binding of the antagonists SB-674042 and SB-334867. The NH<sub>2</sub>-terminal-TM1 region also influences SB-674042 binding. Data from domain exchange and point mutation studies do not fully agree. Domain exchange studies infer that agonist selectivity is also determined by the TM3-TM4 region (287, 356). Assessment of GPCR structures with other techniques, such as NMR (67, 347), has not been applied to orexin receptors.

### Orexin Receptor Variants and Mutants

Mouse OX<sub>2</sub> receptor has been proposed to be expressed in two variants ( $\alpha$  and  $\beta$  isoforms) with different COOH termini due to alternative splicing of the transcript (64). The splicing may be subject to regulation in different tissues and brain nuclei (63, 64). Some receptor sequence variants, such as 167 Gly/Ser, 265 Leu/Met, 279 Arg/Glu, 280 Gly/Ala, 281 Arg/His, and 408 Ile/Val for OX<sub>1</sub> and 10 Pro/Ser, 11 Pro/Thr, 193 Cys/Ser, 293 Ile/Val, 308 Val/Ile, and 401 Thr/Ile for OX<sub>2</sub>, are found in humans (201, 276, 282) and may represent mutations or polymorphisms. Correlation with some disorders (cluster headache, depression, panic disorder) has been found for some genotypes (OX<sub>1</sub><sup>Val408</sup>, OX<sub>2</sub><sup>Ile308</sup>) (14, 248, 289, 290) but causality remains to be shown. The *canarc* mutant narcoleptic dogs harbor frameshift mutations in the OX<sub>2</sub> gene, prematurely truncating the receptor protein (218). Another OX<sub>2</sub> mutation found in a narcoleptic dog, Glu54Lys, does not truncate the receptor or hinder the membrane expression but instead, seems to abrogate orexin binding and receptor signaling (159). Some

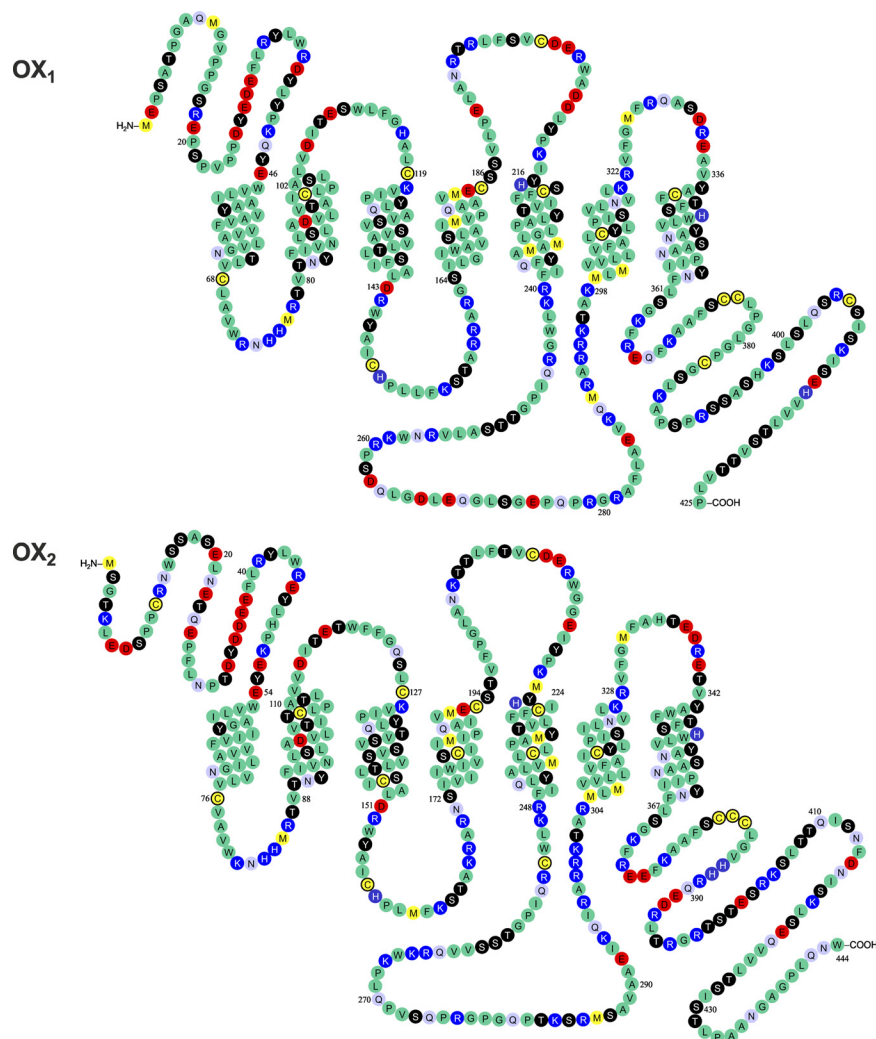


Fig. 2. Peptide sequences of the human orexin receptors utilized in the studies of the author. Note that there are a number of sequence variants known [see *Orexin Receptor Variants and Mutants*]. Amino acids are marked in different color code based on some of their side-chain properties (as in Fig. 1): pure CH-containing (aliphatic and aromatic) in green, OH-containing in black with white lettering, S-containing in yellow, amide-containing in mauve, other N-containing in blue with white lettering, and COOH-containing in red.

receptor mutations have been experimentally introduced to investigate receptor structure/pharmacology or signaling (see above and under *Cell Death*).

## OREXIN RECEPTOR LIGANDS

### Agonists

Orexin-A and -B are usually considered equal ligands for OX<sub>2</sub> receptor but orexin-B is thought to be a 5- to 100-fold weaker ligand than orexin-A at OX<sub>1</sub> receptors. This view originates from studies of binding and Ca<sup>2+</sup> elevation in recombinant CHO cells (307), and in other heterologous expression systems, including CHO, neuro-2a, PC12 and BIM cells by Ca<sup>2+</sup> elevation or phospholipase C (PLC) activation (9, 151, 328, 346, 414). The finding provides a basis for using orexin-A and orexin-B to distinguish between OX<sub>1</sub> and OX<sub>2</sub> receptors in native systems (201). However, even in defined, recombinant expression systems, this distinction is not always valid (Table 1). Certain such systems, e.g., CHO and HEK-293 cells, deviate with respect to the same response like Ca<sup>2+</sup> elevation (288). The results are likely explained by “biased agonism” (selective agonist trafficking of receptor responses; 185–187, 200). Differences between cell lines may result from distinct signal transduction machineries. Also orexin receptor

heteromerization partners (see *Interaction of orexin and endocannabinoid systems*) could impact this. The alanine-scanned orexin peptides differentially activate Ca<sup>2+</sup> influx and release (9), which suggests that orexin peptide-receptor interaction may show biased agonism. A further implication of this is that these two Ca<sup>2+</sup> responses may be regulated by different signaling pathways. The finding that Ca<sup>2+</sup> elevation measured in the same cells shows a different profile if cells are attached or detached (Table 1) indicates that there may be detachment-induced changes in the signaling cascades.

Some studies report orexin peptide mutagenesis. NH<sub>2</sub>-terminal truncation of orexin-A successively reduces the potency (and likely the binding affinity) at OX<sub>1</sub> and OX<sub>2</sub>; however, the extreme NH<sub>2</sub> terminus has not been investigated (9, 74, 205, 206, 275). No data have been published regarding COOH-terminal truncation of orexin-A while removal of the COOH-terminal methionine from orexin-B eliminates its activity (206). Orexin-A and orexin-B are highly homologous in this region (Fig. 1), suggesting the central importance for peptide-receptor interaction; however, the COOH-terminal amino acid is not conserved. The COOH terminus of orexin-A or -B does not allow additions (133; Putula J, Turunen PM, and Kukkonen JP, unpublished observations). Studies using alanine scanning of

Table 1. *Orexin-A- and -B pharmacology with respect to different responses in our clone of human OX<sub>1</sub>-expressing CHO cells*

Response and Method	EC <sub>50</sub> -orexin-B/EC <sub>50</sub> -orexin-A: OX <sub>1</sub>	EC <sub>50</sub> -orexin-B/EC <sub>50</sub> -orexin-A: OX <sub>2</sub>	Reference No.
Ca <sup>2+</sup> measurement with attached cells	2.6	1.3	(288)
Ca <sup>2+</sup> measurement with detached cells	6.9	1.0	(9)
Arachidonic acid release with attached cells	3.3	Not measured	(363)
Arachidonic acid release with detached cells	15	Not measured	(365); Orexin-B data from Putula J, Turunen PM, and Kukkonen JP, unpublished observations
PLC assay with attached cells	14	Not measured	(171)
PLD assay with attached cells	13	Not measured	(171)
ERK phosphorylation with attached cells	18	Not measured	(10)
cAMP response with detached cells in the absence of cholera toxin (G <sub>s</sub> activation)	1.6	Not measured	(149)
cAMP response with detached cells in the presence of cholera toxin (PKCδ activation)	12	Not measured	(149)

the truncated orexin-A peptides point out certain amino acids. Truncation and alanine substitutions have a similar impact on both OX<sub>1</sub> and OX<sub>2</sub>, but the potency of the peptide to activate OX<sub>2</sub> receptor is somewhat less affected (9, 275); this may be predicted, based on the lower selectivity of OX<sub>2</sub> between orexin-A and -B (see above). Whether the disulfide bonds in orexin-A play a role is not clear (205, 206). A truncated orexin-B, orexin-B<sub>6-28</sub>, has equal or higher potency than orexin-B for OX<sub>1</sub> and OX<sub>2</sub> and some substituted orexin-B<sub>6-28</sub> analogs may have high selectivity for OX<sub>2</sub> (205). Ala<sup>11</sup>,DLeu<sup>15</sup>-orexin-B is another agonist with a reputed high selectivity for OX<sub>2</sub> (16). However, in our studies the selectivity of this ligand, assessed by Ca<sup>2+</sup> elevation in recombinant CHO and HEK-293 cells, was less than 10-fold (288).

The agonist profiles of orexin-A and orexin-B or sometimes also Ala<sup>11</sup>,DLeu<sup>15</sup>-orexin-B have been used to determine the involvement of the OX<sub>1</sub> or OX<sub>2</sub> receptor in particular responses in native systems. As discussed above and in previous studies (199, 201), one can question the use of agonists to determine involvement of the orexin receptor subtypes since data with recombinant cells suggest that biased agonism occurs at orexin receptors. In native systems, multiple types of receptors may contribute to the measured output, especially when the responses involve complex neuronal circuitry.

Low-molecular-weight orexin receptor agonists are not known. It has been proposed that the OX<sub>1</sub>-selective antagonist SB-334867 sometimes shows weak agonistic behavior (28; Putula J and Kukkonen JP, unpublished observations).

### Antagonists

Orexin receptor antagonists have been and are commercially developed as drug candidates as reviewed in several publications (69, 300, 314). OX<sub>1</sub> receptor was first targeted with SB-334867 (285); this ligand has been an important tool in orexin research. Other OX<sub>1</sub>-selective ligands include SB-408124, SB-410220, and SB-674042 (also as <sup>3</sup>H-SB-674042) (207). Most of the more recently reported ligands have been OX<sub>2</sub>-selective or nonselective. A major hindrance for orexin research is lack of commercial availability of other ligands than SB-334867, SB-408124, TCS-OX2-29 [compound 29 of (140)], and TCS 1102 [compound 18 of (29)].

### Labeled Ligands

[<sup>125</sup>I]-labeled orexin-A and -B are the only commercially available radioligands but are not well-suited for receptor

studies due to high filter binding, high-energy radiation, etc. (199). Also, the agonistic nature of the ligands makes them problematic; agonist radioligands may only detect the G protein-precoupled receptor population ("agonist high-affinity site"), and in intact cells the agonistic effects (receptor activation including possible desensitization and internalization) are not favorable (200). Custom-made <sup>3</sup>H-SB-674042, <sup>3</sup>H-almorexant, and <sup>3</sup>H-EMPA have been used by pharmaceutical companies (207, 235–237). Fluorescently labeled orexin-A has been used in some studies (74, 96, 181), but their potencies/affinities have not been reported. Orexin-B has been labeled with the toxin saporin (orexin-B-saporin), which can be used to selectively ablate orexin receptor-expressing neurons [see, e.g., (115)]. It is unclear whether only OX<sub>2</sub>-expressing neurons are eliminated.

### CELLULAR SIGNALING OF OREXINS

Orexin responses are mediated by the two G protein-coupled orexin receptors. A wide cellular response repertoire is seen in different cell types, but even a single cell type can have multiple responses (Fig. 3). One of the major challenges in orexin research is the evaluation of the significance of various responses in different contexts.

#### G Protein Coupling of Orexin Receptors

Many reviews indicate that OX<sub>1</sub> receptors couple to G<sub>q</sub> proteins and that OX<sub>2</sub> receptors couple to G<sub>q</sub> and G<sub>i/o</sub> family members. However, no experimental evidence fully supports these conclusions. First, it can be difficult to determine G protein coupling of a GPCR, due to, for instance, the scarcity of specific molecular tools, the difficulty of direct measurements, and the need to disrupt the cellular milieu (200). The only direct measurements of multiple G protein coupling of orexin receptors have been by assessment of <sup>32</sup>P-GTP-azido-anilide-labeling combined with immunoprecipitation. The results reveal that OX<sub>2</sub> receptors in human adrenal cortex activate G<sub>i</sub>, G<sub>s</sub>, and G<sub>q</sub> proteins (181, 294). Mixed orexin receptor populations in rat adrenal cortex or hypothalamus couple to G<sub>i</sub>, G<sub>o</sub>, G<sub>s</sub>, and G<sub>q</sub> (180) (Figs. 3 and 4A). In brain slices, <sup>35</sup>S-GTP-labeling of orexin-activated G proteins also suggests coupling to G<sub>i/o</sub> family G proteins (30, 31). These are the only direct evidence for G protein coupling; little can be concluded from the results. The techniques, although powerful, require disruption of the cellular structure before the receptor stimulation, and they may thus disrupt the signaling complexes, etc.

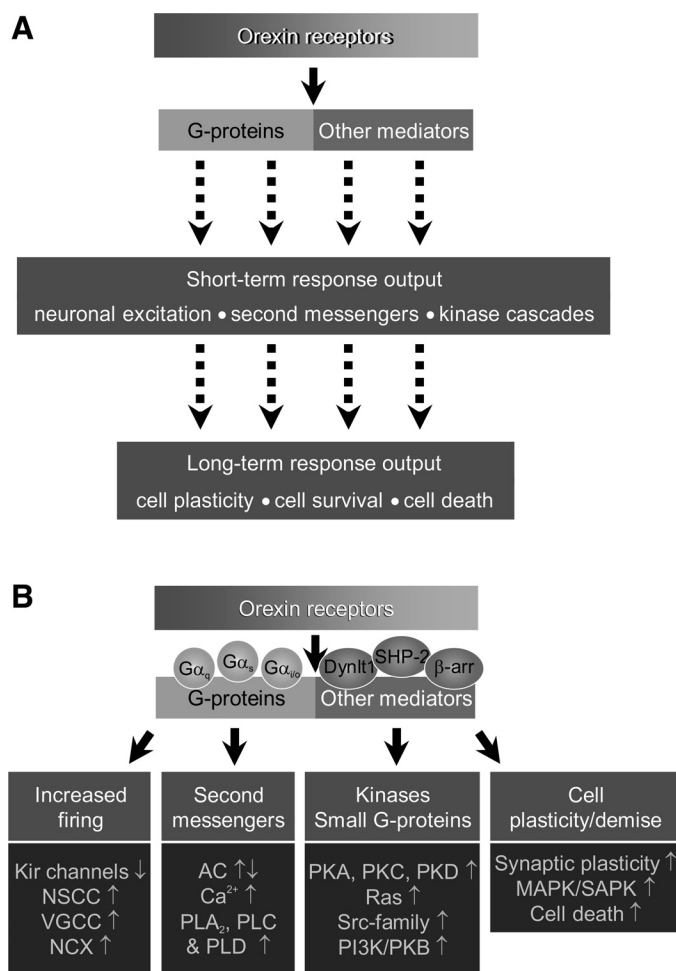


Fig. 3. Overview of cellular responses to orexin stimulation. Some responses have been omitted for the sake of clarity. A: examples of general responses. B: some molecular mechanisms. The relationships between different outputs measured are often not clear (see text and Figs. 4 and 5 for explanations). AC, adenylyl cyclase;  $\beta$ -arr,  $\beta$ -arrestin; Dyntl, dynein light chain Tctex-types 1 and 3; see *Glossary* for other definitions.

Indirect measurements of receptor responses in cell lines or ex vivo tissue samples have been interpreted to indicate coupling of orexin receptors to certain G proteins. However, one must be careful in drawing conclusions from such studies. For example, an elevation or decrease in cellular cAMP levels does not necessarily indicate coupling to  $G_s$  or  $G_i$  proteins, respectively; adenylyl cyclases (ACs) that generate cAMP and phosphodiesterases that hydrolyze cAMP are regulated in many ways (338). PLC activation does not necessarily reflect involvement of  $G_q$ , as PLC isoforms can be regulated in other ways, too (198). Use of inhibitors to infer signaling mechanisms can also lead to erroneous conclusions. AC inhibitors may be toxic at the concentrations needed for inhibition (10), and so is the PLC inhibitor, U-73122 (348). In recombinant cell systems, one must consider artifacts induced by clonal selection, receptor overexpression, and nonnative signaling environment.

Indirect measurements of  $OX_1$  receptor regulation of AC in recombinant CHO cells indicate coupling to  $G_q$ ,  $G_i$ , and  $G_s$  proteins, but this could, in part, reflect artificial coupling from receptor overexpression (149). Many studies show strong PLC

activation by orexin receptors based on PLC activity assays (151, 168, 169, 179, 180, 228, 245, 294) and indirect evidence, i.e.,  $Ca^{2+}$  release (151, 228). PLC-produced  $IP_3$  (Fig. 4A) underlies  $Ca^{2+}$  release in  $OX_1$ -expressing CHO cells (90). This may result from  $G_q \rightarrow PLC\beta$  coupling of the receptors but has not been proven; other PLC isoforms may be involved. Indeed, evidence exists for involvement of at least two different PLC types in  $OX_1$  receptor signaling in CHO cells (168).  $Ca^{2+}$  elevation induced by orexin receptor activity also involves PLC-independent  $Ca^{2+}$  influx, likely via nonselective cation channels (Fig. 4B). In recombinantly  $OX_1$ -expressing HEK-293 cells, results with chimeric  $G_s$ -based G proteins suggested that the receptors couple to  $G_{q/11}$  and  $G_{16}$ , another  $G_q$  family member (231). Heterologous expression of dominant-negative G proteins has been used to determine orexin receptor coupling in recombinant and native cells (292, 346). However, dominant-negative G proteins likely produce less-specific receptor blockade (200), which may explain the  $>100\%$  inhibition observed if one sums the effects of each dominant-negative construct alone (293, 346).

The only “selective” G protein inhibitor available, pertussis toxin (PTx), which inactivates  $G_{i/o}$  proteins and blocks their activation by GPCRs, has been used to block orexin signaling (143, 149, 309, 372). However, inhibition of  $G_{i/o}$  might also have an indirect effect on orexin signaling, and PTx may also affect other cellular targets than  $G_{i/o}$  proteins [e.g., (239)].

In summary, the G protein coupling of orexin receptors is far from clear, but based on the evidence available, both  $OX_1$  and  $OX_2$  receptors are likely to couple to  $G_{i/o}$ ,  $G_s$ , and  $G_q$  family G proteins (Figs. 3 and 4A).

#### Orexin Receptor Coupling to Other Proteins

GPCRs can interact with proteins other than heterotrimeric G proteins, but in many cases the functional significance of such interaction remains elusive (298). Orexin receptors directly interact with dynein light chain Tctex-types 1 and 3 [(87); Fig. 3B], but the functional effect is unclear. Immunoprecipitation revealed that the protein tyrosine phosphatase SHP-2 interacts with activated  $OX_1$  receptors, an interaction that may mediate cell death (see *Cell Death*).  $OX_1$  receptor activation induces  $\beta$ -arrestin translocation, and  $\beta$ -arrestin may contribute to ERK signaling (96, 251), effects akin to those noted for many other GPCRs (291).

#### Cell Plasticity

Many GPCRs activate signaling cascades involved in the regulation of cell growth, plasticity, survival, and death, for instance MAPK/SAPK pathways (84, 123, 124). This may occur via “direct” downstream activity cascades or by trans-activation of tyrosine kinase receptors (84, 124, 322). Orexin receptors activate ERK and p38 MAPK/SAPK cascades in recombinant cells (10, 12, 139, 201, 346) and in some native cells and cell lines (292, 321). Indirect evidence has been obtained by inhibition of p38 or MEK1 (the kinase activating ERK) (12, 320, 321, 333). The upstream activation mechanisms of these cascades are largely unclear, but analysis in  $OX_1$ -expressing CHO cells suggests that Ras is upstream of ERK activation and that it is probably regulated by protein kinase C (PKC), phosphoinositide-3-kinase (PI3K), and Src signaling (10). PI3K, a central cell survival signal, has also

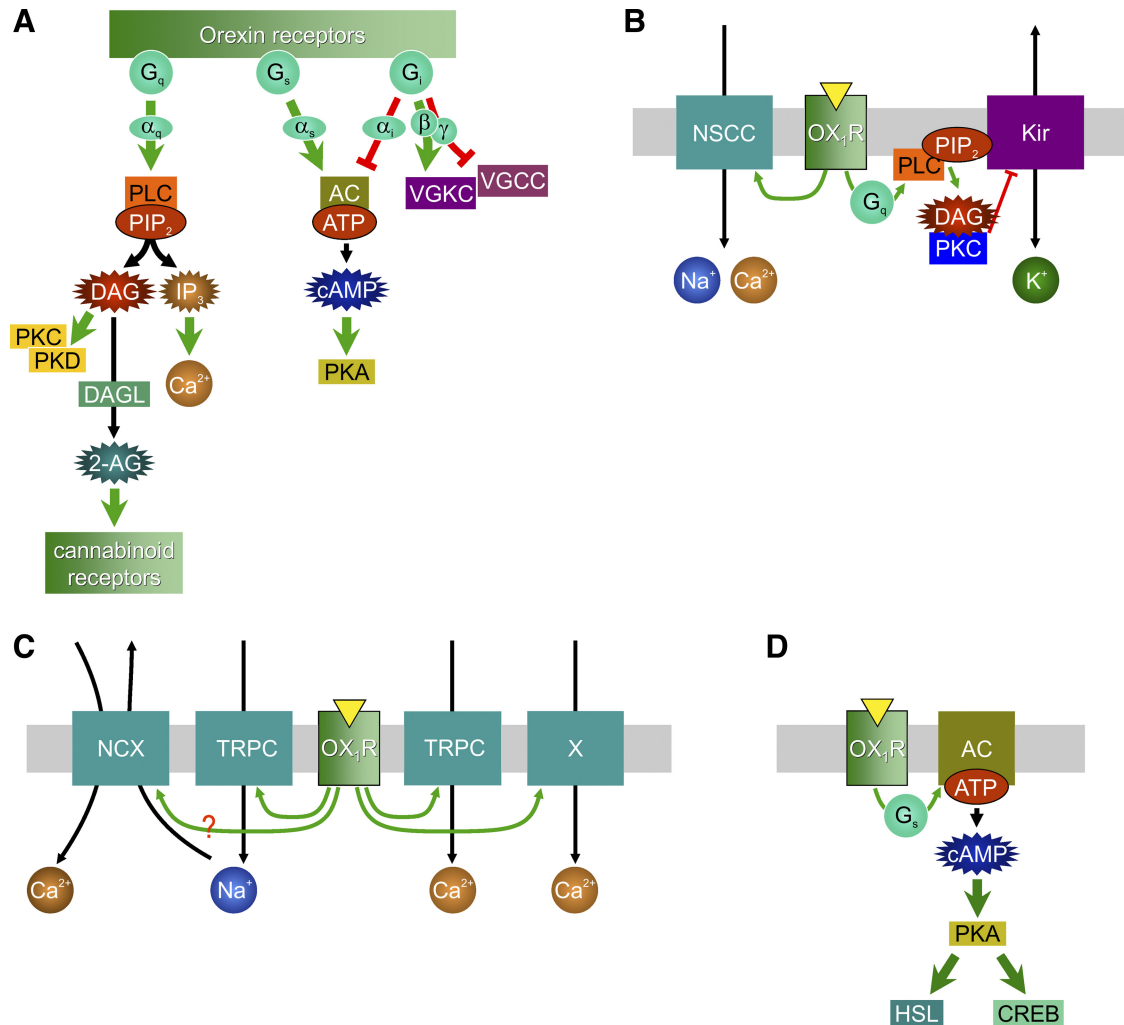


Fig. 4. Some specific orexin signaling mechanisms. *A*: G protein signaling of orexin receptors and the putative targets. As described in the text, most of the responses presented have been shown for orexin receptors, except the Gβγ signaling. Most of the signal cascades have targets in addition to those shown; for instance, cAMP can act on EPAC, a guanine nucleotide exchange factor for Rap1, and NSCC. The pathways are also strongly simplified; e.g., AC activity and cAMP levels are regulated in many additional ways. *B*: in neurons, postsynaptic depolarization is induced upon activation of NSCC and K<sup>+</sup> channels (putatively Kir-type). Some possible mechanisms for Kir activation are depicted. Molecular pathways for NSCC activation are largely unknown. *C*: Ca<sup>2+</sup> influx in native neurons and recombinant cells is obtained via activation of different nonselective cation channels of both the TRPC family and other types (“X”). In addition, VGCC may be targeted (not shown). TRPC (or other NSCC)-mediated Na<sup>+</sup> influx probably most strongly leads to Na<sup>+</sup> influx; the reverse mode of NCX may then mediate Ca<sup>2+</sup> influx (*left*). Whether the activation of NCX is a passive process or stimulated by orexin receptors (e.g., via PKC) is unclear (“?”). *D*: signaling in the adrenal cortex. Orexin-activated cAMP → PKA pathway underlies glucocorticoid release. Orexins may also induce enzymes required for steroid synthesis. Orexin stimulation elevates cAMP putatively via Gα<sub>s</sub> → AC coupling. cAMP activates PKA, which phosphorylates targets, including hormone-sensitive lipase (HSL), which liberates cholesterol for glucocorticoid synthesis, and cAMP response element-binding protein (CREB), which can mediate enzyme induction. VGKC, voltage-gated K<sup>+</sup> channels; see *Glossary* for other definitions.

been implicated in other studies (126, 320, 327). Orexin receptor signaling is involved in long-term potentiation (LTP) in mouse hippocampal slices, a response that is blocked by kinase inhibitors including the PI3K inhibitor wortmannin, the MEK1 inhibitor PD98059, and the p38 inhibitor SB-203580. As all the inhibitors fully block the response alone, there is some doubt as to the specificity or cellular target of the inhibition (320). Orexin-A stimulates (putatively via ERK) AMPA receptor insertion on the cell surface in cocultures of rat prefrontal cortex and striatum neurons; the same process may trigger the long-term enhancement of AMPA currents within the striatum in a slice preparation (323). In the rat ventral tegmental area, orexin-A stimulates NMDA receptor currents by enhanced NMDA receptor translocation to the plasma

membrane via a PKC-dependent pathway (40). In cultured rat adrenocortical cells, the ERK pathway may mediate orexin-stimulated cell division while p38 inhibits cell division (333). In InR1-G9 glucagonoma cells, the PI3K pathway may mediate suppression of glucagon expression and secretion (126). Orexins are indispensable for postnatal development of brown adipose tissue (BAT) (see *Feeding and Metabolism*).

*Cell Death*

Orexin receptor stimulation can induce cell death in recombinant CHO cells and in native colon carcinoma and neuroblastoma cells (11, 12, 301, 385, 387). In recombinant OX<sub>1</sub>-expressing CHO cells, p38 seems to be responsible for orexin



receptor-promoted apoptotic cell death, characterized by caspase activation, nuclear condensation, gene transcription, and protein synthesis (12). Interestingly, the inhibition of caspases does not block the cell death but rather changes the profile of cell death by eliminating the need for gene transcription (12).

Yet another mechanism for  $OX_1$ -mediated cell death has been suggested. Inhibition of tyrosine phosphatases with phenyl arsine oxide (PAO) or Src-family kinases with PP2 fully inhibited cell death in a recombinant CHO-S cell clone (386). Expression of wild-type (wt)  $OX_1$  receptor in native mouse embryonic fibroblasts but not in  $G_{\alpha_q}$ - and  $G_{\alpha_{11}}$ -deficient (MEFQ11) cells induced cell death. The authors concluded that  $G_q$  or  $G_{11}$  mediates the cell death perhaps via Src family kinases but not via PLC, as PLC inhibition did not inhibit the cell death. Both orexin receptors were discovered to express novel motifs, immunoreceptor tyrosine-based inhibitory motif (*ITIM*), IYNFL, at the junction of TM7 and the COOH terminus (386), and immunoreceptor tyrosine-based switch motif (*ITSM*) in the TNYFIV-sequence, at the junction of intracellular loop 1 and TM2 (91). Mutation of the tyrosine in either motif in  $OX_1$  receptor (Y358F and Y83F, respectively) blocked interaction of the activated receptor with the tyrosine phosphatase SHP-2 and orexin-induced cell death. Cell death was also blocked by dominant-negative SHP-2 or the SHP inhibitor NSC-87877. Thus, it was suggested that these motifs are phosphorylated by a Src family kinase at Tyr<sup>358</sup> and Tyr<sup>83</sup>, respectively, at which SHP-2 would bind and induce cell death.

Those results are of great interest but there are some problems with their interpretation. GPCRs contain a highly conserved TM7 NPxxY-motif, which is essential for receptor stability [see, e.g., (98, 173)]; in orexin receptors the tyrosine is the same as that in the postulated *ITIM* motif. Different GPCRs are affected in different ways by substitutions in the NPxxY-motif, in terms of impact on functional activity and trafficking. In addition, substitution of different amino acids for Tyr leads to different phenotypes [(173) and references therein]. Perhaps the structural changes in the  $OX_1$ -Y358F receptor perturb its proper expression and/or signaling. Such effects could explain the apparently contradictory finding that the mutation abolishes  $Ca^{2+}$  signaling by the receptor and drastically reduces high-affinity [<sup>125</sup>I]-labeled orexin-A binding (387), although Y358 phosphorylation should be downstream of G protein activation, and, as we know from our experiments in CHO cells, PLC activation by  $OX_1$  receptors does not require Src activity (10; Ekholm M and Kukkonen JP, unpublished observations). By contrast, Y83F only inhibits cell death but not receptor binding or  $Ca^{2+}$  signaling. In addition, if the two tyrosine phosphorylation sites (*ITIM* and *ITSM*) were independent, as the authors suggest, mutations of either one (Y83F and Y358F) should not fully block tyrosine phosphorylation of  $OX_1$ . Thus, the sole Src-phosphorylation and SHP-2-binding site of the  $OX_1$  receptor may reside in the *ITSM* sequence assuming that it (TNYFIV) is accessible for phosphorylation; a phosphopeptide analysis of  $OX_1$  is needed.

Different signal pathways can induce cell death. We identified the p38 MAPK/SAPK pathway in CHO cell clones (12), whereas others identified SHP-2 as a central player (91, 386) (Fig. 5). These results, as such, are not mutually exclusive, and the outcomes of inhibition of SHP-2 and p38 have not been

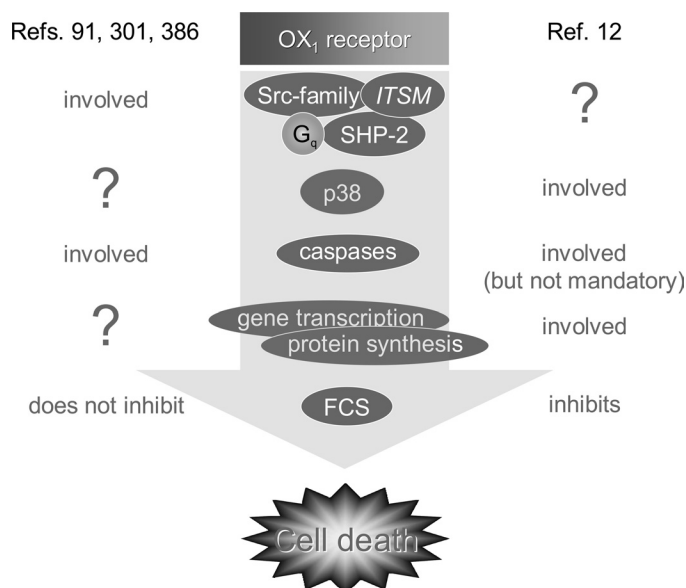


Fig. 5. Comparison of the orexin-mediated cell death pathways determined in the independent studies of two research groups. FCS, fetal calf serum; p38, a MAPK/SAPK; see *Glossary* for other definitions.

tested in the same system; while we have used the classical CHO-K1 cells (12), suspension-adapted CHO-S subclone was used in other studies (91, 386). The signal pathways are not known in the native cells or cell lines where programmed cell death is also seen (301, 385).

Does orexin-induced cell death have a physiological consequence? There is no direct knowledge of this. Despite the fact that the primary signal transduction cascades identified in orexin-mediated cell death are activated in minute-hour scale (12, 301, 386), orexin-induced cell death seems to require a clearly longer exposure time (several hours-days), and, in the lack of good methods to measure physiological orexin levels (see *Antibodies*), we do not know whether such occurs in the body. However, it is not known whether a shorter “priming” exposure or repetitive exposures to orexins could lead to cell death in the long run. No healthy native cells, which express orexin receptors, seem to have been exposed to orexins for longer time periods, with a notable exception of C3H10T1/2 mesenchymal stem cells and HIB1b preadipocytes, which differentiate towards brown adipocytes upon this treatment. Orexin receptors are expressed in some cancer cell types, where they can induce cell death (301, 385), and thus orexin receptor activation could be considered interesting from the perspective of cancer therapy. However, it is difficult to predict the responses of healthy tissues on long-term orexin receptor activation, if such was accomplished by exogenous drugs.

Recently, orexins have been shown to protect immortalized hypothalamic neurons against  $H_2O_2$  toxicity (54). ERK activation is also suggested to be antiapoptotic in  $OX_1$ -expressing CHO cells although cell death via the p38 pathway becomes dominant in the long run (12).

#### *Ion Fluxes in Neurons and Other Cell Types*

Orexins are strongly neuroexcitatory, as shown in the initial study (78) and verified in a large number of subsequent studies on neurons from different brain regions, mainly by the use of

electrophysiological techniques on ex vivo slices. The excitatory action of orexins has been ascribed both to pre- and postsynaptic mechanisms, i.e., enhanced transmitter release and facilitation of depolarization, respectively. The latter have been more frequently reported and investigated in more detail. Two classical mechanisms seem to contribute to the postsynaptic depolarization, namely inhibition of  $K^+$  channels and activation of nonselective cation channels (Fig. 4B). The presynaptic and postsynaptic effects can be distinguished by synaptic isolation protocols [tetrodotoxin (TTx), low  $Ca^{2+}$ -high  $Mg^{2+}$ ] and measurements of synaptic currents.

Inhibition of  $K^+$  channels is indicated when depolarization is accompanied by an increase in input resistance (reduction in membrane conductance); the channel types involved can be identified based on current-voltage ( $I$ - $V$ ) curves, ion manipulations, etc. Inhibition of  $K^+$  channels by orexins occurs in many brain nuclei within cerebrum, diencephalon, and brain stem (23, 27, 37, 47, 83, 117, 118, 144, 155, 158, 160, 167, 195, 261, 392, 406, 407, 410, 411). The signal transduction mechanisms or specific channel identities have generally not been investigated but in some cases the responses are insensitive to PTx (144) and mediated by PKC (407, 411), suggestive of a  $G_q \rightarrow PLC\beta \rightarrow PKC$  pathway. Inward rectifier  $K^+$  (Kir) channels may be orexin targets (Fig. 4B). Kir channels of the subfamily 3 (Kir3 aka GIRK channels) are targets for many inputs from GPCRs; for instance, the PLC pathway may inhibit the channels by depletion of  $PIP_2$  as well as by activation of PKC via DAG (229, 304). Other Kir subfamilies can also be targeted by GPCR signaling (138, 395). Kir3.1/3.2 (GIRK1/2) channels are inhibited in recombinant HEK-293 cells by orexin receptor activation via a PTx-insensitive pathway (143).

Activation of cation influx is suggested by a decrease in input resistance. For orexins, this has been seen in various regions of cerebrum, diencephalon, and brain stem (53, 94, 158, 160, 220, 392, 393, 405, 406). An obvious candidate for these responses is nonselective cation channels; some studies suggest their involvement (Fig. 4B).  $Na^+/Ca^{2+}$ -exchanger (NCX), an electrogenic exchanger carrying 3  $Na^+$  for 1  $Ca^{2+}$ , has also been implicated, based on blockade of the current by  $Ni^{2+}$  or KB-R7943 (3, 51, 94, 392, 393, 410). NCX can be active in normal ( $Na^+$  in,  $Ca^{2+}$  out) and reverse mode ( $Na^+$  out,  $Ca^{2+}$  in) and thus may depolarize or hyperpolarize cells. However, the conclusions are not always straightforward. Fully reliable  $I$ - $V$  curves are difficult to obtain in native neurons due to space-clamp problems, and pharmacological inhibitors are not highly selective. For instance, KB-R7943 is often used at concentrations that inhibit both modes of NCX in addition to ion channels (166);  $Ni^{2+}$  is even less selective. Intracellular signal pathways that trigger orexin-mediated cation fluxes have not been investigated in neurons.

Studies in recombinant systems may support the findings in neurons.  $OX_1$  receptor stimulation in CHO cells activates a receptor-operated  $Ca^{2+}$  influx pathway (208, 228, 363). TRPC family channels may mediate at least part of the current (208) (Fig. 4C). TRPC channels also contribute to orexin responses in  $OX_1$ -expressing HEK-293 cells and IMR-32 neuroblastoma cells (274, 280). NCX seems to take part in the response in recombinant cells (223), making conclusions more difficult. TRPC channels likely conduct more  $Na^+$  than  $Ca^{2+}$  under physiological conditions (377), and one of the proposed response pathways of orexins involves TRPC-mediated primary

$Na^+$  influx and secondary  $Ca^{2+}$  influx via reversely working NCX (223) (Fig. 4C). The activation mechanism of nonselective cation channels is unclear, but the phospholipase  $A_2$  ( $PLA_2$ ) pathway is a likely candidate in  $OX_1$ -expressing CHO cells (363, 364).

$Ca^{2+}$  measurements have sometimes been made in neurons [e.g., (164, 192, 193, 204, 358, 367, 368, 370, 371)]. PKC-mediated activation of L-type (or N-type) voltage-gated  $Ca^{2+}$  channels (VGCC) has been implicated in some cases (192, 193, 367, 370). Signaling to L- and N-type channels has been observed in some nonneuronal cells (334, 398).

TTx often eliminates action potential-dependent transmitter release and thus also contribution from a long-range upstream network. A direct presynaptic site of action has been suggested on the basis of results of TTx-insensitive enhancement of inhibitory or excitatory postsynaptic current (IPSC or EPSC) frequency upon orexin stimulation (77, 103, 331). Also some TTx-sensitive responses may be presynaptic: orexins may act by enhancing action potential-dependent responses and not on their own. Since TTx does not block retrograde synaptic transmission by, e.g., endocannabinoids (142), care is needed in drawing conclusions regarding the site of orexin action (pre- or postsynaptic).

#### Lipid Signals

GPCRs can couple to multiple lipid signaling systems via phospholipases (198). Orexin receptors activate PLC (228, 245, 294), and this signaling has physiological significance, e.g., in neuronal excitation (see *Ion Fluxes in Neurons and Other Cell Types*). Our studies of lipid signaling pathways activated by recombinant human  $OX_1$  receptor-expressing CHO cells have revealed coupling to several PLC signals, PLD and  $PLA_2$  (Figs. 3B and 6). Thus, orexin receptors may be extremely "plastic" in their ability to couple to phospholipase cascades, although for many of the responses the physiological role is unknown.

**Phospholipase C.** PLC hydrolyzes phosphoinositides (PI, PIP,  $PIP_2$ ) to diacylglycerol (DAG) and  $IP_3$  (if  $PIP_2$  is the substrate) (Figs. 4A and 6). The PLC family is divided into  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\zeta$  subfamilies (198).  $PLC\beta$  is regulated by GPCRs via  $G_{\alpha_{q/11}}$  (or more weakly via  $G\beta\gamma$ ), but  $PLC\eta$  and  $PLC\delta$  can also be regulated by  $G\beta\gamma$ , and the other PLC isoforms are targeted by GPCRs via, e.g.,  $Ca^{2+}$ , phosphorylation or Ras or Rho family G proteins (198). PLCs require  $Ca^{2+}$  for activity, but  $Ca^{2+}$  is not a major stimulant for isoforms other than  $PLC\delta$  and  $PLC\zeta$ . Roles for different PLC isoforms are difficult to separate as there are no selective inhibitors and RNAi can be difficult due to multiple isoforms. The most used inhibitor, U-73122, is thought to inhibit all PLC isoforms, though this has not been scrutinized. It also has serious off-target effects, including cellular toxicity (348), and it may be ineffective, due to, for instance, instability.

Both orexin receptors activate PLC, as has been shown by direct measurements in recombinant cell lines, including CHO (168, 228; unpublished observations), HEK-293 (286), neuro-2a (151) and PC12 cells (151; Putula J, Jäntti M, and Kukkonen JP, unpublished observations) and by using U-73122 or  $Ca^{2+}$  measurements in the absence of extracellular  $Ca^{2+}$ . Direct measurements have seldom been done on native cells but,  $IP_3$  generation upon orexin receptor activation has

been shown in human pheochromocytomas and rat adrenal cortex and hypothalamus (180, 245).

It is widely assumed that orexin receptors couple to PLC $\beta$  via G $\alpha_q$  but no direct evidence has shown this. In CHO cells, orexin receptors couple to PLC stimulation very effectively, much better than another G $_q$ -coupled receptor, M $_1$  muscarinic receptor, suggesting that several PLC isoforms could be involved (169). Additional evidence for this has been obtained by analyzing the products of PLC in these cells (168). The results suggest that two different PLC activities act in concert downstream of orexin receptor signaling, one hydrolyzing PI or PIP and the other PIP $_2$ . Thus far these PLC activities have not been molecularly identified. Ca $^{2+}$  influx also seems to regulate PLC activities in CHO cells in an unusual manner (169, 228).

PLC signaling is generally thought to lead to Ca $^{2+}$  release via IP $_3$  receptors on the endoplasmic reticulum and to activation of PKC by DAG. Other proteins are also targeted by DAG (198), including protein kinase D (PKD), which has been identified in OX $_1$  receptor signaling in HEK-293 cells (281). PKC mediates a number of orexin receptor responses in native neurons and recombinant cells (see above). Involvement of PLC in orexin signaling has been proposed based on use of the inhibitor D609 (189, 367, 394, 407). D609 (318) is a putative inhibitor of bacterial and protozoan phosphatidylcholine-specific PLC (PC-PLC), for which no mammalian counterpart has been isolated (198). D609 also inhibits mammalian sphingomyelin synthase (226). Might a PC-PLC or sphingomyelin synthase be involved in orexin receptor signaling? This is an interesting possibility but, given D609's multiple effects, it should not be used in signaling studies until its inhibitory profile is fully characterized.

**Phospholipase D.** PLD family enzymes PLD1 and PLD2 hydrolyze phosphatidylcholine to choline and phosphatidic acid (Fig. 6). Phosphatidic acid is an intracellular messenger with a number of protein targets, and it can also be hydrolyzed by phosphatidic acid phosphohydrolase activity to DAG (Fig. 6) (198). We have recently shown that OX $_1$  receptor stimulation activates PLD1 in CHO cells (Fig. 6) (168, 171). Evidence is lacking from other cell types, but since we observe a strong and potent response in CHO cells, we speculate that this is likely to take place. Activation of PLD occurs via a novel PKC isoform, most likely PKC $\delta$ , but without requirement of PLC-dependent DAG generation (171). The targets of PLD in orexin signaling are unknown.

**Phospholipase A $_2$ .** PLA $_2$  refers to a large family of enzymes (80). Although well known for their role in liberation of arachidonic acid from the *sn*2-position of phospholipids for eicosanoid production (Fig. 6), very few PLA $_2$  isoforms are specific for arachidonic acid or have only *sn*2-hydrolysis activity (198).

We find that OX $_1$  receptor stimulation in recombinant CHO cells activates arachidonic acid release by cPLA $_2$  [cytosolic (Ca $^{2+}$ -sensitive) PLA $_2$ , likely cPLA $_2\alpha$ ] in CHO cells (Fig. 6) (363, 364). The cPLA $_2$  enzyme activity is important for orexin receptor-operated Ca $^{2+}$  influx in these cells (363, 364). Arachidonic acid is also released upon orexin receptor stimulation in OX $_1$ -expressing HEK-293 cells, and this may contribute to Ca $^{2+}$  oscillations (280). However, it is unclear whether the release occurs via PLA $_2$  or another pathway (364).

**Interaction of orexin and endocannabinoid systems.** Endocannabinoids are arachidonic acid-containing messengers pro-

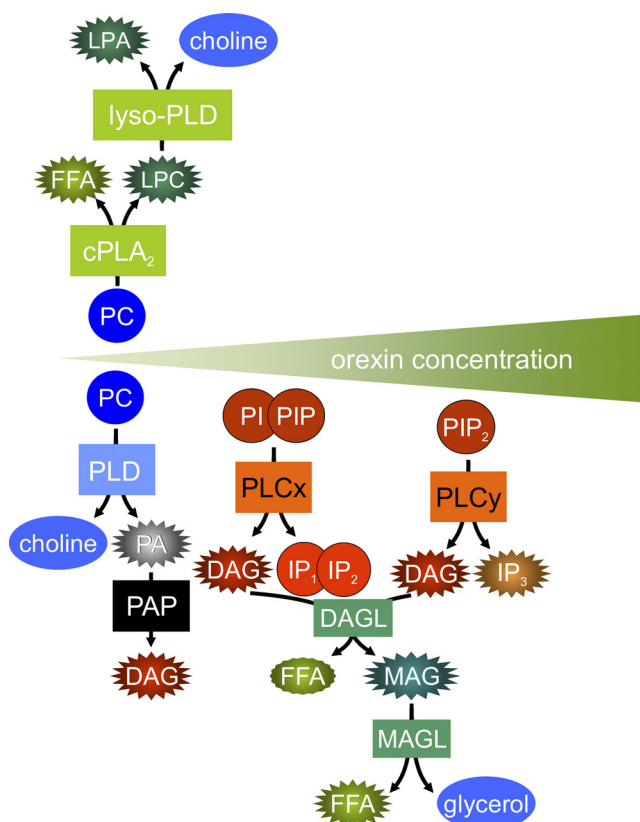


Fig. 6. Lipid signaling pathways of orexin receptors. Membrane phospholipids are shown by circles, less active lipid metabolites (in signaling) as ovals, more active ones as stars, and enzymes as rectangles. FFA, free fatty acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAG, monoacylglycerol (like 2-AG); MAGL, monoacylglycerol lipase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PI, PIP, and PIP $_2$ , phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate, respectively. The pathways are simplified and lack many possible metabolic connections. FFAs are considered more active as a messengers (sharper stars) when they are polyunsaturated, such as arachidonic acid. We find two apparently different PLC activities in human OX $_1$  receptor-expressing CHO cells (168), here denoted PLCx and PLCy, because of the lack of knowledge of their molecular identity. IP $_1$  and IP $_2$ , inositol mono- and bisphosphate(s), respectively.

duced by (phospho)lipase action. The originally identified, and possibly most important, endocannabinoids are 2-arachidonoylglycerol (2-AG) and *N*-arachidonylethanolamine (anandamide) (175). Endocannabinoids are ligands for the GPCRs CB $_1$  and CB $_2$ . CB $_1$  is widely expressed in CNS neurons (175). Endocannabinoids in the CNS regulate appetite, nociception, memory, and mood (175). Endocannabinoids are thought to be released postsynaptically and act on presynaptic, inhibitory CB $_1$  receptors by a process termed retrograde synaptic transmission (175) (Fig. 7). The system may act in both a homosynaptic and a heterosynaptic manner. Presynaptic inhibition likely occurs by G $_i$  family protein-derived G $\beta\gamma$ -mediated inhibition of VGCC and/or activation of Kir channels [see, e.g., (156, 175, 230, 366)].

Diacylglycerol lipase (DAGL) removes the *sn*1-fatty acid from DAG – likely produced by PLC – generating 2-AG (198). The regulation of DAGL is not clear, but it may be dually activated in G $_q$ -coupled GPCR signaling by substrate (DAG) availability and via Ca $^{2+}$  or PKC (198). Intracellularly

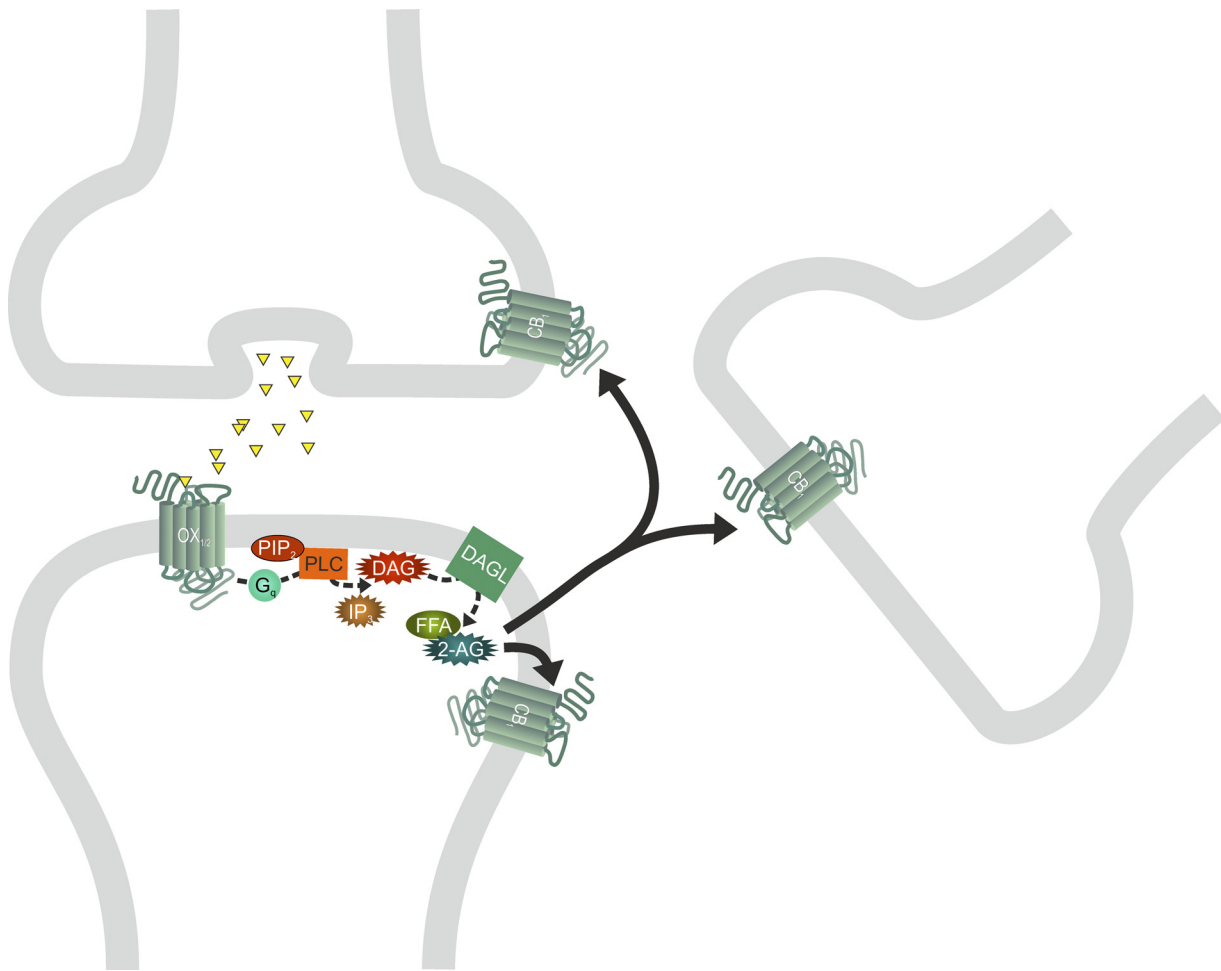


Fig. 7. Synaptic 2-AG in orexin signaling. Orexins (yellow triangles) released upon action potential activate postsynaptic orexin receptors. Activity of these receptors leads to PLC- and DAGL-dependent 2-AG production, which can act in a paracrine and autoocrine manner. Similar scheme applies to other “stimulatory” receptor signaling. FFA, free fatty acid; see *Glossary* for other definitions.

produced 2-AG is released to the extracellular space via an unknown mechanism and is inactivated by hydrolysis to arachidonic acid and glycerol by a number of enzymes (175, 313).

There is an apparent overlap between the putative functions of endocannabinoids and orexins, especially in the regulation of appetite and analgesia (57, 175, 299, 307). Overlap is also seen in their neuroanatomic distribution, but, at least in part due to problems related to orexin antibodies (see *Antibodies*), direct synaptic connections are not well defined. Some functional studies indicate orexin-endocannabinoid interaction, for example, in the stimulation of appetite (71): intracerebroventricular orexin-A-induced feeding in rats is abolished by CB<sub>1</sub> receptor blockade with rimonabant. Some analgesic effects of orexins occur via ventrolateral periaqueductal gray matter (18), a site where orexin-A (presynaptically) inhibits GABAergic signaling, apparently via OX<sub>1</sub> receptor stimulation of 2-AG generation and action on CB<sub>1</sub> receptors (142, 242, 283). In the dorsal raphé, inhibition by orexin-B of the glutamate release occurs via 2-AG-mediated CB<sub>1</sub> receptor activation (130). Conversely, stimulation of upstream cannabinoid systems inhibits excitatory glutamatergic drive to orexinergic neurons in the lateral hypothalamus (157). CB<sub>2</sub> receptors are also expressed in the CNS microglia, but whether orexins have a role in the

regulation of these cells via endocannabinoids, or by other means, is unknown.

Studies with heterologous coexpression have suggested that OX<sub>1</sub> and CB<sub>1</sub> receptors form heteromers (dimers or oligomers) (93, 139, 374). GPCRs can make heteromeric complexes, at least upon overexpression [reviewed in (36, 302)], and CB<sub>1</sub> receptors may interact with dopamine D<sub>2</sub>,  $\mu$  opioid, and AT<sub>1</sub> angiotensin II receptors (241, 296, 303). FRET (Förster/fluorescence resonance energy transfer) studies have presented direct evidence for the existence of OX<sub>1</sub>-CB<sub>1</sub> receptor complexes (374). As a suggested consequence, OX<sub>1</sub> signaling to ERK was potentiated 100-fold in CHO cells; this potentiation was inhibited by blocking CB<sub>1</sub> receptors. In contrast, CB<sub>1</sub> signaling to ERK and OX<sub>1</sub> signaling to PLC were unaffected by receptor coexpression (139). It has also been suggested that OX<sub>1</sub> receptor trafficking is altered by oligomerization (93). The studies thus suggest a direct molecular interaction between OX<sub>1</sub> and CB<sub>1</sub> receptors, but there are some possible pitfalls. For example, most, if not all, of the findings in the coexpression systems might result from orexin receptor-stimulated production of endocannabinoid, which act on CB<sub>1</sub> receptors, a possibility not rigorously tested. Indeed, we have found that OX<sub>1</sub> receptor stimulation in CHO cells strongly activates

DAGL and releases 2-AG, which is able to activate CB<sub>1</sub> receptors in neighboring cells (364). DAGL-dependent 2-AG release also occurs in OX<sub>1</sub>-expressing HEK-293 and neuro-2a cells (364). Pharmacological analysis suggests a similar concept for synaptic signaling (130, 142). If orexin receptor activity can do this in a paracrine fashion, the same may also occur in an autocrine manner. Future studies should assess this possibility.

Orexins thus show substantial versatility in their cellular signaling (Fig. 3), but how these signaling events link to functional responses is poorly understood. Research on signal transduction by orexin receptors may help to answer general questions regarding GPCR signaling pathways and the coupling to distinct signaling mechanisms in different tissues.

#### TOOLS AVAILABLE

##### *Receptor Ligands*

Numerous small molecule orexin receptor antagonists have been reported but very few are commercially available. The known orexin receptor agonists, on the other hand, are solely based on the native orexin peptides (see above); this also includes the commercially available radioligands (see OREXIN RECEPTOR LIGANDS ABOVE).

##### *Antibodies*

I have recently noted problems associated with the antibodies against orexin receptors (199) that lead me to question results with any of them unless receptor expression is independently verified (e.g., by mRNA, functional responses, or knockdown). Cross-reactivity with other antigens seems to occur with anti-orexin peptide antibodies, such as ones used in radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) kits (199). I thus seriously question results of orexin determinations in plasma and CSF and urge caution in immunohistochemistry (IHC) studies.

##### *Transgenic Animal Models*

Conventional knockouts of orexin peptides (PPO-KO) and orexin receptors [OX<sub>1</sub>-KO, OX<sub>2</sub>-KO, and the double-knockout (DKO)] in mice are available, as also is a conditionally restorable OX<sub>2</sub>-KO (Table 2). An OX<sub>2</sub>-ablation phenotype is found in familial narcoleptic canines (218).

The proximal (~3.2 kb) PPO promoter (PPO-pro) directs expression of genes to orexinergic neurons of mice/rats (306) but may not have all the regulatory properties of the native PPO promoter. PPO-pro has been widely used to express proteins in orexinergic neurons (Tables 2 and 3). A mouse with ectopic PPO overexpression has also been produced (250).

The orexinergic system has also been subjected to viral manipulation (Table 3). A number of other native or transgenic mutants—not directly affecting orexinergic neurons—have been utilized in orexin receptor studies [see, e.g., (13, 112, 224, 384)].

The findings obtained with the transgenic and other animal models (Tables 2 and 3) are described in other sections of this review.

##### *Medical Use*

While the very first indication for orexin receptor antagonists may have been appetite suppression, drug development

has recently focused on hypnotics. Blockade of OX<sub>1</sub> with SB-334867 or SB-408124 in rats and OX<sub>1</sub>-KO in mice only weakly modulates wakefulness and sleep [e.g., (86, 165, 249, 330)]. Knockout of both receptor subtypes is required for the classical narcoleptic phenotype (174, 249). In contrast, elimination of OX<sub>2</sub> induces a narcoleptic phenotype in dogs (218). See also *Regulation of Wakefulness and Sleep* and Table 2 for the full details of genetic evidence. In humans, the situation is not clear. OX<sub>2</sub>-selective and nonselective antagonists have been developed, some of which are in phase II or III trials (69, 300). Almost no results of these studies have been published. Recently, the dual orexin receptor blocker (DORA), almorexant, failed in phase III trials; we may thank this unfortunate incident for the fact that some results of the human studies with almorexant have been presented. The grounds for the withdrawal of almorexant from further development have not been reported, but its long half-life (145, 146) is not advantageous. Almorexant and another DORA, SB-649868, are effective inducers of sleep in a number species including humans (33, 44, 147, 148). Whether inhibition of both orexin receptor subtypes or OX<sub>2</sub> only is more advantageous has been investigated in rat; unfortunately, the results seem contradictory (86, 258). Suvorexant (MK-4305) may be the only DORA currently in phase III trials. Suvorexant is an effective sleep inducer and maintainer in rats, dogs, and rhesus monkeys (383). This year, Merck has announced the results of two phase III studies ([http://www.merck.com/newsroom/news-release-archive/research-and-development/2012\\_0613.html](http://www.merck.com/newsroom/news-release-archive/research-and-development/2012_0613.html)), suggesting that this compound is effective also in long-term treatment in humans and well-tolerated during the treatment and with respect to discontinuation. According to the press release, results of two more phase III trials are going to be published this year and a New Drug Application filed in the US. Suvorexant is thus close to becoming the first orexin receptor antagonist in the clinic for insomnia. This will also supply very interesting information on the orexin functions in humans and possibly pave the way for additional indications.

Narcolepsy (and perhaps other sleep/wakefulness disorders) is the most obvious use of orexin receptor agonists. Because low-molecular-weight agonists for orexin receptors are not known, orexin-A itself has been used in some attempts at orexin replacement therapy. In a sporadically narcoleptic dog, only very high intravenous doses produced mild amelioration of the symptoms (109). As discussed above (*Orexin Peptides*), blood-brain barrier penetration of orexin-A may be limited. Intranasal administration of orexin-A does not appear to be a promising approach either (19). Perhaps the absence of orexins in narcolepsy affects the levels of orexin receptor expression and thereby impact on the outcome of the studies. Healthy subjects thus need to be tested as well. Nevertheless, a peptide such as orexin-A lacks optimal ADME (absorption, distribution, metabolism, excretion) properties of a drug, and thus, a small molecule agonist would be preferable. Another possible therapeutic use of orexin receptor agonists would be cancer since orexin receptor stimulation has been suggested to induce programmed cell death in some cancer and other cell types (see *Cell Death*).

The absence of published studies on orexin receptor antagonists and the lack of agonistic ligands make it difficult to predict, on one hand, possible shortcomings (lack of effect,

Table 2. Transgenic animal models directly targeting the orexin system

Abbreviation	Explanation	Use (when not obvious)	(Homozygote) Phenotype	Reference No.
PPO-KO mouse	Preproorexin knockout		- Narcolepsy with cataplexy - Feeding/metabolic phenotypes: see text	(61, 110, 132, 321, 382)
OX <sub>1</sub> -KO mouse	OX <sub>1</sub> receptor knockout		- Only weak disturbances in sleep/wakefulness cycles - Reduced wakefulness-promoting effect of icv orexin-A	(112, 249, 382)*
OX <sub>2</sub> -KO mouse	OX <sub>2</sub> receptor knockout		- Largely similar weight and fat mass as wt mice on regular and high-fat diet - Narcolepsy with mild cataplexy - Fragmented sleep - Reduced wakefulness-promoting effect of icv orexin-A (more strongly than in OX <sub>1</sub> -KO)	(112, 249, 381, 382)
OX <sub>2</sub> -TD mouse	Reversible OX <sub>2</sub> -KO; the knockout can be reversed by expression of CRE recombinase	Conditional (place- or time-dependent) reinstatement of OX <sub>2</sub> expression	- Largely similar weight and fat mass as wt mice on regular and high-fat diet - Same as OX <sub>2</sub> -KO - Introduction of CRE using adeno-associated virus in posterior hypothalamus (including tuberomammillary nucleus) reverses sleepiness but not fragmented sleep pattern	(254)
DKO mouse	Orexin receptor double-knockout (both OX <sub>1</sub> and OX <sub>2</sub> )		- Narcolepsy similar to PPO-KO - Full abolition of effect of icv orexin-A on wakefulness and non-REM and REM sleep	(153, 174, 249, 382)
OX <sub>1</sub> -H <sub>1</sub> -KO mouse	Dual knockout of OX <sub>1</sub> receptor and histamine H <sub>1</sub> receptor	Investigation of interaction of orexinergic and histaminergic pathways	- Similar to OX <sub>1</sub> -KO (not compared in parallel)	(153)
PPO-pro-GFP mouse	Expression of GFP under the proximal PPO promoter	Identification of orexinergic neurons		(212)
PPO-pro-TTC-GFP mouse	Expression of fusion of C-terminal tetanus toxin fragment and GFP under the proximal PPO promoter	Mapping of the upstream neuronal pathways for orexinergic neurons (retrograde transport)		(308)
PPO-pro-YC2.1 mouse	Expression of the Ca <sup>2+</sup> indicator YC2.1 under the proximal PPO promoter	Selective Ca <sup>2+</sup> measurements in orexinergic neurons		(358)
PPO-pro-CRE	Expression of CRE recombinase under the proximal PPO promoter	Selective CRE-based recombination in orexinergic neurons (for knockout or knock in, etc.)	- If mated with <i>floxed-GABA<sub>B</sub></i> receptor 1 mice → elimination of GABA <sub>B1</sub> receptor in orexinergic neurons → constitutive GABA <sub>A</sub> -mediated inhibition of orexinergic neurons and fragmented sleep and wakefulness pattern	(243, 312)

Continued

Table 2.—Continued

Abbreviation	Explanation	Use (when not obvious)	(Homozygote) Phenotype	Reference No.
PPO-pro-halorhodopsin	Expression of the light-activated Cl <sup>-</sup> transporter halorhodopsin under the proximal PPO promoter		<ul style="list-style-type: none"> <li>- If used together with <i>floxed-M<sub>3</sub>D<sub>q</sub></i>-adeno-associated virus, CRE-recombinase-dependent expression of mutated M<sub>3</sub>-muscarinic receptor, that only responds to some synthetic ligands and not acetylcholine; orexinergic neurons can be selectively activated</li> <li>- If used together with <i>floxed-M<sub>4</sub>D<sub>1</sub></i>-adeno-associated virus, CRE-recombinase-dependent expression of mutated M<sub>4</sub>-muscarinic receptor that is only sensitive to some synthetic ligands and not acetylcholine; orexinergic neurons can be selectively inhibited</li> </ul>	(360)
PPO-pro-melanopsin/mCherry	Expression of the light-activated G protein-coupled receptor melanopsin and the red fluorescent protein mCherry under the proximal PPO promoter	Selective optogenetic inhibition of orexinergic neurons	<ul style="list-style-type: none"> <li>- Light stimulation induces slow-wave-sleep during the inactive period but not during the active phase</li> <li>- Light stimulation induces awaking in sleeping mice</li> </ul>	(361)
PPO-ataxin-3 rat	Expression of the poly-Gln-repeat protein ataxin-3 under the proximal PPO promoter	Selective postnatal destruction of the orexinergic neurons	<ul style="list-style-type: none"> <li>- Elimination of orexinergic neurons</li> <li>- Narcolepsy with cataplexy</li> <li>- Feeding/metabolic phenotype not reported</li> </ul>	(34)
PPO-ataxin-3/CAG-PPO mouse	Expression of PPO under β-actin-CMV hybrid promoter in PPO-ataxin-3 mice	Ubiquitous PPO overexpression in PPO-ataxin-3 mice	<ul style="list-style-type: none"> <li>- Reversal of PPO-ataxin-3 phenotype on sleep/wakefulness</li> </ul>	(250)

\*The details for construction of the OX<sub>1</sub>-KO mouse have not been published; although first reported in an abstract in 2000, subsequent publications with it or the DKO reference this abstract or a review (382).

Table 3. *Viral targeting of the orexin system*

Construct	Use (when not obvious)	Reported Use	Results	Reference No.
PPO-pro-channelrhodopsin-2 lentivirus	Expression of the light-activated nonspecific cation channel channelrhodopsin-2 under the proximal PPO promoter	Selective optogenetic stimulation of orexinergic neurons upon injection in lateral hypothalamus	- Light stimulation induces awaking in sleeping mice - Activation of histaminergic neurons in mouse slice preparations is driven by glutamate released from orexinergic neurons	(4, 316)
CMV-pro-PPO adeno-associated virus	General expression of PPO under the CMV promoter	Orexin replacement in PPO-ataxin-3 mice	- Injection in lateral hypothalamus or zone incerta in PPO-ataxin-3 mice blocks cataplexy	(219)
MCH-pro-PPO adeno-associated virus	Expression of PPO under the proximal MCH promoter	Orexin replacement in PPO-ataxin-3 mice	- No effect on the sleep/wakefulness phenotype of PPO-ataxin-3 mice	(219)
Orexin-lentivirus	Expression of orexin (?) under which promoter?	Orexin overexpression	- Stimulation of mesenchymal C3H10T1/2 cell differentiation to brown adipocytes	(321)
OX <sub>1</sub> -shRNA-lentivirus	Expression of shRNA against the mouse OX <sub>1</sub> receptor	Knockdown of OX <sub>1</sub>	- Block of orexin-A-stimulated HIB1 brown preadipocyte differentiation	(321)

See Table 2 for viruses used in connection with transgenic animals.

side-effects, etc.), and on the other, possible novel applications for these ligands.

#### PHYSIOLOGICAL FUNCTIONS IN THE CNS

##### *Orexinergic Neurons*

**Molecular and regulatory signatures.** The anatomy of orexinergic neurons and projections has been mapped in numerous mammalian and nonmammalian species, in greatest detail in mice and rats, but there is little information for humans. However, the similarity between other species may allow some extrapolation of the knowledge.

Orexinergic neuron cell bodies are found solely in the lateral hypothalamic area and nearby regions. They do not form a distinct nucleus but are mixed with other neurons, such as melanin-concentrating hormone (MCH)-expressing neurons (201). The number of orexin neurons in humans is estimated to be  $\leq 80,000$  (105, 106, 350, 351). Prodynorphin, secretogranin II, neuronal activity-regulated pentraxin (NARP), and nociceptin/orphanin FQ (26, 39, 68, 72, 120, 121, 240, 297) are likely also expressed in all of the orexinergic cells, but, unlike orexins, those peptides/proteins are also expressed in other brain areas. Galanin and glutamate may be cotransmitters in a subpopulation of orexinergic neurons (1, 154, 161, 163, 247, 316, 355). The role of the other putative transmitters in the functions of orexinergic neurons is rather unclear. Glutamatergic transmission of orexinergic neurons may be important for excitation of histaminergic neurons (316), and dynorphins may impact orexin effects both positively and negatively (214). However, the narcoleptic phenotype of orexinergic neurons is the same in mice that only lack orexins (PPO-ko mice) and that lack the entire orexinergic neuron population (PPO-pro-ataxin-3 mice).

Orexin neurons seem to be regulated by many transmitter systems, as determined by IHC, in situ hybridization, tracing, and functional studies. These putative regulatory inputs are depicted in Fig. 8; however, the figure is both incomplete and speculative, especially with respect to the source of transmitters and the physiological significance; expression of a receptor or response to an exogenous ligand need not imply a physiological action. In addition, IHC studies may suffer from issues related to specificity of antibodies or antagonists, and func-

tional studies of problems with synaptic block [see *Interaction of orexin and endocannabinoid systems* and discussion in (199)].

Orexinergic neurons may also be regulated by blood components, like glucose (49, 125, 378, 380, 402) and dietary amino acids (177), and paracrine messengers, like endocannabinoids (see *Interaction of orexin and endocannabinoid systems*) and adenosine. Whether leptin directly acts on orexinergic neurons or upstream of them is disputed (154, 161, 210, 211, 224, 402). Glucose hyperpolarizes and inhibits orexinergic neurons via opening of an unidentified K<sup>+</sup> channel (50, 176). Orexin neuron activity (measured by c-fos expression) and PPO mRNA production increase in fasting and glucopenia (45, 57, 81, 222, 259, 307), probably via the forkhead box transcription factor Foxa2 (325). Insulin inhibits this cascade (325). Dietary amino acids depolarize and activate orexinergic neurons by closure of tolbutamide-sensitive K<sub>ATP</sub> channels and Na<sup>+</sup> influx via amino acid transporters (177).

**Upstream and downstream projections.** PPO-IHC was originally used to map the downstream projections of orexinergic neurons (265, 283). In situ hybridization of orexin receptor mRNA provided additional evidence (242, 357). Caution is required in assessing studies with orexin receptor antibodies as sole evidence for receptor expression (see *Antibodies*). Orexinergic neurons project to well-defined CNS areas [(242, 265, 283, 357); reviewed in (201)]. Downstream target transmitter systems are shown in Fig. 8. Orexinergic neurons terminate not only on dendrites (or cell bodies) but also on axon terminals; thus the anatomical mapping may be partly misleading. Also, the production of endocannabinoids in response to orexin receptor activity may spread the signal in the target nucleus (see *Interaction of orexin and endocannabinoid systems*).

Antero- and retrograde tracing with injected biotinylated dextrans (97, 137, 409), Diamine Yellow (368), Fluorogold (97), cholera toxin subunit b (247, 409) or pseudorabies virus (362) or transgenic expression of TTC-GFP (308) to and from orexin neurons have also been performed. Each technique has its own advantages and disadvantages. Orexinergic neuron-directed expression of the tracer may be advantageous, since the region (e.g., lateral hypothalamus) contains other types of





### Feeding and Metabolism

The role of orexins in feeding and metabolic regulation is complex. The original discovery was that injection of orexins intracerebroventricularly or into specific CNS nuclei in rats increased short-term feeding (85, 307, 341, 354). Central injection of orexins also elevates metabolic rate, at least in part by activating the sympathetic nervous system [(201); see below]. The major role of orexins is currently considered to be the regulation of metabolism rather than feeding, as recently discussed in another review (199); thus, I shall only present a short overview here.

Important evidence linking orexins to metabolic regulation was that mice on a high-fat diet are protected against body weight increase by ectopic overexpression of PPO acting by increasing energy consumption (112). Central administration of orexins increases metabolic rate in rodents [see, e.g., (227, 373)]. Orexin-deficient animal models (PPO-KO mice, PPO-pro-ataxin-3 mice) show somewhat contradictory phenotypes. Some studies suggest normal growth or even obesity despite low caloric intake (131, 132, 382), but recently PPO-KO and PPO-pro-ataxin-3 mice were found to be more sensitive to high-fat diet-induced obesity than wt mice (132, 321). However, these models differ from one another. PPO-KO mice lack the orexin peptides during development whereas ataxin-3 under PPO-pro is expressed only postnatally. Thus, the PPO-KO animals may show compensatory changes that are less likely to occur in PPO-pro-ataxin-3 mice. On the other hand, since orexinergic neurons express other transmitters (see *Orexinergic Neurons*), elimination of these transmitters together with orexins in PPO-pro-ataxin-3 mice may give a different phenotype. The genetic background of the mice also seems to play a part (110, 132). Orexins affect the wakefulness pattern and physical activity, which are parameters not fully considered in these studies. By extrapolating these data, one might predict that human narcoleptics would have a lower metabolic rate, but that does not seem to be the case (107).

Orexinergic neurons are regulated by glucose levels. Food deprivation or acute hypoglycemia induces PPO mRNA expression and activates orexinergic neurons; also hypothalamic mRNA for orexin receptors has been reported to increase in fasting (180). Electrophysiological studies show activation of orexinergic neurons by glucose and inhibition by dietary amino acids. Leptin and insulin may display direct or indirect inhibitory effect on these neurons. See *Molecular and regulatory signatures* for details.

*Metabolic rate and thermogenesis.* Central injection of orexins elevates metabolic rate (201), while PPO-KO mice are defective in elevating metabolic rate to compensate for caloric intake (321). In rodents, BAT is a major heat producer (and dissipant upon calorie excess). BAT is regulated by sympathetic nervous system projections from raphé pallidus (260). CNS administration of orexins stimulates these projections and increases BAT thermogenesis (32, 277, 362, 413). Orexinergic neurons may also be important for native activation of BAT; however, the transmitter is suggested not to be orexin as indicated by normal regulation in PPO-ko mice (412). Placentally derived orexins are indispensable for embryonic BAT differentiation (PPO-KO mice) (321). The signaling may occur via OX<sub>1</sub> receptors on brown adipocyte precursors, utilizing p38 MAPK and bone morphogenic protein receptor 1A (BMPRIA)

pathways. These findings represent a new aspect of orexin research, but there are still many open questions. An interesting finding in one study (412) is that PPO-ko mice are capable of normal BAT thermogenesis, whereas this was found to be grossly impaired in another study (321), despite the apparently same C57BL/6J background and source. However, if placental orexins drive the differentiation of normal BAT, then the phenotype of the pups, with respect to the BAT differentiation, should be defined by the maternal genotype and not the genotype of the pups themselves. This actually seems to be the case since in Zhang et al.'s study (412) heterozygous mothers were used while the mothers in Sellayah et al.'s study (321) were homozygous (personal communication with Dr. T. Kuwaki and Dr. D. Sikder). For a more detailed discussion on orexins and BAT, please see Kukkonen (199).

### Regulation of Wakefulness and Sleep

Various animal models, including PPO-KO and DKO mice and PPO-pro-ataxin-3 mice and rats, have disrupted regulation of wakefulness and sleep and a narcoleptic phenotype (Table 2). Single knockout of OX<sub>1</sub> or OX<sub>2</sub> in mice yields milder phenotypes while ablation of OX<sub>2</sub> alone in dogs is highly effective (Table 2); thus orexin receptor subtypes may have different roles in different species. The roles played by the receptor subtypes in humans are not known, but the issue is highly relevant for the development of orexin receptor antagonists for insomnia (see *Medical Use*). Ablation of the lateral hypothalamic orexinergic neurons (and MCHergic and possibly other neurons) by localized injection of orexin-B-saporin also induces a narcoleptic phenotype (115).

By contrast, intracerebroventricular injection of orexin-A reduces REM and deep sleep and increases wakefulness (41, 129). Optogenetic stimulation of orexinergic neurons in PPO-pro-channelrhodopsin-2 lentivirus-transduced mice (Table 3) has a similar effect whereas inhibition of orexinergic neurons in PPO-pro-halorhodopsin mice gives a mild sleep-inducing effect (Table 2). It is possible that optogenetic techniques cannot produce a very efficient inhibition of these neurons.

Current thinking is that orexin from orexinergic neurons stimulates histaminergic (tuberomamillary nucleus), noradrenergic (locus coeruleus), serotonergic (raphé nuclei), and cholinergic (basal forebrain) neurons to activate, e.g., the cerebral cortex. Sleep-on GABAergic neurons (ventrolateral preoptic nucleus [VLPO]), when active, send inhibitory output to orexinergic neurons and to these aminergic nuclei. The wake-on neurons (e.g., in dorsal raphé) also send projections that inhibit firing of orexinergic neurons (262, 358). As the aminergic neurons also inhibit VLPO firing, there may be mutual inhibitory regulation. The regulation of REM sleep may be more complex. See Refs. 15, 127, 217, 268, 305, and 311 for detailed reviews.

*Narcolepsy.* With one known exception (282), human narcolepsy is a sporadic disease. It yet has a genetic component, strong association with particular MHC/HLA type II haplotypes, most notably HLA DQB1\*0602 (and its "partners" DRB1\*1501 and DQA1\*0102) (269, 278), implicating a possible autoimmune origin.

Two important pieces of evidence as to the mechanism, though not to the etiology, of narcolepsy were published in 1999. Hereditarily narcoleptic canines were shown to harbor

inactivating mutations in the OX<sub>2</sub> receptor gene (218), and PPO-KO to lead to a narcoleptic phenotype in mice (61). Soon, human narcoleptics with cataplexy were demonstrated to show nil or very low levels of orexin-A in the CSF (270). Ablation of orexinergic neurons (or orexin target neurons; PPO-pro-ataxin-3 mice or rats, saporin-orexin-B injections in the rat lateral hypothalamus) or knockout of both orexin receptor types in mice (DKO mice) lead to narcolepsy (115, 174, 249). It is currently believed that orexinergic neurons die in human narcolepsy, reducing orexin levels and thus their ability to sustain wakefulness and stabilize the wakefulness and sleep circuitry. Limited postmortem studies support this view (39, 350–352). However, the mechanism of the cell death is unknown. Autoimmunity has been hypothesized but the evidence is circumstantial, such as the association with HLA DQB1\*0602 and an apparent narcolepsy epidemic that followed swine flu vaccinations in Finland and Sweden (267, 340). Autoantibodies have been, during the years, sought for in human narcoleptics without any very definitive answer. It seems that even the positive findings, like that concerning the autoantibodies against Tribbles homolog 2 (73), are difficult to conclusively explain with respect to narcolepsy as the antibody target proteins are not exclusively expressed in orexinergic neurons or may not be easily accessible for antibodies. However, autoantibodies may not be the only determinant of autoimmunity, and, in the case of HLA type II, some effects might be triggered independent of antibody production (104). One should also consider the possibility that the actual determinant of the “narcolepsy genotype” is another gene cosegregating with HLA DQB1\*0602.

In the absence of much proof, many different hypotheses for the mechanisms of the cell death (if that indeed is taking place) can be presented (104, 199). Death of orexinergic neurons might occur immunologically triggered or by other means. The actual target of dysregulation could also lie upstream of orexinergic neurons; orexinergic neurons could be maintained by some upstream neurons, death of which might be enough to induce decline of orexinergic neurons. On the other hand, overactive upstream circuitry might induce an excitotoxic death of orexinergic neurons. Orexinergic neurons themselves may be particularly sensitive or accessible to an assault. Orexinergic neurons might end up dead upon an assault targeted on other nearby cells (bystander killing). Finally, although maybe far-fetched, the orexinergic projections meeting orexin neurons (see *Upstream and downstream projections*) could be hypothesized to induce neuronal cell death as orexins can induce death in some other cell types (see *Cell Death*).

#### *Sympathetic Activation and Stress Response*

Orexins can activate sympathetic neurons (368, 372). Intracerebroventricular orexin or administration in brain stem sites induces sympathetic activation associated with elevated heart rate, blood pressure, sympathetic neuron activity, glucose uptake in skeletal muscle, catecholamine release in the circulation, and stereotypic behavior suggestive of stress response [(201); see also (79, 141, 255, 324, 331, 335, 406) and *Metabolic rate and thermogenesis* for BAT]. PPO-KO and PPO-pro-ataxin-3 mice show lower basal and stress-induced sympathetic activation (203, 412).

Orexins may activate the hypothalamo-pituitary-adrenal axis both via pituitary ACTH release and direct stimulation of adrenal cortex (see *Adrenal Gland* and *Pituitary Gland*). However, the dual orexin receptor antagonist, almorexant, does not affect ACTH or corticosterone levels nor the hormone responses to stressful conditions or CRH exposure in rat, suggesting that the role of orexin in this regulation at the central level is less significant (337).

#### *Other Functions*

Endogenous orexins contribute to native analgesia, and analgesia can be induced by central (intracerebroventricular, specific brain sites), intrathecal, and intravenous (but not intraperitoneal) orexin administration. This is observed in the models for acute thermal (hotplate, tail-flick, paw withdrawal), mechanical (tail-pressure), or chemical/inflammatory (subcutaneous formalin, capsaicin or carrageenan, intraperitoneal acetic acid or MgSO<sub>4</sub>) pain (18, 35, 253, 376, 399). Analgesia was blocked by SB-334867 but not the opioid antagonist naloxone (35, 376). Nociceptin/orphanin FQ induces pain and inhibits orexinergic neurons and analgesia, but the analgesia can still be obtained by orexin injection (253, 397). Adenosine A<sub>1</sub> receptor signaling in the brain may act in concert or downstream of orexins in the antinociceptive responses (253, 376), and histamine H<sub>1</sub> or H<sub>2</sub> receptor inhibition or knockout enhances the effect of orexin (252). In rat ventrolateral periaqueductal gray, orexins are suggested to be antinociceptive via endocannabinoid production and release (18, 142). Orexins are also effective in models of allodynia (400, 401). Orexinergic neurons are activated in mice by long-lasting stress and pain (375). PPO-KO and PPO-pro-ataxin-3 mice show less stress-induced antinociception; central orexin-A administration restores stress-induced antinociception in the latter (375, 397). SB-334867 enhances thermal nociception (376) and inflammatory pain (35).

A number of studies [initially in (43, 134)] show a role for orexins in addiction, and possibly in motivation/reward/reinforcement-linked processes [reviewed in (17, 42, 60, 353)].

Intracerebroventricular orexin-A enhances anxiety-like behavior in mice and rats, as assessed by behavior in elevated plus-maze and light-dark tests (339). Similar response is elicited by direct injection of orexins in the thalamic paraventricular nucleus (213). Nicotine-induced anxiogenic effect is absent after SB-334867 administration or in PPO-KO mice (284) while footshock-induced anxiety is abolished by TCS-OX<sub>2</sub>-29 in the paraventricular nucleus (213). In contrast, others (326) suggest orexin-A to be anxiolytic. No difference in anxiety was detected between wt, OX<sub>1</sub>-KO, or OX<sub>2</sub>-KO mice (319). SB-334867 eliminated the panic response in a rat panic anxiety model (170). Use of the forced swim model for depression implied opposite roles for OX<sub>1</sub> and OX<sub>2</sub> receptors in mice (319).

#### **OREXIN PHYSIOLOGY IN THE PERIPHERY OF THE BODY**

Peripheral expression of orexins and orexin receptors has been investigated mainly in rats using mRNA detection (RT-PCR, in situ hybridization) and IHC. For the reasons I note above and previously (199), I have doubts regarding IHC detection of orexins and orexin receptors. Functional responses to orexins occur in a number of tissues, although in most cases

it is unclear whether these are physiologically relevant. Recent reviews discuss peripheral actions; readers are encouraged to consult these and the original studies for more in-depth views (136, 201, 273, 332, 388).

#### *Gastrointestinal Tract*

In addition to discovery of orexin peptides and receptors in the gastrointestinal tract, functional responses have been observed both on the cellular level and in the organ scale (100, 136, 201, 388). mRNA for PPO as well as for OX<sub>1</sub> and OX<sub>2</sub> are expressed in rat myenteric plexi (191). Intracerebroventricular orexin-A stimulates gastric acid secretion (275) while duodenal intraarterial orexin-A stimulates duodenal bicarbonate secretion in a feeding status-dependent manner (101). Fasting reduces mucosal expression of OX<sub>1</sub> and OX<sub>2</sub> mRNA (28). Positive and negative effects on gastrointestinal motility of central or intravenous orexins have been observed (136), possibly because of the numerous targets of orexins (CNS, parasympathetic and enteric ganglia, neuronal circuitry of the intestine, muscle). Orexins promote depolarization and contraction in isolated mouse duodenal muscle (334), thus verifying suggestions based on RT-PCR (191). In guinea pig ileal strips, orexin-A-induced contraction and acetylcholine overflow were considered indirect based on sensitivity to TTx (244). Guinea pig ileal submucosal and myenteric neurons are excited by low or mid-nanomolar orexin-A (184, 191). Vagal afferent neurons (nodose ganglia) of humans and rats express orexin receptor mRNA, and orexin-A inhibits cholecystokinin response; orexins may thus contribute to gut-to-CNS signaling (52).

#### *Endocrine Pancreas*

Expression of orexin-A, orexin-B, and PPO in the pancreatic  $\beta$ -cells has been suggested, based on IHC in rat and guinea pig (6, 190, 264). PPO has been detected by RT-PCR in human pancreas (264). Orexin receptor expression has also been reported by both IHC and RT-PCR in pancreatic neurons or endocrine cells (5, 89, 126, 190, 191, 272). Altered hormone secretion in responses to orexins has been observed in isolated cells or islets in some studies. However, there are often problems related to methodology or interpretation, and contradictory results have been obtained. Subcutaneous orexins stimulate insulin secretion in rats, but the response is strongly reduced in pancreatic explants, possibly suggesting an indirect effect (271). OX<sub>1</sub> immunoreactivity is suggested to increase in spontaneously diabetogenic Goto-Kakizaki rats and in streptozotocin-induced diabetes in Wistar rats upon diabetes progression, especially in dying cells (5).

#### *Adipose Tissue*

OX<sub>1</sub> and OX<sub>2</sub> receptor mRNA have been identified in human subcutaneous and omental white adipose tissue (82). Exposure to a high concentration (100 nM) of orexin-A and -B induced peroxisome proliferator-activated receptor- $\gamma$ 2 (PPAR $\gamma$ 2) mRNA in subcutaneous adipocytes. By contrast, orexins strongly reduced mRNA for hormone-sensitive lipase in omental adipocytes and weakly reduced baseline lipolysis (82). Preadipocyte-like 3T3-L1 cells express mRNA for OX<sub>1</sub> and OX<sub>2</sub> receptors (327). Orexin-A (100 nM) stimulated glucose uptake via PI3K-mediated GLUT4 glucose transporter

translocation, reduced hormone-sensitive lipase mRNA and lipolysis, and stimulated triglyceride synthesis via PI3K and PPAR $\gamma$ . Orexin-A also stimulated adiponectin secretion from 3T3-L1 cells (327). Results of these two studies thus suggest that orexin-A may stimulate lipid storage and inhibit lipolysis in adipocytes, and possibly stimulate proliferation and differentiation [see also (415)]. Switonska et al. (342) show that subcutaneous orexin-A and orexin-B injections elevate plasma leptin, but the physiological mechanism is unknown. The effect of orexin on BAT is discussed under *Metabolic rate and thermogenesis*.

#### *Adrenal Gland*

Orexin receptor expression and the stimulatory effect of orexins on rat and human adrenal cortex has been shown in isolated cells, explants, and intact animals (180, 181, 232, 234, 246, 294). OX<sub>1</sub> and OX<sub>2</sub> receptor mRNA is expressed in rats (172, 180, 233), while humans may express mainly OX<sub>1</sub> mRNA (181, 246, 294). Orexin receptor stimulation increases cortisol and corticosterone secretion (depending on the species) via the classical cAMP/PKA-dependent pathway (232, 234, 246) (Fig. 4D). Some studies have noted increased aldosterone secretion (232, 266). AC stimulation likely takes place via activation of G<sub>s</sub> protein (Fig. 4D), although the contributions of the simultaneous activation of G<sub>q</sub>  $\rightarrow$  PLC  $\rightarrow$  IP<sub>3</sub> cascade and G<sub>i/o</sub> (180, 181, 294) have not been assessed. Orexin receptor expression and signaling have also been observed in the adrenocortical cell line H293R (292, 293). In these cells, orexins induce enzymes required for steroidogenesis (293, 379). However, contradictory results have been published in this cell line, and the signaling seems to differ from that of native cells (293, 379).

Orexin receptor expression (mainly OX<sub>1</sub> mRNA) has been detected in rat adrenal medulla in some (221, 246) but not other studies (172). While most of the very few studies on functional responses show no responses or unconvincing ones, clear responses to orexin-A, in a SB-334867-sensitive manner, have been noted in amperometric recordings of catecholamine secretion in a mouse preparation (65). Primary human pheochromocytomas express OX<sub>2</sub> mRNA and exhibit catecholamine secretion in response to orexin-A in a PLC  $\rightarrow$  PKC cascade-dependent manner (245).

#### *Hematopoietic Cells*

Three reports on effects of orexin on hematopoietic cells have been published. Ichinose et al. (162) reported that orexin-B activates a K<sup>+</sup> conductance in mouse macrophages. Extremely high concentrations were required, orexin-A was less efficacious than orexin-B, and the experimental setup suffered from some limitations; therefore it is difficult to assess the significance of these findings. Two other studies have described expression of OX<sub>1</sub> and OX<sub>2</sub> receptors in normal and malignant human hematopoietic CD34-positive stem/progenitor cells (197, 336). No receptor mRNA expression was seen expressed, but the receptor proteins were detected with antibodies. In addition, it is unclear how an antibody directed against an intracellular epitope (such as the one for OX<sub>1</sub>) could detect the receptor in nonpermeabilized cells (197, 336). The authors also report a functional response, i.e., cAMP reduction, in response to rather high orexin concentrations (336). How-

ever, this response is seen without any “background” AC stimulation (with, e.g., forskolin or  $G\alpha_s$  activation); thus this may not represent a receptor-mediated response. In conclusion, although hematopoietic cells would represent a very interesting target for orexin responses, there is no clear proof for receptor expression among cells of this lineage.

### Pituitary Gland

Responses regarding pituitary hormone secretion are very complex and depend on diurnal variation, estrous cycle, etc. (201).  $OX_1$  receptor mRNA has been identified in rat pituitary gland (172) while in another study, both receptor mRNAs were detected, although the levels of  $OX_1$  mRNA were higher (75). All regions of the hypophysis showed expression, but levels were greatest in the intermediate lobe. In human pituitary, both receptor mRNAs have been detected (38).

Intracerebroventricular orexin-A strongly stimulated ACTH release in rats in CRH-dependent manner (7, 48, 202, 310), while, in isolated rat corticotropes, orexin-A inhibited CRH-stimulated ACTH release, an effect mediated by a PKC-dependent pathway downstream of AC (309). PKC stimulation would otherwise be expected to stimulate ACTH secretion. Orexin-A potentiated somatotrope growth hormone secretion in response to growth hormone-releasing hormone (GHRH) in sheep, probably through PKC-dependent stimulation of L-type VGCC (62), while in pig pituitary, orexin-B potently stimulated luteinizing hormone (LH) secretion (20). In human narcoleptics, ACTH secretion is blunted (194) and diurnal rhythm of growth hormone secretion is distorted (279).

If the orexin receptors in the pituitary are to be considered to have a functional role, there should be access of orexins to these receptors. Orexinergic projections have only been observed in the posterior pituitary (265). It is not clear whether orexinergic neurites project to the median eminence, a site from which orexins could be directly released to the pituitary portal circulation (75, 370). Thus, the physiological role of the anterior and intermediate lobe orexin receptors is ill-defined. If not directly innervated by orexinergic neurons, they might respond to circulating orexins from the hypothalamus or from another (peripheral?) source.

Elevated levels of CRH and vasopressin mRNAs are observed in hypothalamic paraventricular nucleus after intracerebroventricular orexin-A (7, 48), and orexin-A directly stimulates these neurons (76, 310), suggesting an alternative, indirect link to regulation of hypophyseal release of ACTH.

### Reproductive Tract

$OX_1$  but not  $OX_2$  mRNA has been detected by RT-PCR in rat testis and ovaries (172) and in human male reproductive tract in the testis, epididymis, penis, and seminal vesicle (179).  $OX_1$  expression may be under hypophyseal control (22). Orexin-A has functional responses in rat testis, stimulating testosterone production (22) and regulating testicular gene expression (21).

### Peripheral Orexin Sources

If orexin receptors expressed in various peripheral tissues are physiologically functional, what is the source of the orexin needed to stimulate these receptors? In principle, the source could be 1) the CNS, if central orexins enter the blood, such as

orexin-A found in CSF; or 2) a peripheral organ in either an endocrine or paracrine manner. Studies that have investigated penetration of orexin from the blood to the rat, mouse, or dog brain have yielded contradictory results (35, 183); only two studies (in two species) have assessed the CNS exit of orexin-A to the plasma (183, 315). Together with the uncertain methods to measure orexin levels, the data do not yet provide compelling evidence for significant transport of orexin from the CNS to the blood.

The testis is the only peripheral location in rats where both PPO mRNA and protein expression have been verified by Northern blotting, RT-PCR, and IHC (172, 307, 343); in contrast, no expression has been seen in the ovaries. PPO mRNA has been detected in human kidney, placenta, stomach, ileum, colon, adrenal gland, and pancreas (264), and epididymis and penis of male reproductive tract (179). PPO mRNA has also been detected in mouse placenta (321) and rat small intestine (longitudinal muscle or myenteric plexi) (191). Since measurement of plasma orexin is unreliable (see *Antibodies*), it is difficult to evaluate circulating orexin levels.

In case we would fail to find orexins with access to some of the receptors, we should consider the possibility of other functions of orexin receptors. One possibility is that they are regulated by ligands other than orexins. Alternatively, the receptors may have ligand-independent functions, such as constitutive activity or ability to act as scaffold proteins. Finally, receptor expression in some tissues may represent an evolutionary or developmental remnant of insignificant functional consequence.

### CONCLUSIONS AND FUTURE PERSPECTIVES

Significant progress in mapping of the orexin physiology has been made thanks to the use of modern techniques (e.g., transgenic mice and viral vectors). Some novel findings have been made, as exemplified by the role of orexin in BAT development. For transgenic animal studies, inducible transgenic constructs or local viral vectors may find more use in orexin studies compared with the use of conventional transgenes. Development of orexin receptor agonists is likely to soon yield novel findings, especially in studies with humans.

At the same time, progress has been slow in some key areas of orexin research. One such area is orexin peptide and orexin receptor protein determinations. These would have important research and even clinical diagnostic value, but there are as yet no established protocols. Studies of orexin receptor function at the cellular level are not simple and have been performed by a small number of research groups. Therefore the progress has been slow, and the studies have not always been conducted with a sufficient amount of rigor. I hope that future efforts will show improvement of these areas and that the cause of human idiopathic narcolepsy will be revealed so as to develop both preventive and better palliative treatment.

Looking back, the first 5 years of orexin research gave us many seminal findings, and the 10 following years, reviewed here, were not very much less intriguing. It will be exciting to see what is discovered regarding orexins and their receptors during the next 10 years!

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

## AUTHOR CONTRIBUTIONS

J.P.K. conception and design of the research; analyzed the data; interpreted the results of the experiments; prepared the figures; drafted the manuscript; edited and revised the manuscript; approved the final version of the manuscript.

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