

Orexin and Orexin Receptors

Takeshi Sakurai, MD, PhD

1. INTRODUCTION

“Reverse pharmacological” approaches, i.e., ligand identification using cell lines expressing orphan receptors, combined with genetic engineering techniques, have increased our understanding of novel signaling systems in the body (1). Orexin/hypocretin is the first example of the factors that were successfully applied using such approaches (2). Our group initially identified orexin-A and orexin-B as endogenous peptide ligands for two orphan G-protein–coupled receptors found as human expressed sequence tags (ESTs) (2). This chapter discusses structures and functions of orexin neuropeptides and their receptors.

2. IDENTIFICATION OF OREXIN

Most neuropeptides work through G-protein–coupled receptors (GPCRs). There are numerous (approx 100–150) “orphan” GPCR genes in the human genome; the cognate ligands for these receptor molecules have not been identified yet. We performed an approach, so-called reverse pharmacology, that aims to identify ligands for orphan GPCRs. We expressed orphan GPCR genes in transfected cells and used them as a reporter system to detect endogenous ligands in tissue extracts that can activate signal transduction pathways in GPCR-expressing cell lines. We identified orexin-A and orexin-B as endogenous ligands for two orphan GPCRs found as human ESTs (2).

Structures of orexins were chemically determined by biochemical purification and sequence analysis by Edman sequencing and mass spectrometry (2). Orexins constitute a novel peptide family, with no significant homology with any previously described peptides. Orexin-A is a 33-amino-acid peptide of 3562 Dalton, with an N-terminal pyroglutamyl residue and C-terminal amidation (Fig. 1). The molecular mass of the purified peptide as well as its sequencing analysis indicated that the four Cys residues of orexin-A formed two sets of intrachain disulfide bonds. The topology of the disulfide bonds was chemically determined to be [Cys6-Cys12, Cys7-Cys14]. This structure is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog, and pig). On the other hand, rat orexin-B is a 28-amino-acid, C-terminally amidated linear peptide of 2937 Dalton, which was 46% (13/28) identical in sequence to orexin-A (Fig. 1A). The C-terminal half of orexin-B is very similar to that of orexin-A, whereas the N-terminal half is more variable. Mouse orexin-B was predicted to be identical to rat orexin-B. Human orexin-B has two amino acid substitutions from the rodent sequence within the 28-residue stretch. Pig and dog orexin-B have one amino acid substitution from the human or rodent sequence.

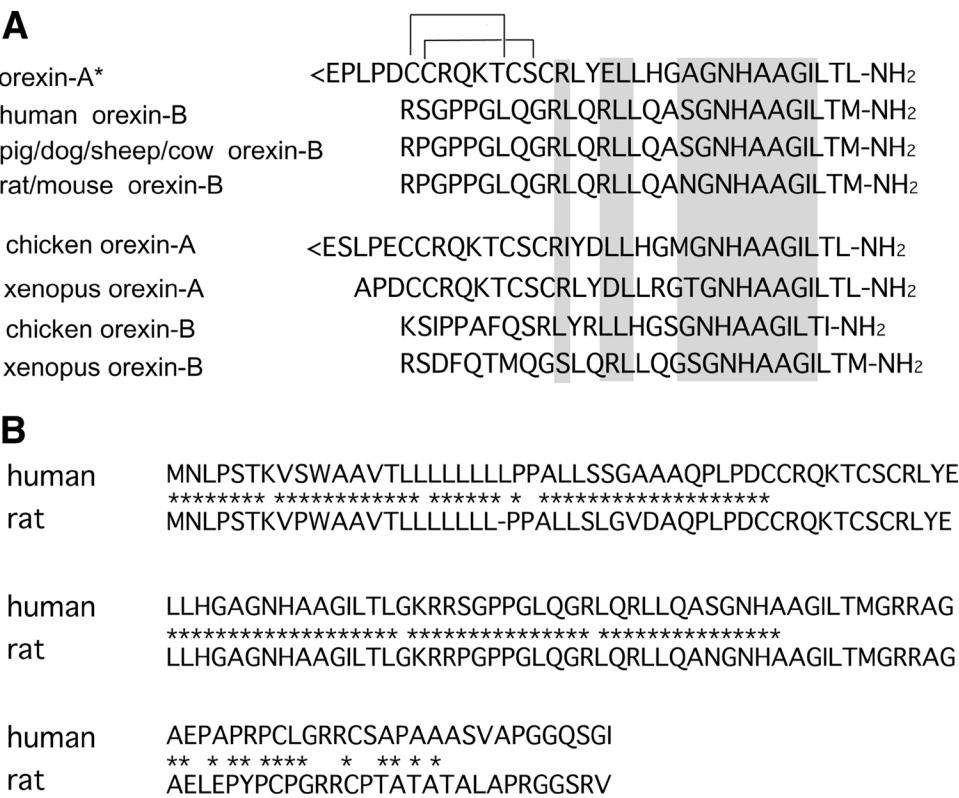


Fig. 1. (A) Structures of mature orexin-A and -B peptides. The topology of the two intrachain disulfide bonds in orexin-A is indicated above the sequence. Amino acid identities are indicated by shaded boxes. **(B)** Amino acid sequences of human and rat prepro-orexin. Asterisks indicate the identical amino acids between human and rat sequences.

Other than mammalian species, structures of *Xenopus* and chicken orexin-A and orexin-B, which also have conserved structures compared with mammalian sequences, have been elucidated (Fig. 1A).

The prepro-orexin cDNA sequences revealed that both orexins are produced from the same 130-residue (rodent) or 131-residue (human) polypeptide, prepro-orexin, by proteolytic processing. The human and mouse prepro-orexin sequences are 83 and 95% identical to the rat counterpart, respectively (Fig. 1B) (2). The majority of amino acid substitutions were found in the C-terminal part of the precursor, which appears unlikely to encode another bioactive peptide (Fig. 1B).

An mRNA encoding the same precursor peptide was independently isolated by de Lecea et al. (3) as a hypothalamus-specific transcript. They predicted that this transcript encodes two neuropeptides, named hypocretin-1 and -2. The terms “hypocretin” and “orexin” are currently used as synonyms.

We reported orexins initially as orexigenic peptides (2). Subsequently, they have been reported to have a variety of pharmacological actions (*see* other chapters). In particular, recent observations implicate orexins/hypocretins in sleep disorder narcolepsy and in the regulation of the normal sleep process. The biological activities of orexins are discussed in other chapters of this book.

3. THE PREPRO-OREXIN GENE: STRUCTURE AND REGULATION OF EXPRESSION

The human prepro-orexin gene, which is located on chromosome 17q21, consists of two exons and one intron distributed over 1432 bp (4). The 143-bp exon 1 includes the 5'-untranslated region and the coding region that encodes the first seven residues of the secretory signal sequence. Intron 1, which is the only intron found in the human prepro-orexin gene, is 818-bp long. Exon 2 contains the remaining portion of the open reading frame and the 3'-untranslated region.

The human prepro-orexin gene fragment, which contains the 3149-bp 5'-flanking region and the 122-bp 5'-noncoding region of exon 1, was reported to have an ability to express *lacZ* in orexin neurons without ectopic expression in transgenic mice, suggesting that this genomic fragment contains most of the necessary elements for appropriate expression of the gene (4). This promoter is useful to examine the consequences of expression of exogenous molecules in orexin neurons of transgenic mice, thereby manipulating the cellular environment in vivo (4–6). For example, this promoter was used to establish several transgenic lines, including orexin neuron-ablated mice and rats and mice in which orexin neurons specifically express green fluorescent protein (GFP) (5,6).

The regulation of expression of the prepro-orexin gene still remains unclear. Prepro-orexin mRNA was shown to be upregulated under fasting conditions, indicating that these neurons somehow sense the animal's energy balance (2). Several reports have shown that orexin neurons express leptin receptor- and STAT-3-like immunoreactivity, suggesting that orexin neurons are regulated by leptin (7). We consistently found that continuous infusion of leptin into the third ventricle of mice for 2 wk resulted in marked downregulation of prepro-orexin mRNA level (5). Therefore, reduced leptin signaling may be a possible factor that upregulates expression of prepro-orexin mRNA during starvation. Prepro-orexin levels were also increased in hypoglycemic conditions, suggesting that expression of the prepro-orexin gene is also regulated by plasma glucose levels (8). These observations are consistent with our electrophysiological study of GFP-expressing orexin neurons in transgenic mice, which showed that orexin neurons are regulated by extracellular glucose concentration and leptin (5).

4. STRUCTURES AND PHARMACOLOGY OF OREXIN RECEPTORS

The actions of orexins are mediated by two G-protein-coupled receptors termed orexin-1 receptor (OX₁R) and orexin-2 receptor (OX₂R) (Fig. 2) (2). Among various classes of G-protein-coupled receptors, OX₁R is structurally more similar to certain neuropeptide receptors, most notably to the Y2 neuropeptide Y (NPY) receptor (26% similarity), followed by the thyrotropin-releasing hormone (TRH) receptor, cholecystokinin type-A receptor, and NK2 neurokinin receptor (25,23, and 20% similarity, respectively).

The amino acid identity between the deduced full-length human OX₁R and OX₂R sequences is 64%. Thus, these receptors are much more similar to each other than they are to other GPCRs. Amino acid identities between the human and rat homologs of each of these receptors are 94% for OX₁R and 95% for OX₂R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays using Chinese hamster ovary (CHO) cells expressing OX₁R suggested that orexin-A is a high-affinity agonist for OX₁R. The concentration of cold orexin-A required to displace 50% of specific radioligand binding (IC₅₀) was 20 nM. Human orexin-B also acted as a specific agonist on CHO cells expressing OX₁R. However, human orexin-B has significantly lower affinity compared with

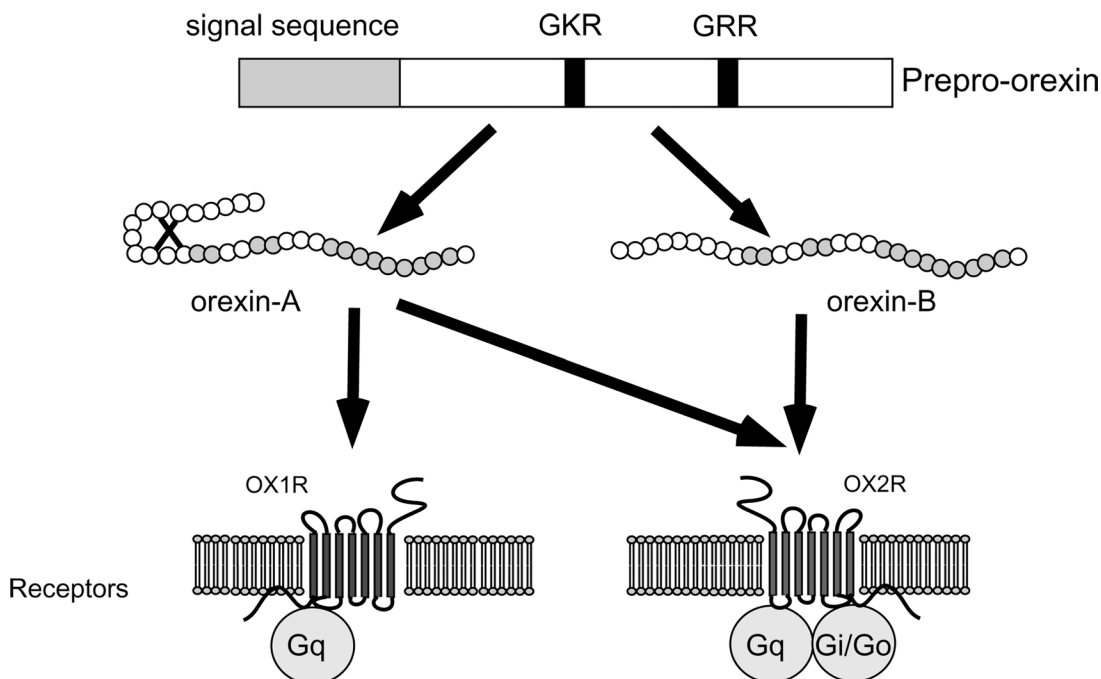


Fig. 2. Schematic representation of the orexin system. Orexin-A and -B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G-protein-coupled receptors named orexin-1 (OX₁R) and orexin-2 (OX₂R) receptors. OX₁R is selective for orexin-A, whereas OX₂R is a nonselective receptor for both orexin-A and orexin-B.

human OX₁R: the calculated IC₅₀ in a competitive binding assay was 250 nM for human orexin-B, indicating two orders of magnitude lower affinity compared with orexin-A (Fig. 2).

On the other hand, binding experiments using CHO cells expressing the human OX₂R cDNA demonstrated that OX₂R is a high-affinity receptor for human orexin-B with an IC₅₀ of 20 nM. Orexin-A also had high affinity for this receptor with an IC₅₀ of 20 nM, which is similar to the value for orexin-B, suggesting that OX₂R is a nonselective receptor for both orexin-A and orexin-B (Fig. 2).

5. MOLECULAR GENETIC STUDIES OF OREXIN RECEPTORS

Earlier genetic studies revealed that dogs with a mutation of the OX₂R gene or OX₂R-knockout mice displayed a narcolepsy-like phenotype (9,10), whereas OX₁R knockout mice did not reveal any obvious abnormality in the sleep/wake states (10). These studies provide strong evidence for the roles of OX₂R in regulating the vigilance state in human and animals. However, double receptor knockout (OX₁R- and OX₂R-null) mice appear to be a phenocopy of prepro-orexin knockout mice, suggesting that OX₁R also has additional effects on sleep/wakefulness. Consistent with this, the behavioral and electroencephalographic phenotype of OX₂R knockout mice is less severe than that found in prepro-orexin knockout mice (9). These findings suggest that loss of signaling through both receptor pathways is necessary for severe narcoleptic characteristics. Indeed, OX₂R knockouts are only mildly affected with cataplexy-like attacks of REM sleep, whereas orexin knockout mice are severely affected (9).

The phenotypes of orexin receptor knockout mice are discussed more precisely in another chapter.

6. HOW MANY OREXIN RECEPTOR GENES?

Two genes for orexin receptors have been identified in mammalian species thus far. The phenotypes of OX_1R and OX_2R double-deficient mice were analyzed and shown to have sleep state abnormality, which was indistinguishable from that of prepro-orexin gene-deficient mice. This observation suggests that only two receptors for orexins might exist in mammals, at least in vigilance state control. However, it is possible that there are other subtypes of receptors produced from OX_1R or OX_2R genes by alternative splicing. In fact, two alternative C-terminus splice variants of the murine OX_2R , termed m $OX_2\alpha R$ (443 amino acids) and m $OX_2\beta R$ (460 amino acids) have been identified (11). However, orexin-A and orexin-B showed no difference in binding characteristics between the splice variants.

7. DISTRIBUTION OF OREXIN RECEPTORS

Although orexin receptors are expressed in a pattern consistent with orexin projections, mRNA for OX_1R and OX_2R were shown to be differentially distributed throughout the brain. For instance, within the hypothalamus, a low level of OX_1R mRNA expression is observed in the dorsomedial hypothalamus (DMH), whereas a higher level of OX_2R mRNA expression is observed in this region. Other areas of OX_2R expression in the hypothalamus are the arcuate nucleus, paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and, most significantly, the tuberomammillary nucleus (TMN) (12). In these regions, there is little or no OX_1R signal. In the hypothalamus, OX_1R mRNA is abundant in the anterior hypothalamic area and ventromedial hypothalamus (VMH). Outside the hypothalamus, high levels of OX_1R mRNA expression are detected in the tenia tecta, hippocampal formation, dorsal raphe nucleus, and, most prominently, the locus ceruleus (LC). OX_2R mRNA is abundantly expressed in the cerebral cortex, nucleus accumbens, subthalamic nucleus, paraventricular thalamic nuclei, anterior pretectal nucleus, and the raphe nuclei.

Within the brain, OX_1R is most abundantly expressed in the LC, whereas OX_2R is most abundantly expressed in the TMN, regions highly important for maintenance of arousal. The raphe nuclei contain both receptor mRNAs. These observations suggest strong interaction between orexin neurons and the monoaminergic systems. More precise distribution of orexin receptors is discussed in Chapter 3.

8. STRUCTURE-ACTIVITY RELATIONSHIPS OF OREXINS

Activities of synthetic orexin-B analogs in cells transfected with either OX_1R or OX_2R were examined to define the structural requirements for activity of orexins on their receptors (13). The ability of N- or C-terminally truncated analogs of orexin-B to increase cytoplasmic Ca^{2+} levels in the cells showed that the absence of N-terminal residues had little or no effect on the biological activity and selectivity of both receptors. Truncation from the N-terminus to the middle part of orexin-B resulted in moderate loss of activity, in the order of peptide length. In particular, deletion of the conserved sequence between orexin-A and orexin-B caused a profound loss of biological activity, and the C-terminally truncated peptides were also inactive for both receptors. These results suggest that the consensus region between orexin-A and orexin-B is important for the activity of both receptors.

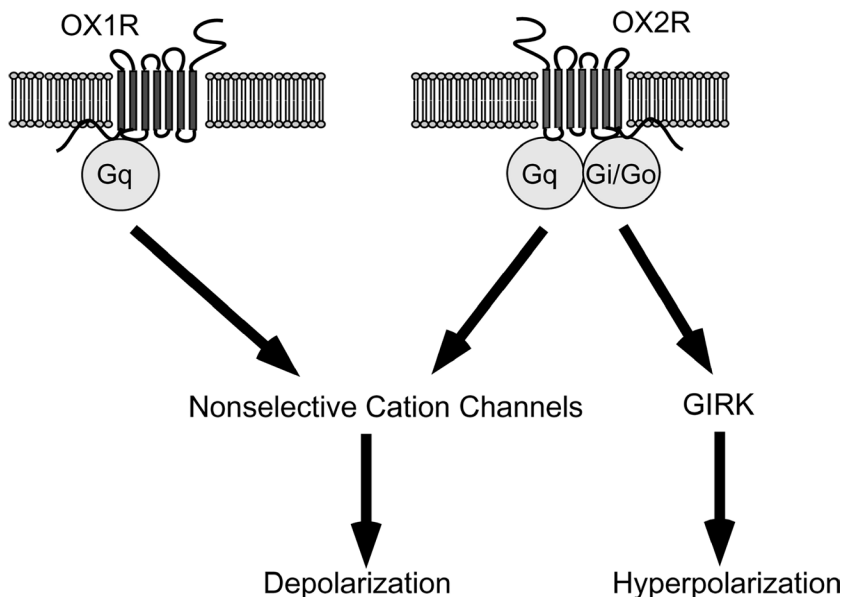


Fig. 3. Schematic representation of the intracellular signal transduction systems of orexin receptors. OX₁R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX₂R may couple to G_{i/o}, and/or G_q. GIRK, G-protein-gated inwardly rectifying potassium channel.

Substitution of each amino acid of the natural sequence of orexin-B by L-alanine revealed that the residues in the N-terminal region could be substituted by L-alanine without loss of activity of both receptors. However, substitution in the C-terminal region (especially at positions 24–28) decreased the activity, just as C-terminal truncation did. Substitution of each amino acid of orexin-B by the corresponding D-amino acid also showed that the C-terminal region is highly important for the activity of orexin-B.

Orexin-A (positions 15–33), the C-terminal half of orexin-A, and orexin-B (positions 10–28) have similar sequences, however, their selectivity to OX₁R and OX₂R is different. This finding indicates that not only the activity but also the ligand/receptor selectivity is closely related to the C-terminal half of the orexin sequence.

9. SIGNAL TRANSDUCTION SYSTEM

Both OX₁R and OX₂R are G-protein-coupled receptors, which transmit information into cells by activating heterotrimeric G proteins. Activation of the signaling pathways associated with distinct G proteins may contribute to the diverse physiological roles of orexin in particular neurons. Although many G-protein-coupled neurotransmitter receptors are potentially capable of modulating both voltage-dependent calcium channels and G-protein-gated inwardly rectifying potassium channels (GIRKs), there might be a substantial degree of selectivity in the coupling to one or other of these channels in neurons (Fig. 3). The signal transduction pathways of orexin receptors were examined in cells transfected with OX₁R or OX₂R. In OX₁R-expressing cells, forskolin-stimulated cAMP was not affected by orexin administration. In addition, PTX treatment did not show any effects on orexin-induced increases in [Ca²⁺]_i.

These results suggest that OX₁R does not couple to PTX-sensitive G_{i/o} proteins (14). In contrast, orexin inhibited forskolin-stimulated cAMP production in a dose-dependent manner

in OX₂R-expressing cells. The effect was abolished by pretreatment with PTX. However, orexin-induced increases in $[Ca^{2+}]_i$ were not affected by PTX treatment in OX₂R-expressing cells. These results indicate that the OX₂R couples to PTX-sensitive G proteins that were involved in the inhibition of adenylyl cyclase by orexin. They also suggest that OX₁R couples exclusively to PTX-insensitive G proteins, and OX₂R couples to both PTX-sensitive and -insensitive proteins. The relative contribution of these G proteins in the regulation of neuronal activity remains unknown.

Orexins have been shown to have an excitatory activity in many types of neurons in vivo. For instance, noradrenergic cells of the LC (15), dopaminergic cells of the ventral tegmental area (16), and histaminergic cells from the TMN (17) have been shown to be activated by orexins. Because LC neurons exclusively express OX₁R, whereas TMN neurons exclusively express OX₂R, these observations suggest that both OX₁R and OX₂R signaling are excitatory on neurons. However, these studies only examined the effect of orexins on receptor-expressing cell bodies. There is a possibility that orexin receptors locate on presynaptic terminals, because Li et al. (18) reported that orexin increases local glutamate signaling by facilitation of glutamate release from presynaptic terminals. Therefore, it is possible that activation of PTX-sensitive G proteins downstream of OX₂R might be involved in functions other than activation of neurons, such as in the tips of developing neurites and on presynaptic nerve terminals, leading to growth cone collapse and enhanced synaptic release of the transmitter. Alternatively, OX₂R-mediated activation of Gi might result in inhibition of some populations of neurons. In fact, orexin was recently reported to inhibit prepro-melanocortin neurons in the arcuate nucleus in vitro (19).

REFERENCES

1. Wise, A., Jupe, S.C., and Rees, S. (2004) The identification of ligands at orphan G-protein coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **44**, 43–66.
2. Sakurai, T., Amemiya, A., Ishii, M., et al. (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* **92**, 573–585.
3. de Lecea, L., Kilduff, T.S., Peyron, C., et al. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc. Natl. Acad. Sci. U S A* **95**, 322–327.
4. Sakurai, T., Moriguchi, T., Furuya, K., et al. (1999) Structure and function of human prepro-orexin gene. *J. Biol. Chem.* **274**, 17771–17776.
5. Yamanaka, A., Beuckmann, C.T., Willie, J.T., et al. (2003) Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* **38**, 701–713.
6. Hara, J., Beuckmann, C.T., Nambu, T., et al. (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30**, 345–354.
7. Hakansson, M., de Lecea, L., Sutcliffe, J.G., Yanagisawa, M., and Meister, B. (1999) Leptin receptor- and STAT3-immunoreactivities in hypocretin/orexin neurons of the lateral hypothalamus. *J. Neuroendocrinol.* **11**, 653–663.
8. Griffond, B., Risold, P.Y., Jacquemard, C., Colard, C., and Fellmann, D. (1999) Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. *Neurosci. Lett.* **262**, 77–80.
9. Willie, J.T., Chemelli, R.M., Sinton, C.M., and Yanagisawa, M. (2003) Distinct narcolepsy syndromes in orexin receptor-2 and orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* **38**, 715–730.
10. Willie, J.T., et al. (2001) To eat or to sleep? Orexin in the regulation of feeding and wakefulness. *Annu. Rev. Neurosci.* **24**, 429–458.
11. Chen, J. and Randeva, H.S. (2004) Genomic organization of mouse orexin receptors: characterization of two novel tissue-specific splice variants. *Mol. Endocrinol.* **18**, 2790–2804.

12. Marcus, J.N., Aschkenasi, C.J., Lee, C.E., et al. (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. *J. Comp. Neurol.* **435**, 6–25.
13. Asahi, S., et al. (1999) Structure-activity regulation studies on the novel neuropeptide orexin. *Peptide Sci.* 37–40.
14. Zhu, Y., Miwa, Y., Yamanaka, A., et al. (2003) Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins. *J. Pharmacol. Sci.* **92**, 259–266.
15. Hagan, J.J., Leslie, R.A., Patel, S., et al. (1999) Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc. Natl. Acad. Sci. U S A* **96**, 10911–10916.
16. Nakamura, T., Uramura, K., Nambu, T., et al. (2000) Orexin-induced hyperlocomotion and stereotypy are mediated by the dopaminergic system. *Brain Res.* **873**, 181–187.
17. Yamanaka, A., Tsujino, N., Funahashi, H., et al. (2002) Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem. Biophys. Res. Commun.* **290**, 1237–1245.
18. Li, Y., Gao, X. B., Sakurai, T., and van den Pol, A. N. (2002) Hypocretin/orexin excites hypocretin neurons via a local glutamate neuron-A potential mechanism for orchestrating the hypothalamic arousal system. *Neuron* **36**, 1169–1181.
19. Muroya, S., Funahashi, H., Yamanaka, A., et al. (2004) Orexins (hypocretins) directly interact with neuropeptide Y, POMC and glucose-responsive neurons to regulate Ca²⁺ signaling in a reciprocal manner to leptin: orexigenic neuronal pathways in the mediobasal hypothalamus. *Eur. J. Neurosci.* **19**, 1524–1534.

The Orexin/Hypocretin System

Physiology and Pathophysiology

Nishino, S.; Sakurai, T. (Eds.)

2005, 416 p. 151 illus., 9 illus. in color., Hardcover

ISBN: 978-1-58829-444-9

A product of Humana Press