The Orexin/Hypocretin System

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Contemporary Clinical Neuroscience

The Orexin/Hypocretin System

Physiology and Pathophysiology

Edited by

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Cover illustration: Figure 7 from Chapter 21, "Hypocretin Status in Neurological Disorders in Relation to Excessive Sleepiness and Cataplexy," by Takashi Kanbayashi, Junko Arii, Hideaki Kondo, Testuo Shimizu, and Seiji Nishino.

Cover design by Patricia F. Cleary.

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ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data
The orexin/hypocretin system / edited by Seiji Nishino and Takeshi Sakurai.
p. cm.
Includes bibliographical references and index.
ISBN 1-58829-444-7 (alk. paper) e-ISBN 1-59259-950-8
1. Orexins. 2. Narcolepsy. I. Nishino, Seiji. II. Sakurai, Takeshi.
QP572.O74O74 2005
612.4'05—dc22 2005000318

PREFACE

Orexin/hypocretin research began in 1998, as a result of the discovery of a new hypothalamic neuropeptide. In 1999, it was found that mutations in the orexin/hypocretin-related genes caused a sleep disorder (narcolepsy) in dogs and mice. These findings were soon followed by the discoveries of orexin/hypocretin ligand deficiency in human narcolepsy.

The finding of the major pathophysiological mechanisms of human narcolepsy resulted in its reclassification as a neurological, not a psychiatric, disorder. The importance of early diagnosis and initiation of treatment for human narcolepsy has been repeatedly emphasized because the disease typically starts around puberty (when social and school influences become important). Orexin/hypocretin deficiency in narcolepsy subjects can be detected clinically in cerebrospinal fluid (CSF) orexin/hypocretin measures (low CSF orexin/hypocretin levels are strongly associated with narcolepsy–cataplexy among various neurologic and sleep disorders). Thus, the CSF orexin/hypocretin measurements are expected to be included as a diagnostic test for narcolepsy–cataplexy in the second revision of international diagnostic criteria (ICSD). This positive diagnostic test is very useful for establishing an early diagnosis for narcolepsy–cataplexy, and many patients will likely receive immediate benefits. Cerebrospinal orexin/hypocretin measurements are also informative for the nosological classification of hypersomnia. Because orexin/hypocretin deficiency is observed in most human narcolepsy–cataplexy, orexin/hypocretin replacement therapy is now a promising new choice for the treatment of human narcolepsy, and research in this area is actively in progress.

The Orexin/Hypocretin System: Physiology and Pathophysiology examines these exciting discoveries and presents new findings such as ligand replacement and gene therapies in animal models of narcolepsy. The next important step for narcolepsy research is to discover the pathological mechanisms for the loss of orexin/hypocretin neurons in humans. This information is critical to prevention or cure of the disease, and another breakthrough in this area is expected in the not too distant future.

How the orexin/hypocretin system physiologically regulates sleep and wakefulness remains largely unknown. It is not fully understood how and why the symptoms of narcolepsy occur when orexin/hypocretin neurotransmission is impaired. Sleep is a complex physiological phenomenon, and multiple systems are involved in its regulation. Because we were reluctant to request just one author to cover the roles of orexin/hypocretin in sleep regulation, we invited several contributors who are working in this field to freely discuss their opinions; as a result we could not avoid significant overlaps among these chapters. Because we did not instruct the authors to unify their hypotheses, controversies may also exist. However, there is room for readers to actively participate in these debates and to carry out the experiments to prove or disprove these hypotheses.

The orexin/hypocretin system is also of exceptional interest in neuroscience research. In addition to its involvement in vigilance control and narcolepsy, the system likely regulates various hypothalamic functions such as neuroendocrine functions, stress reactions, and autonomic functions necessary for human survival. Numerous researchers have initiated multidisciplinary approaches in order to understand the various aspects of the physiological functions of the orexin/hypocretin system. In the same way, narcolepsy is a useful disease model for understanding the link between vigilance control with other fundamental hypothalamic functions, such as regulation of feeding behavior and autonomic function. Similarly, clinical applications of orexin/hypocretin agonists and antagonists for various diseases are suggested.

We introduced several experimental methods for orexin/hypocretin research and discussed the use and limitations of these methods that are useful for the multidisciplinary approaches in the orexin/hypocretin research field, as well as for other neuropeptidergic systems.

Finally, we would like to emphasize that rapid, significant success in narcolepsy research has not been achieved without careful observations in the appropriate animal models of the disease. These approaches, which are used to study narcolepsy, will now encourage researchers to initiate genetic linkage and positional cloning experiments, as well as to generate various genetically engineered animal models. A link between orexin/hypocretin ligand deficiency and narcolepsy in orexin/hypocretin knockout mice could not have been made without excellent scientific acumen combined with a modicum of luck. Tenacious efforts by researchers, together with the application of modern technologies, made these breakthroughs possible in a timely manner.

Living in a post-genome era, the success of the orexin/hypocretin story is driving many researchers to search novel bioactive peptides and their receptors for further discoveries in physiology, and these are likely to lead to novel opportunities for clinical treatments. Orexins/hypocretins are one of the first endogenous ligands discovered for orphan G protein–coupled receptors. Since 1995, about 70 ligands and/or orphan receptors have been identified or re-recognized. There are still about 100 orphan receptors, and the search for the endogenous ligands for these receptors is actively in progress. It is therefore possible that the abnormal function of some of the unknown ligands and uncharacterized receptors are directly involved in the etiology and/or pathophysiology of several neurologic and psychiatric disorders. The study of the orexin/hypocretin system is a good example of what can be achieved.

We would like to acknowledge Dr. Shuji Hoshino (Hoshino Surgical Clinic, Hiroshima) for the generous gift to support the project, and Edward Tuan for his editorial and clerical assistance.

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I
History

1

History and Overview of Orexin/Hypocretin Research

From Orphan GPCR to Integrative Physiology

Michihiro Mieda, PhD, and Takeshi Sakurai, MD, PhD

1. INTRODUCTION

Since its discovery in 1998, the field of orexin/hypocretin biology has grown rapidly. In the last 6 yr, more than 900 articles on orexin/hypocretin research have been published. Information on the role of the orexin/hypocretin system in narcolepsy–cataplexy has had a huge impact on the study of sleep and wakefulness. Scientists are now using a multidisciplinary approach to understand various aspects of the physiological functions of orexins/hypocretins in order to apply orexin/hypocretin biology to the diagnosis and treatment of sleep-related disorders.

2. DISCOVERY OF OREXIN/HYPOCRETIN

Identification of orexin/hypocretin peptides in 1998 was the result of a beautiful convergence of two independent research groups utilizing completely different methodologies. de Lecea et al. *(1)* utilized a molecular biological technique to isolate a series of cDNA clones that are expressed in the hypothalamus but not in the cerebellum and the hippocampus by subtractive hybridization. One of these was expressed exclusively by a bilaterally symmetric structure within the posterior hypothalamus. They subsequently cloned cDNAs covering the entire coding region, which encodes a putative secretory protein of 130 amino acids. According to its primary sequence, they predicted that this protein gives rise to two peptide products that are structurally related to each other. Because these predicted peptides were expressed in the *hypo*thalamus and had similarity to se*cretin*, they named them hypocretin-1 and -2 *(1)*.

It was later learned that hypocretins (orexins) are, in fact, not relatives of the incretin family; receptors for these peptides have considerably different structures from those of the incretin recepor superfamily, which usually couple to the Gs family of G proteins. *Hypocretin* mRNA was detected only in the brain, and antibodies raised against prepro-hypocretin identified hypocretin-positive cell bodies exclusively in the perifornical area of the hypothalamus and hypocretin-positive nerve fibers in many brain areas. It was further demonstrated by electron microscopy and electrophysiology studies that hypocretin is present in synaptic vesicles, and also that hypocretin-2 has excitatory effects on hypothalamic neurons but not on hippocampal dentate granule neurons. Although their prediction of mature peptides processed from prepro-hypocretin later turned out to be inexact, their study strongly suggested that hypocretin peptides are novel neurotransmitters exclusively expressed in a population of neurons in the perifornical area.

From: *Contemporary Clinical Neuroscience: The Orexin/Hypocretin System: Physiology and Pathophysiology*
Edited by: S. Nishino and T. Sakurai © Humana Press Inc., Totowa, NJ

Around the same time as the report by de Lecea et al., Sakurai et al. *(2)* reported identification of two peptides (orexin-A and orexin-B) that were endogenous ligands of two orphan G-protein-coupled receptors (GPCRs) whose cognate ligands had not been identified, which they named orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R). These peptides are cleaved from a single-precursor polypeptide prepro-orexin, which is expressed by a select population of neurons clustered around the perifornical lateral hypothalamus. It was later learned that prepro-orexin and prepro-hypocretin were identifical and that orexin-A and -B corresponded to hypocretin-1 and -2, respectively. However, the original structures of predicted hypocretin-1 and -2 were not identical to purified orexin-A and -B because of an incorrect prediction of proteolytic sites, as well as the loss of two intrachain disulfate bonds and N-terminal pyroglutamylation in hypocretin-1, which were found in orexin-A. Since these peptides were exclusively expressed in neurons of the lateral hypothalamus, which has been implicated as a "feeding center," intracerebroventricular (icv) administration of orexin-A or -B in the lateral ventricle was examined and found to increase food intake in rats dose dependently; this was the reason these peptides were named "orexin" after the Greek word *orexis*, meaning appetite. Furthermore, expression of *prepro-orexin* mRNA was upregulated more than twofold upon fasting, just like *neuropeptide Y* (*NPY*) mRNA, whose product is a well-known feeding peptide.

3. LOSS OF OREXIN/HYPOCRETIN SIGNALING CAUSES NARCOLEPSY–CATAPLEXY

Soon after the discovery of orexin/hypocretin, two independent studies utilizing dog forward genetics and mouse reverse genetics dramatically increased our understanding of orexin/hypocretin biology. Human narcolepsy–cataplexy is a debilitating neurological disease characterized by excessive daytime sleepiness, premature transitions to REM sleep (so-called sleep-onset REM periods), and cataplexy (sudden bilateral skeletal muscle weakness without impairment of consciousness) *(3,4)*. Scientists at Stanford University had established and been maintaining canine breeds with autosomal recessive inheritance of a narcolepsy syndrome for decades. In 1999, Lin et al. *(5)* at Stanford succeeded in identifying mutations in the *OX_2R/hypocretin* receptor 2 *(hcrtr2)* gene responsible for canine narcolepsy–cataplexy by positional cloning.

In the same month, Chemelli et al. *(6)* reported *prepro-orexin* knockout mice that exhibited a phenotype strikingly similar to human narcolepsy–cataplexy, characterized by cataplexy-like abrupt behavioral arrests, sleep/wake state fragmentation, non-REM sleep, and direct transitions from wakefulness to REM sleep. Orexin/hypocretin-immunoreactive nerve terminals were found on neurons implicated in arousal regulation, thus demonstrating the important role of orexin/hypocretin in sleep/wake regulation.

Subsequently, disruptions of the orexin/hypocretin system in human narcolepsy–cataplexy were confirmed. Nishino et al. *(7,8)* found that orexin-A/hypocretin-1 was undetectable in the cerebrospinal fluid (CSF) of up to 95% of narcolepsy–cataplexy patients but was readily detected in normal control individuals. Drastic reductions of *orexin/hypocretin* mRNA and immunoreactivity in postmortem brains of narcolepsy–cataplexy patients were also shown by Peyron et al. *(9)* and Thannickal et al. *(10)*. Furthermore, an unusually severe, early-onset case of human narcolepsy–cataplexy was associated with a mutation in the *orexin/hypocretin* gene that impairs peptide trafficking and processing *(9)*. These studies of human and animals alike established that the failure of signaling mediated by orexin/hypocretin neuropeptides causes narcolepsy–cataplexy.

4. MORE THAN JUST SLEEP/WAKE REGULATION

Even before the discovery of their linkage to narcolepsy–cataplexy, a detailed description of projection patterns of orexin/hypocretin–positive neurons suggested their involvement in a wide range of physiological functions, such as feeding, autonomic regulation, neuroendocrine regulation, and sleep/wake regulation *(11,12)*. Distribution of orexin/hypocretin–responsive neurons, marked with Fos expression induced by icv administration of orexin/hypocretin peptides, supported this prediction *(12)*.

Orexin/hypocretin administration experiments have demonstrated their multiple pharmacological effects. Since the initial report of orexin/hypocretin as feeding peptides *(2)*, increased food intake by laboratory animals following icv administration of orexin/hypocretin peptides has been reproduced. In addition to their effect on feeding, central administration of orexin/hypocretin peptides has been reported to cause a wide variety of physiological responses, such as an increase in motor activity, wakefulness (and suppression of sleep), energy expenditure, sympathetic outflow, gastric acid secretion and gastric motility, and hypothalamus–pituitary–adrenal (HPA) axis activity, as well as suppression of secretion of some hormones such as growth hormone, prolactin, and thyroid-stimulating hormone *(13–18)*.

Subsequently, many microinjection experiments into local brain areas identified putative target neurons that mediate effects of central orexin/hypocretin administrations. A confounding factor of microinjection experiments has been the dual innervation of orexin/hypocretin neurons to both excitatory and inhibitory areas as regards a certain physiological function. For example, REM sleep-related muscle atonia can be facilitated or inhibited by local orexin/hypocretin administration depending on the site of injection in brainstem areas *(19–21)*. Similarly, orexin/hypocretin neurons make excitatory input connections to both autonomic excitatory and inhibitory areas of the heart rate: the rostral ventrolateral medulla and the rostral ventromedial medulla, which mediates sympathetic tachycardia; and the nuclei of the solitary tract and ambiguus, which mediate sympathoinhibition and vagal bradycardia *(22–25)*. The balance between inputs into excitatory and inhibitory areas seems important, and therefore the dynamics of the action of the orexin/hypocretin system, as well as its interactions with other neuronal systems, should be considered *(26)*.

Orexin/hypocretin peptides have always been characterized as neurotransmitters *(1,27)*, and examination of neuronal responses to orexin/hypocretin application by in vitro electrophysiological recordings has also been carried out to identify downstream neurons of the orexin/hypocretin system and their signaling mechanisms. Site of action (pre or post), receptor subtype responsible, and downstream intracellular signaling cascade depend on the types of neuron examined, but the actions reported so far are mostly excitatory on postsynaptic cells and facilitatory on presynaptic release.

5. INTEGRATIVE PHYSIOLOGY OF THE OREXIN/HYPOCRETIN SYSTEM

As described above, central administration of orexin/hypocretin peptides causes a wide variety of effects. The critical question is whether these pharmacological effects are relevant to functions of endogenous orexin/hypocretin neurons in physiological conditions. The essential role of orexin/hypocretin on regulation of the sleep/wake cycle has been supported by several different approaches and seems valid. As described above, loss of orexin/hypocretin signaling causes narcolepsy–cataplexy in the human, dog, and mouse. Icv administration of orexin/hypocretins increases wakefulness and suppresses both non-REM and REM sleep. Orexin/hypocretin–positive nerve terminals and orexin/hypocretin receptors are found in

nuclei previously implicated in sleep/wake regulation, such as the tuberomammillary nucleus, locus ceruleus, dorsal raphe nucleus, pedunculopontine tegmental nucleus/laterodorsal tegmental nucleus, and basal forebrain. Microinjection of orexin/hypocretin into these areas in vivo increases wakefulness and suppresses sleep, and application of orexin/hypocretins excites neurons of these areas in vitro *(28)*. Nevertheless, several researchers are now claiming that orexin/hypocretin peptides regulate motor activity rather than wakefulness *per se*, and maintenance of wakefulness by orexin/hypocretins may be secondary to increased activity *(26,29,30)*. Whether orexin/hypocretins regulate wakefulness and/or motor activity or even higher functions, such as alertness and attention, requires further study.

So what about other functions of orexin/hypocretins proposed by pharmacological studies? One pharmacological effect of orexin/hypocretin initially reported was stimulation of food intake *(2)*. Although the efficacy of orexin/hypocretin was lower than that of NPY, a well-known feeding peptide, it was as potent as other appetite-stimulating peptides such as melanin-concentrating hormone (MCH), and this effect of orexin/hypocretin was reproduced in several laboratories *(31,32)*. In support of this idea, administration of anti-orexin/hypocretin antibody or an OX_1R-selective antagonist reduced food intake *(33,34)*, and *prepro-orexin* knockout mice and transgenic mice lacking orexin/hypocretin neurons ate less than control wild-type mice *(35,36)*. However, some researchers claim that the increase in food intake caused by orexin/hypocretin administration is secondary to an increase in wakefulness and activity *(37)*. However, other arousal-promoting substances do not always increase food intake. For example, corticotropin-releasing factor has a strong arousal effect yet suppresses appetite.

The "feeding peptides" such as NPY usually decrease energy expenditure while increasing food intake, which is more reasonable under the situations animal need to save energy *(38)*. In contrast, orexin/hypocretin increase energy expenditure. Therefore, orexin does not simply act as a system that maintains long-term body weight homeostasis. The function of orexins might be necessary for food seeking and feeding behaviors, especially when animals are faced with scarcity. Food seeking and food intake require more vigilant states and more energy expenditure. Recent evidence has suggested that orexin/hypocretin neurons are capable of sensing indicators of energy balance such as glucose and leptin; negative energy balance activates orexin/hypocretin neurons *(39–41)*. These findings raised a hypothesis that orexin/hypocretin neurons link energy homeostasis and sleep/wake regulation so that they allow animals to increase arousal under negative energy balance *(41)*. Similarly, other proposed roles of orexin/hypocretins, such as regulation of autonomic and endocrine systems, need to be studied further. Detailed characterization of disturbances in these systems in animals lacking orexin/hypocretin signaling pathways (including human narcolepsy–cataplexy patients) is needed.

Overall, it is likely that the orexin/hypocretin system not only promotes arousal but also regulates autonomic and endocrine systems so that physiological conditions of the whole body are well coordinated on demands of external and internal circumstances to awake and execute behaviors. We know that the orexin/hypocretin system regulates many downstream targets, but we have little knowledge about when orexin/hypocretin neurons are activated or about the upstream signals that regulate activities of orexin/hypocretin neurons. These questions have been addressed by counting Fos expression in orexin/hypocretin neurons, monitoring release of orexin/hypocretin by measuring orexin/hypocretin content in the CSF or microdialysis perfusates, and in vitro recording of activities of orexin/hypocretin neurons. These studies have suggested several factors as regulators of orexin/hypocretin neurons, such as energy balance, stress, and the circadian pacemaker *(39–44)*. It is very helpful to

systematically describe afferent pathways to orexin/hypocretin neurons. In addition, monitoring activities of orexin/hypocretin neurons in vivo with high temporal resolution, such as single-unit recording in unanethetized animals, would be essential to understand regulation of orexin/hypocretin neurons, as well as to understand their physiological roles.

The orexin/hypocretin system seems to have multiple upstream activators and downstream targets. Thus, it is important to ask whether physiological responses induced by activation of orexin/hypocretin neurons are stereotyped or variable depending on the natural conditions that cause activation of orexin/hypocretin neurons. It might be the combination of the orexin/hypocretin system and the other neuronal systems that determines the specific pattern of responses upon a certain circumstance, or the orexin/hypocretin system might have some heterogeneity within itself so that each subpopulation of orexin/hypocretin neurons can elicit physiological responses different from each other (for example, *see* ref. *45*).

6. CLINICAL POTENTIALS OF OREXIN/HYPOCRETIN PEPTIDES

In addition to providing a better understanding of the regulation of the sleep/wake cycle, discovery that the cause of human narcolepsy–cataplexy correlates with the loss of orexin/hypocretin production has had a tremendous impact on our clinical understanding of human narcolepsy–cataplexy. Narcolepsy–cataplexy is thought to be an autoimmune disease because of its strong association with certain human leukocyte antigen alleles *(46)*. Narcolepsy–cataplexy may result from selective autoimmune degeneration of orexin/hypocretin neurons. Indeed, residual gliosis was observed in the perifornical area of postmortem brains of narcolepsy–cataplexy patients *(10)*. Thus, we can now concentrate on orexin/hypocretin neurons as a target of research to understand the molecular and genetic mechanisms surrounding the development of narcolepsy–cataplexy, which might lead to accurate estimation of disease risk and eventually to prevention of disease onset.

This research on orexin/hypocretin peptides has led to the development of a novel, definitive diagnostic test for human narcolepsy–cataplexy. Mignot and his colleagues at Stanford *(7)* found that low orexin-A/hypocretin-1 levels in CSF is specifically correlated to patients with narcolepsy–cataplexy. The current nosology of narcolepsy–cataplexy is controversial. Measurements of CSF orexin-A/hypocretin-1 levels are expected to complement current diagnosis of narcolepsy–cataplexy.

Finally, the discovery of orexin/hypocretin has brought about the possibility of novel therapies for narcolepsy–cataplexy. Currently, excessive sleepiness is treated using psychostimulants, and cataplexy is treated with tricyclic antidepressants.γ-Hydroxybutyrate (sodium oxybate) is also used to consolidate nocturnal sleep and reduce cataplexy *(47)*. This therapeutic regimen is problematic owing to limited effectiveness, undesirable side effects such as insomnia or symptom rebounds, and the potential for abuse. We recently showed that ectopic expression of a *prepro-orexin/hypocretin* transgene or administration of orexin-A/hypocretin-1 in the brain of orexin/hypocretin neuron-ablated mice suppressed the narcoleptic phenotype of these mice *(48)*. These results indicate that orexin/hypocretin neuron-ablated mice retain the ability to respond to orexin/hypocretin neuropeptides and that a temporally regulated and spatially targeted secretion of orexin/hypocretins is not necessary to prevent narcoleptic symptoms. A similar result was also obtained by Fujiki et al. *(49)*, demonstrating that orexin-A/hypocretin-1 administered intravenously at an extremely high dose induces a very short-lasting anticataplectic effect in an orexin/hypocretin-deficient narcoleptic dog. Thus, an orexin/hypocretin receptor agonist would be of potential value for treating human narcolepsy–cataplexy. The development of stable, blood-brain barrier-permeable agonists for orexin/hypocretin receptors is still

awaited. Such agonists might also be useful in the treatment of other conditions involving excessive daytime sleepiness in humans. In addition, orexin/hypocretin receptor antagonists may also prove useful as safe hypnotics and antiobesity drugs *(50).*

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II

Overview of the Orexin/Hypocretin Neuronal System

2
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.

From: *Contemporary Clinical Neuroscience: The Orexin/Hypocretin System: Physiology and Pathophysiology*
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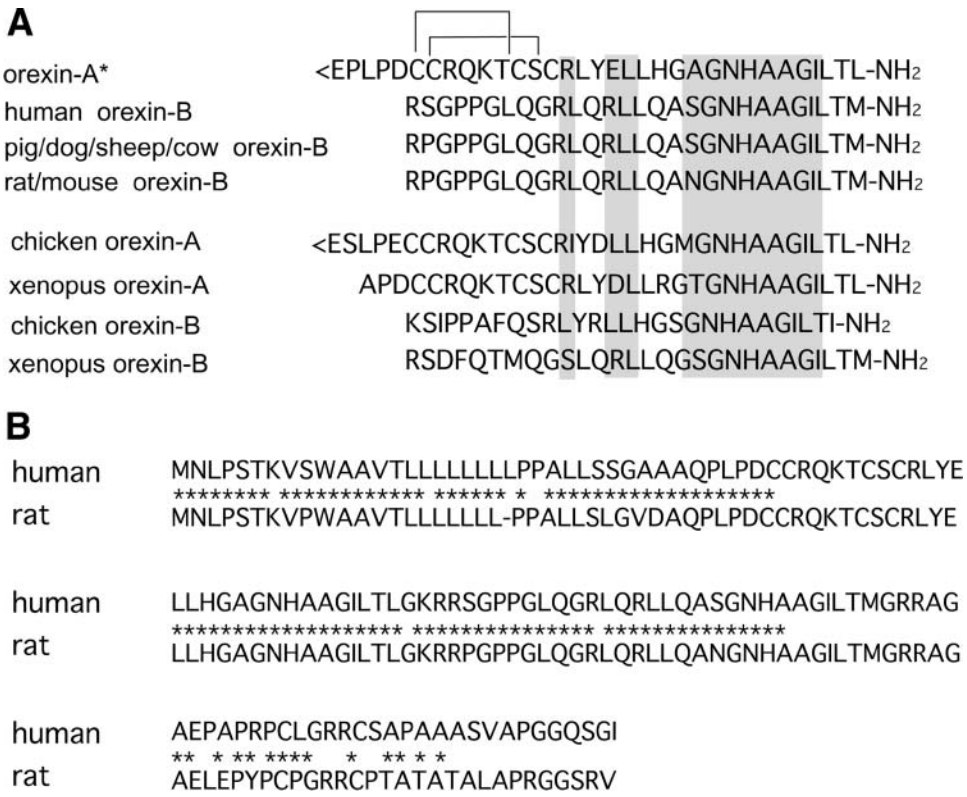

Fig. 1. **(A)** Structures of mature orexin-A and -B peptides. The topology of the two intrachain disulfide bonds in orexin-A is indicated above the sequence. Amino acid identities are indicated by shaded boxes. **(B)** Amino acid sequences of human and rat prepro-orexin. Asterisks indicate the identical 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 (OX_1R) and orexin-2 receptor (OX_2R) (Fig. 2) *(2)*. Among various classes of G-protein–coupled receptors, OX_1R 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, cholesystokinin type-A receptor, and NK2 neurokinin receptor (25,23, and 20% similarity, respectively).

The amino acid identity between the deduced full-length human OX_1R and OX_2R 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 OX_1R and 95% for OX_2R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays using Chinese hamster ovary (CHO) cells expressing OX_1R suggested that orexin-A is a high-affinity agonist for OX_1R. The concentration of cold orexin-A required to displace 50% of specific radioligand binding (IC_{50}) was 20 n*M*. Human orexin-B also acted as a specific agonist on CHO cells expressing OX_1R. However, human orexin-B has significantly lower affinity compared with

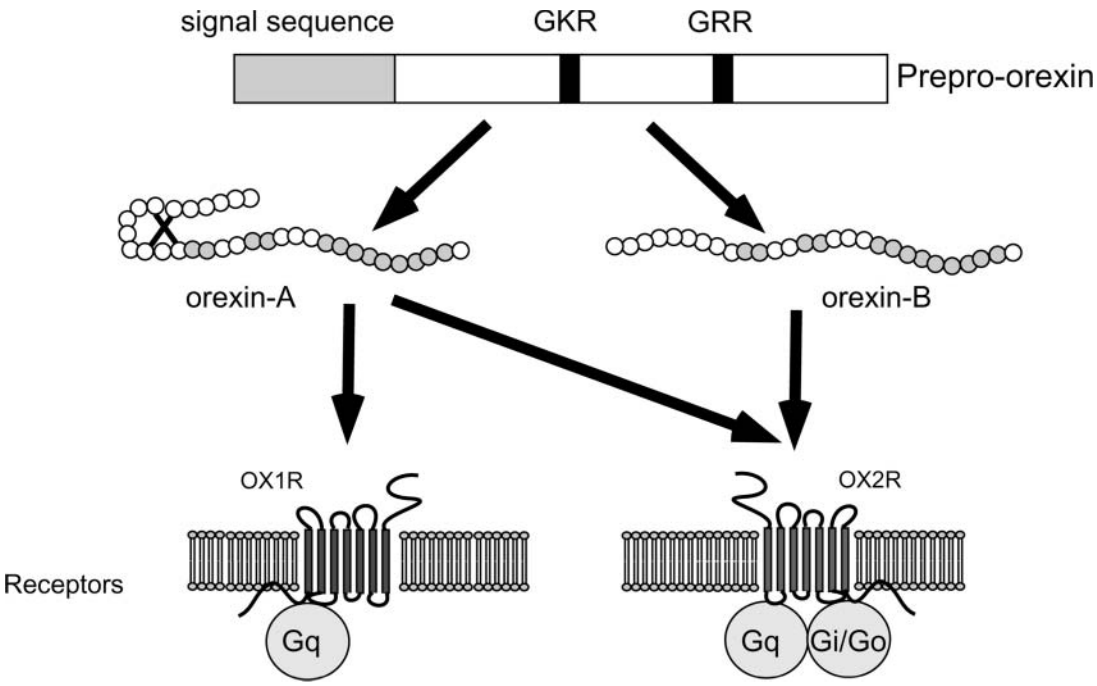

Fig. 2. Schematic representation of the orexin system. Orexin-A and -B are derived from a common precursor peptide, prepro-orexin. The actions of orexins are mediated via two G-protein–coupled receptors named orexin-1 (OX_1R) and orexin-2 (OX_2R) receptors. OX_1R is selective for orexin-A, whereas OX_2R is a nonselective receptor for both orexin-A and orexin-B.

human OX_1R: the calculated IC_{50} in a competitive binding assay was 250 n*M* 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 OX_2R cDNA demonstrated that OX_2R is a high-affinity receptor for human orexin-B with an IC_{50} of 20 n*M*. Orexin-A also had high affinity for this receptor with an IC_{50} of 20 n*M*, which is similar to the value for orexin-B, suggesting that OX_2R 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 OX_2R gene or OX_2R-knockout mice displayed a narcolepsy-like phenotype *(9,10)*, whereas OX_1R knockout mice did not reveal any obvious abnormality in the sleep/wake states *(10)*. These studies provide strong evidence for the roles of OX_2R in regulating the vigilance state in human and animals. However, double receptor knockout (OX_1R- and OX_2R-null) mice appear to be a phenocopy of prepro-orexin knockout mice, suggesting that OX_1R also has additional effects on sleep/wakefulness. Consistent with this, the behavioral and electroencephalographic phenotype of OX_2R 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, OX_2R 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 recepters 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.

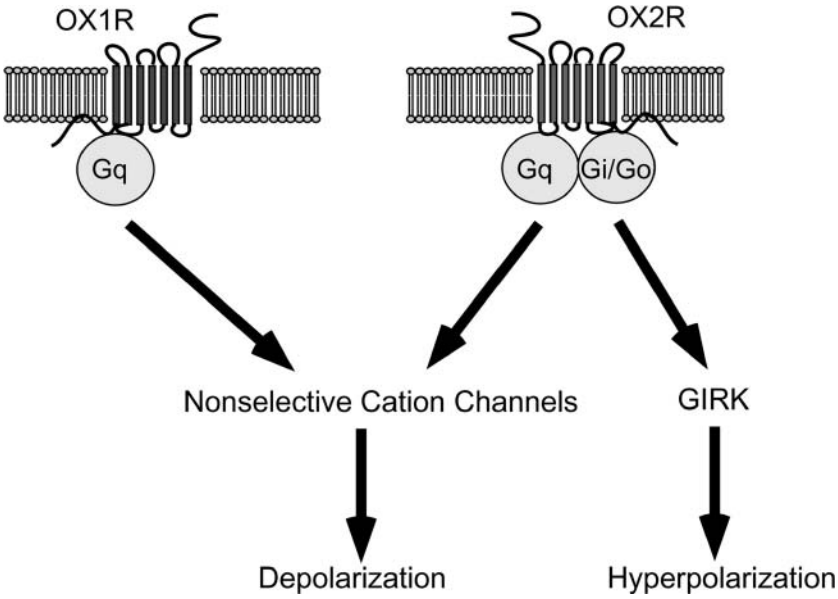

Fig. 3. Schematic representation of the intracellular signal transduction systems of orexin receptors. OX_1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX_2R may couple to $G_{i/o}$, and/or G_q. GIRK, G-protein-gated inwardly rectifying potassium channel.

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 OX_1R and OX_2R 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 OX_1R and OX_2R 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 OX_1R or OX_2R. In OX_1R-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 $[Ca^{2+}]_i$.

These results suggest that OX_1R 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 OX_2R-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 OX_2R-expressing cells. These results indicate that the OX_2R couples to PTX-sensitive G proteins that were involved in the inhibition of adenylyl cyclase by orexin. They also suggest that OX_1R couples exclusively to PTX-insensitive G proteins, and OX_2R 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 OX_1R, whereas TMN neurons exclusively express OX_2R, these observations suggest that both OX_1R and OX_2R 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 OX_2R 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, OX_2R-mediated activation of Gi might result in inhibition of some populations of neurons. In fact, orexin was recently reported to inhibit preopio-melanocortin neurons in the arcuate nucleus in vitro *(19)*.

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3

Orexin Projections and Localization of Orexin Receptors

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1. INTRODUCTION

The past several years have provided important insights into the physiological significance of the central orexin system. An important component of these is an increased understanding of the unique neuroanatomy of the orexin/hypocretin system. The orexin peptides, orexin-A and orexin-B, are produced in a restricted region of the central nervous system, the neurons of the lateral hypothalamic area. From this small neuronal source, on the order of a few thousand neurons, orexin-expressing neurons project over virtually the entire brain and spinal cord. At the terminals of these projections, orexin interacts with two distinct receptors, the orexin-1 receptor and the orexin-2 receptor. Given the diffuse projection pattern of orexin-containing axons, it is not surprising that orexin receptor expression has been described in a large number of brain areas. This chapter briefly outlines the brain regions innervated by hypothalamic orexin neurons and provides an overview of the distribution of each receptor subtype.

The location of orexin neurons combined with the orexin fiber innervation and receptor distribution pattern have been used to formulate anatomic hypotheses as to the likely physiological roles of the orexin system. These roles include the control of wake and sleep states, feeding and drinking behavior, neuroendocrine and autonomic regulation, locomotor activity, and many others. Notably, several of these models have been supported by physiological, pharmacological, and genetic evidence. The physiological importance of orexins in these different systems is addressed in detail by others in this text; in this chapter we consider the anatomical evidence for the role of orexins in these multiple systems.

2. LOCATION AND NEUROCHEMICAL PROPERTIES OF OREXIN NEURONS

The discovery of the orexin (hypocretin) peptides was met with enthusiasm, in part because of the unique expression pattern of the peptides *(1,2)*. It was immediately evident that neurons expressing orexin peptides are found in a very small region of the central nervous system. These neurons are located predominantly in the perifornical region and the lateral hypothalamus, at the tuberal level of the hypothalamus where the median eminence is evident. This region of the lateral hypothalamus has classically been implicated in a wide variety of behavioral and homeostatic regulatory systems, and thus the simple location of orexin-expressing neurons generated hypotheses as to their physiological relevance *(3,4)*.

From: *Contemporary Clinical Neuroscience: The Orexin/Hypocretin System: Physiology and Pathophysiology*
Edited by: S. Nishino and T. Sakurai © Humana Press Inc., Totowa, NJ