

## THE REGULATION OF SLEEP AND WAKEFULNESS BY THE HYPOTHALAMIC NEUROPEPTIDE OREXIN/HYPOCRETIN

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### ABSTRACT

Orexins, also known as hypocretins, are neuropeptides that are exclusively expressed by neurons in the lateral hypothalamic area. Although originally recognized as regulators of feeding behavior, orexins are now mainly regarded as key modulators of the sleep/wakefulness cycle. In addition, anatomical studies of neural networks and analyses of transgenic mice have revealed integrated roles for orexin neurons in the coordination of emotion, energy homeostasis, and the reward system. A functional link between the limbic system and orexin neurons may be important for increasing vigilance in response to emotional stimuli. These findings suggest that orexin neurons relay information about an organism's environment to maintain the proper amount of sleep and wakefulness in animals.

Key Words: orexin/hypocretin, sleep, hypothalamus, optogenetics, neuropeptide

### INTRODUCTION

Studies in genetically modified mice clearly indicate a critical role for orexin neurons in the regulation of sleep and wakefulness. Prepro-orexin knockout mice, orexin-2 receptor knockout mice, and orexin neuron-ablated transgenic mice all show fragmentation in sleep cycles<sup>1-3</sup>. Additionally, degeneration of orexin neurons was found in the narcoleptic brain post-mortem<sup>4-6</sup>. Orexins (orexin-A and -B) are neuropeptides expressed exclusively by neurons in the lateral hypothalamic area (LHA), an area that controls feeding and arousal. Although the number of orexin neurons is small (approximately 4,000 cells in the mouse brain), these neurons project axons throughout the central nervous system<sup>7,8</sup> and consequently affect various homeostatic functions. Dense projections from orexin neurons are observed in the serotonergic dorsal raphe nucleus (DR), noradrenergic locus coeruleus (LC), and histaminergic tuberomammillary nucleus (TMN), and all of these nuclei are involved in promoting arousal<sup>9</sup>. In this review, we discuss the basic features of orexins and orexin receptors. We describe how the orexin system regulates sleep and wakefulness, briefly addressing other physiological functions that are regulated by orexin neurons.

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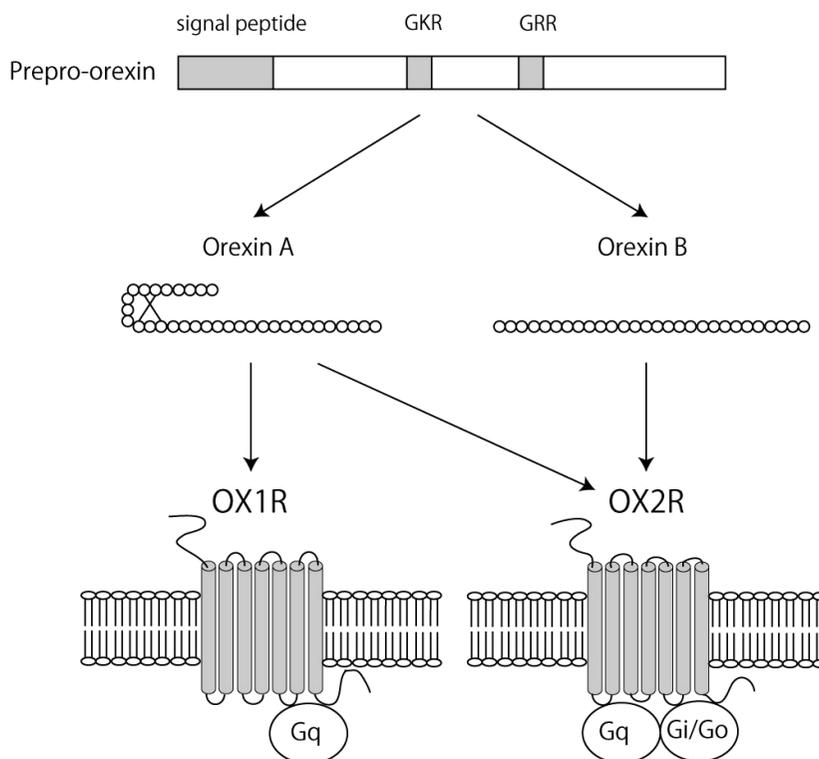
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## OREXINS AND OREXIN RECEPTORS

Two independent groups identified orexins in 1998. Using reverse pharmacology, Sakurai and colleagues identified a novel family of neuropeptides that bind to two closely related orphan G-protein coupled receptors (GPCRs). Intracerebroventricular injection of the synthetic neuropeptides induced feeding behavior<sup>10</sup>, and the ligands were therefore named “orexin” after the Greek word *orexis*, which means appetite. At the same time, de Lecea and colleagues isolated complementary DNA (cDNA) expressed exclusively in the hypothalamus. Two peptides corresponding to the cDNAs showed substantial amino acid sequence homology to the gut peptide secretin and were therefore named “hypocretins.”<sup>11</sup> It is now known that orexin and hypocretin are synonymous for the same set of peptides.

Orexin-A and -B are produced by proteolysis of a common precursor polypeptide, prepro-orexin (Fig. 1). In rats, orexin-A is a 33 amino acid peptide with an N-terminal pyroglutamyl residue and C-terminal amidation. The four cysteine residues in orexin-A form two intra-chain disulfide bonds, and this structure is conserved among mammalian species. Orexin-B is a 28 amino acid peptide with an amidated C-terminus that is 46% identical in sequence to orexin-A. The 3.2 kb fragment of the 5'-upstream region of the human prepro-orexin gene is utilized to express genes of interest in orexin neurons<sup>12,13</sup>. Prepro-orexin mRNA has been shown to be



**Fig. 1** Schematic representation of orexins and orexin receptors. Prepro-orexin is proteolyzed into two mature neuropeptides, orexin-A and orexin-B. Orexin-A acts on both OX<sub>1</sub>R and OX<sub>2</sub>R, while orexin-B mainly acts on OX<sub>2</sub>R. OX<sub>1</sub>R is coupled exclusively to the Gq subclass of G proteins, whereas OX<sub>2</sub>R is coupled to either Gi or Gq.

upregulated under fasting conditions<sup>10</sup>, and the forkhead box transcription factor *Foxa2*, a downstream target of insulin signaling, is reported to be involved in this transcriptional regulation<sup>14</sup>. These findings suggest that the activities of orexin neurons are regulated by metabolic balance.

Orexins bind two GPCRs, orexin receptor-1 and -2 ( $OX_1R$  and  $OX_2R$ ). Orexin-A acts on both  $OX_1R$  and  $OX_2R$ , while orexin-B acts selectively on  $OX_2R$ <sup>10</sup>. Although orexin neurons are localized within the LHA, they have widespread projections throughout the brain<sup>7,8</sup>, and orexin receptors are expressed in many regions. *In situ* hybridization of  $OX_1R$  and  $OX_2R$  shows differences in their distribution<sup>15</sup>.  $OX_1R$  mRNA is observed in the hippocampus, paraventricular thalamic nucleus (PVN), ventromedial hypothalamic nucleus, DR, and LC.  $OX_2R$  mRNA is found primarily in the cerebral cortex, hippocampus, DR, and many hypothalamic nuclei, including the PVN and TMN. Of these regions, the DR, LC, and TMN are well known to be involved in the onset of wakefulness. Orexin receptors are also found in many hypothalamic regions that are strongly implicated in the modulation of feeding, including the LHA, PVN, and the arcuate nucleus.

### INPUT TO OREXIN NEURONS

Orexin neurons not only send projections to various brain regions, but they also receive multiple innervations. Retrograde tracers, such as the nontoxic C-terminal fragment of tetanus toxin (TTC) and the cholera toxin B subunit (CT-B), were utilized to show synaptic connections involving orexin neurons. Sakurai *et al.* (2005) generated a transgenic mouse line expressing a green fluorescent protein (GFP)-labeled TTC. The construct was expressed exclusively in orexin neurons using the human prepro-orexin promoter. Using this mouse line, they identified several brain regions including the basal forebrain cholinergic neurons, GABAergic neurons in the ventrolateral preoptic nucleus, and serotonergic neurons in the median raphe and paramedian raphe nucleus<sup>16</sup>. The regions associated with emotion, including, the amygdala, and the shell region of the nucleus accumbens, and the bed nucleus of the stria terminalis (BST), were also found to innervate orexin neurons. Yoshida *et al.* (2006) injected CT-B into the LHA and counted labeled cells in rats. Projections from the lateral septum, preoptic area, BST, and posterior hypothalamus were identified. Interestingly, it was shown that hypothalamic regions preferentially innervate orexin neurons in the medial and perifornical parts of the field, and that most projections from the brainstem target the lateral part of the field<sup>17</sup>. This indicates that subpopulations of orexin neurons have different physiological functions.

Electrophysiological experiments have been used to identify factors that regulate orexin neurons. Recordings from transgenic mice expressing GFP in orexin neurons demonstrate that agonists of ionotropic glutamate receptors activate orexin neurons, while glutamate antagonists inhibit their activity<sup>18,19</sup>. These results indicate that orexin neurons are tonically activated by glutamate. In addition, monoamine neurotransmitters such as dopamine, noradrenaline, and serotonin (5-HT) hyperpolarize and inhibit orexin neurons via alpha 2-adrenergic and 5-HT1A receptors<sup>18,20,21</sup>. Other factors that reportedly influence the activity of orexin neurons include corticotrophin-releasing factor<sup>23</sup>, ATP<sup>24</sup>, neuropeptide Y<sup>25</sup>, and physiological fluctuations in acid and carbon dioxide levels<sup>26</sup>. It should be noted that factors involved in feeding (such as glucose, ghrelin, and leptin) inhibit the activity of orexin neurons<sup>27</sup>. The large variety of factors that regulate the activity of orexin neurons demonstrates the role of these neurons in diverse processes, such as circadian rhythms, energy balance, and vigilance level.

## FUNCTIONS IN SLEEP AND WAKEFULNESS

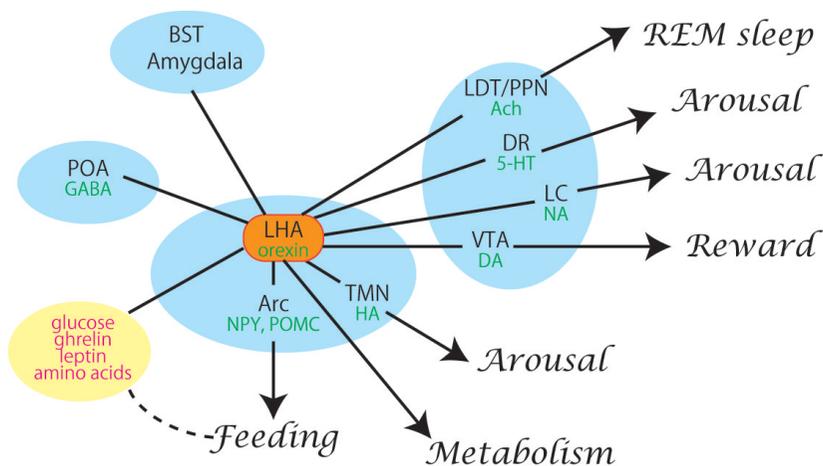
Among the multiple projections from orexin neurons, dense innervations to the DR, LC, and TMN are important for the regulation of sleep and wakefulness. Noradrenergic neurons of the LC<sup>28)</sup>, serotonergic neurons of the DR<sup>29,30)</sup>, and histaminergic neurons of the TMN<sup>31,32)</sup> are activated by orexins, and OX<sub>1</sub>R and/or OX<sub>2</sub>R are expressed in these regions. These findings suggest that the activity of monoaminergic neurons in the brainstem and the hypothalamus are at least partly regulated by orexins. Orexins also have a strong, direct, excitatory effect on cholinergic neurons of the basal forebrain<sup>33)</sup>, which is hypothesized to play an important role in arousal. Cholinergic neurons of the pedunculopontine tegmental nucleus (PPN) and the laterodorsal tegmental nucleus (LDT) play a pivotal role in the regulation of REM sleep and wakefulness. These areas are also strongly innervated by orexin neurons<sup>7,8)</sup>. Microinjection of orexin-A into the LDT increases awake time and decreases REM sleep in cats<sup>34)</sup>. Additionally, injection of orexin-A into the PPN in cats, causes the PPN to require increased stimulus to induce muscle atonia<sup>35)</sup>. It should be noted that the LDT and PPN contain neuronal subtypes other than cholinergic neurons that show activity associated with sleep/wake cycles<sup>36)</sup>.

The physiological importance of the orexin system in sleep is clearly demonstrated in genetically modulated mice. Prepro-orexin knockout mice<sup>2)</sup>, OX<sub>2</sub>R knockout mice<sup>3)</sup>, and orexin neuron-ablated transgenic mice (*orexin/ataxin-3* mice)<sup>1)</sup> show severe sleep fragmentation similar to narcolepsy. Additionally, it has been reported that the number of orexin neurons is greatly reduced and that orexin peptide levels are decreased to undetectable levels in the cerebrospinal fluid of narcoleptic patients<sup>4-6)</sup>. *Orexin/ataxin-3* mice are a well-studied model for narcolepsy; however, orexin neurons are absent from birth in *orexin/ataxin-3* mice. Therefore, other neuronal mechanisms likely compensate for the function of orexin neurons during development. Indeed, the frequency of cataplexy is low in these mice. To address this problem, timing-controlled neuronal ablation models, such as the tetracycline transactivator regulatable system, may be useful.

Recently, new techniques in optogenetics made it possible to manipulate the activity of orexin neurons in freely moving animals. Selective photostimulation of orexin neurons expressing channelrhodopsin-2 increases the probability of transition from non-REM or REM sleep to wakefulness<sup>37)</sup> and activates downstream wake-promoting nuclei such as the LC and TMN<sup>38)</sup>. Consistently, photoinhibition of orexin neurons expressing halorhodopsin induces non-REM sleep<sup>39)</sup>. Furthermore, optogenetic stimulation of the LC produces immediate sleep-to-wake transitions, whereas the inhibition causes a decrease in wakefulness<sup>40)</sup>. These findings indicate that the LC is a major effector of orexin neurons in the regulation of sleep and wakefulness. However, it should be noted that LC noradrenergic neurons only express OX<sub>1</sub>R, and that OX<sub>1</sub>R knockout mice show only weak fragmentation in sleep and no cataplexy<sup>41)</sup>.

Designer receptors exclusively activated by designer drugs (DREADDs) are another approach for regulating the activity of specific neuronal circuits *in vivo*. This method employs modified muscarinic receptors (hM3Dq for excitation, and hM4Di for inhibition) that have lost their affinity for endogenous acetylcholine but can be activated by the synthetic ligand, clozapine-N-oxide<sup>42,43)</sup>. Because stimulation of GPCRs has a longer effect on cellular signaling than optical stimulation, DREADDs are suitable for observing the chronic effects of modulating the activity of specific neurons. Using the DREADD technique, it was reported that excitation of orexin neurons significantly increases the amount of wakefulness time, and decreases both non-REM and REM sleep times. Additionally, it was shown that inhibition of orexin neurons decreases wakefulness time and increases non-REM sleep time<sup>44)</sup>.

## OREXIN NEURONS IN SLEEP AND WAKEFULNESS



**Fig. 2** A schematic diagram illustrating the integrative physiological roles of orexin neurons. Orexin neurons regulate the hypothalamic nuclei involved in feeding behavior. At the same time, they promote wakefulness through monoaminergic nuclei and other sleep-related nuclei in the brain stem. Energy levels influence orexin neuronal activity to coordinate arousal and energy homeostasis. Input from the limbic system may be important for regulating the activity of orexin neurons to evoke emotional arousal or fear-related responses. Abbreviations: 5-HT, serotonin; ACh, Acetylcholine; Arc, arcuate nucleus; BST, bed nucleus of the stria terminalis; DA, dopamine; DR, dorsal raphe nucleus; GABA, gamma-aminobutyric acid; HA, histamine; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; NA, noradrenaline; NPY, neuropeptide Y; POA, preoptic area; POMC, proopiomelanocortin; PPN, pedunculopontine tegmental nucleus; TMN, tuberomammillary nucleus; VTA, ventral tegmental area

## CONCLUDING REMARKS

In this review, we mainly discuss how orexin neurons regulate sleep and wakefulness, yet orexin neurons are also implicated in the reward system, the autonomic nervous system, stress response and feeding behavior (which are discussed in detail in other reviews<sup>45-47</sup>). Orexin neurons in the LHA provide an anatomical link between the limbic system, energy homeostasis, and brain stem monoaminergic, or cholinergic neurons. Similar to the hypothalamus where orexin neurons exist, orexin neurons monitor physiological conditions and coordinate various behaviors in response to environmental changes (Fig. 2). These findings show that the orexin system regulates vigilance states according to internal and external cues.

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