Review

Reverse pharmacology of orexin: from an orphan GPCR to integrative physiology

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Abstract

Orexins, which were initially identified as endogenous peptide ligands for two orphan G-protein coupled receptors (GPCRs), have been shown to have an important role in the regulation of energy homeostasis. Furthermore, the discovery of orexin deficiency in narcolepsy patients indicated that orexins are highly important factors for the sleep/wakefulness regulation. The efferent and afferent systems of orexin-producing neurons suggest interactions between these cells and arousal centers in the brainstem as well as important feeding centers in the hypothalamus. Electrophysiological studies have shown that orexin neurons are regulated by humoral factors, including leptin, glucose, and ghrelin as well as monoamines and acetylcholin. Thus, orexin neurons have functional interactions with hypothalamic feeding pathways and monoaminergic/cholinergic centers to provide a link between peripheral energy balance and the CNS mechanisms that coordinate sleep/wakefulness states and motivated behavior such as food seeking.

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1. Introduction

“Reverse pharmacology”, i.e., endogenous ligand screening using cell lines which express orphan G-protein coupled receptors (GPCRs), combined with genetic engineering techniques, has increased our understanding of novel signaling systems in the body [1]. Perhaps the most
successful example of such approach is discovery of orexin. Many works clearly suggested that orexins are highly important molecules in the regulation of sleep/wakefulness states as well as feeding behavior. This review summarizes recent relevant findings on orexins, and discusses physiological roles of these peptides.

2. Identification of orexins

Most neuropeptides work though G protein–coupled receptor (GPCRs). There are numerous (approximately 100–150) “orphan” GPCR genes in the human genome; the cognate ligands for these receptor molecules have not been identified yet. We performed a 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 ligand substances in tissue extracts that can activate signal transduction pathways in GPCR-expressing cell lines. In this process, we identified orexin-A and orexin-B as endogenous ligands for two orphan GPCRs found as human expressed sequence tags [2]. Orexins constitute a novel peptide family, with no homology with any previously described peptides.

Mammalian orexin-A is a 33-amino-acid peptide with two intrachain disulfide bonds, while mammalian orexin-B is a 28-amino-acid, C-terminally amidated linear peptide (Fig. 1B). Strong homology between orexin-A and orexin-B is found in their C terminal halves. Orexin-A and orexin-B are both derived from a common precursor (prepro-orexin), which is encoded by a gene composed of two exons and an intervening intron located at 17q21 in human [2,3]. Prepro-orexin is a 130–131-residue (depending on the species) polypeptide, which has a typical secretory signal sequence at its N-terminus, and is proteolytically cleaved to form mature orexin-A and -B (Fig. 1A). An mRNA encoding the same precursor peptide was independently isolated by de Lecea et al. as a hypothalamus-specific transcript [4]. They predicted that this transcript encodes two neuropeptides, named hypocretin-1 and -2. The names “hypocretin” and “orexin” are currently used as synonyms.

3. Orexin receptors

Two orexin receptor subtypes, which have 64% amino-acid identity with each other, named orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), have been identified in mammals [2]. We initially identified orexin peptides using cells expressing OX1R, which has a 1-order-of-magnitude greater affinity for orexin-A compared with orexin-B. BLAST search for EST data bases with OX1R sequence as a query led us identification of another subtype of orexin receptor, OX2R, to which both orexin-A and orexin-B bind with similar affinity (Fig. 1A). OX1R is coupled exclusively to the Gq subclass of heterotrimeric G proteins, whereas OX2R is coupled to both Gi/o and Gq when expressed in cell lines [5].

In situ hybridization studies have demonstrated that orexin receptors are expressed in regions in which dense orexin immunoreactive fibers are observed (discussed below). OX1R and OX2R show a markedly different and complementary distribution [6]. For instance, within the hypothalamus, a low level of OX1R mRNA expression is observed in the dorsomedial hypothalamus (DMH), while high level of OX2R mRNA expression is observed in this region. Other areas of OX2R expression include the arcuate nucleus, paraventricular nucleus (PVN), LHA, and most significantly, the tuberomammillary nucleus (TMN) [6]. In these regions, there is little or no OX1R signal. In the hypothalamus, OX1R mRNA is abundant in the anterior hypothalamic area and ventromedial hypothalamus (VMH). Outside the hypothalamus, high levels of OX1R mRNA expression are detected in the tenia tecta, hippocampal formation, dorsal raphe nucleus, and most prominently, the locus coeruleus (LC). OX2R 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, OX1R is most abundantly expressed in the
LC, while OX2R is most abundantly expressed in the TMN, regions highly important for maintenance of arousal.

4. Orexin-producing neurons

Orexin-producing neurons (orexin neurons) are exclusively localized to the LHA [7–9]. These cells diffusely project to the entire neuroaxis, excluding the cerebellum [7–9] (Fig. 2). The densest staining of orexin-immunoreactive nerve endings in the brain was found in the arcuate nucleus of the hypothalamus, raphe nuclei, TMN and LC. Together with the tissue distribution of both orexin receptors, these observations suggest that these regions are major effector sites of orexin.

Melanin-concentrating hormone (MCH) neurons show very similar localization with orexin neurons in the LHA [10]. However, orexin and MCH neurons are distinct and independent neuronal populations [11,12]. Orexin does not colocalize with cocaine and amphetamine-regulated transcript (CART) or nitric oxide synthase, either [13]. However, orexin colocalizes with dynorphin [14], galanin [15], and glutamate [16]. Recently, Eriksson et al. [17] reported that dynorphin suppressed GABAergic input and thus disinhibited histaminergic neurons in the TMN. Therefore, co-localized dynorphin and orexin might synergistically activate TMN histaminergic neurons [17].

5. Orexin deficiency in narcolepsy

The importance of orexin in the regulation of sleep/wakefulness is highlighted by the discovery of orexin deficiency in human narcolepsy patients [18,19]. Approximately 90% of patients with narcolepsy show decreased orexin-A levels in the cerebrospinal fluid [20]. Narcolepsy is a common sleep disorder characterized by a primary disorganization of behavioral states. This disorder affects approximately 1 in 2000 individuals in the United States. Most cases of human narcolepsy usually start during adolescence. A cardinal symptom of the disorder is excessive daytime sleepiness (an insurmountable urge to sleep), manifested particularly as attacks of somnolence at inappropriate times. Nocturnal sleep is also sometimes disturbed by hypnagogic hallucinations, vivid dreaming, and sleep paralysis. Narcolepsy patients often suffer from cataplexy, which is an attack characterized by sudden muscle weakness, which can range from jaw dropping and speech slurring to a complete bilateral collapse of the postural muscles. These attacks of muscle weakness are most often triggered by strong emotional stimuli. Consciousness is preserved during cataplexy. The latency for REM sleep is notably reduced in narcolepsy patients, and the presence of sleep-onset REM period is a diagnostic criterion for narcolepsy. It is generally accepted that the symptoms of narcolepsy can be considered as a pathological intrusion of factors of sleep, and especially REM sleep-related phenomena, into the state of wakefulness. Thus, narcolepsy can be viewed as a behavioral state boundary disorder [21].

The clues suggesting the possible involvement of orexin in narcolepsy initially came from animal models; mice lacking either the orexin gene (prepro-orexin knockout mice) or orexin neurons (orexin/ataxin-3 transgenic mice), as well as mice and dogs with null mutations in the OX2R gene, all have phenotypes remarkably similar to narcolepsy [22–25] (Table 1). Prepro-orexin knockout mice and orexin/ataxin-3 mice showed a similar phenotype, which is...
characterized by behavioral arrest similar to cataplexy, occasional direct transition to REM sleep from wakefulness, and highly fragmented behavioral states, resulted from behavioral instability [23,24] (Fig. 3).

Consistently, a postmortem study in narcoleptic subjects showed undetectable levels of orexin peptides in projection sites such as the cortex and pons and an 80–100% reduction in the number of orexin-containing neurons in the hypothalamus [18,19], supporting an earlier report showing undetectable CSF orexin-A peptide levels in most narcolepsy patients [26]. These results suggest either a loss of orexin-containing neurons or a lack of orexin production in these neurons if still present. One of the predisposing factors is a specific class II HLA haplotype on human chromosome 6, with HLA DQB1*0602 and DQA1*0102 alleles, which were found in more than 85% of all narcoleptic patients.

Table 1
Rodent narcolepsy models produced by genetic engineering

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Reference</th>
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<tr>
<td>Prepro-orexin knockout</td>
<td>[23]</td>
</tr>
<tr>
<td>Sleep/wake fragmentation (severe)</td>
<td></td>
</tr>
<tr>
<td>OX1R knockout</td>
<td>[43]</td>
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<tr>
<td>Cataplexy (+), sleep attack (+)</td>
<td></td>
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<tr>
<td>Sleep/wake fragmentation (mild)</td>
<td></td>
</tr>
<tr>
<td>OX2R knockout</td>
<td>[25]</td>
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<tr>
<td>Cataplexy (+), sleep attack (+)</td>
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<tr>
<td>Sleep/wake fragmentation (severe)</td>
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<tr>
<td>Orexin/ataxin-3 mouse</td>
<td>[24]</td>
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<td>Cataplexy (+), sleep attack (+)</td>
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<td>Sleep/wake fragmentation (severe)</td>
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<tr>
<td>Orexin/ataxin-3 rat</td>
<td>[56]</td>
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<tr>
<td>Cataplexy (+), sleep attack (+)</td>
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<td>Sleep/wake fragmentation (severe)</td>
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Fig. 3. Summary of vigilance state parameters recorded from orexin/ataxin-3 hemizygous transgenic and wild-type littermate control (WT) mice. Upper panels: Total time spent in each state (minutes, mean±S.E.M.), itemized separately for light (left panel) and dark (right panel) periods. Lower panels: Mean episode duration of each vigilance state observed in light (left panel) and dark (right panel) periods. Significant differences (P<0.05; repeated measurements ANOVA) between Tg and WT mice are indicated with an asterisk.
[27]. This suggests a possibility that narcolepsy may result from selective autoimmune degeneration of orexin neurons. The decreased CSF orexin-A peptide level in most narcolepsy patients also suggests that measuring CSF orexin-A might be a definitive diagnostic test [28].

Since narcolepsy is a disorder of sleep–wake cycle organization resulting from absence of orexin, replacement therapy using orexin receptor agonists may provide treatment of narcolepsy. Our recent study showed that chronic over-production of both orexin-A and orexin-B peptides from an ectopically expressed transgene prevented the development of narcolepsy syndrome in orexin neuron-ablated (orexin/ataxin-3) mice [29]. Similarly, acute administration of orexin-A maintained wakefulness, suppressed sleep, and inhibited cataplectic attacks in narcoleptic mice [29]. Together, these findings provide strong evidence for a specific relationship between absence of orexin peptides in the brain and the development of narcolepsy syndrome.

6. Mechanisms of regulation of behavioral states by orexins

The finding of orexin deficiency in narcoleptic patients suggests that orexin has an important role in the normal regulation of sleep/wakefulness. Orexin neurons might be especially important for stabilization of behavioral states, because the major symptom in narcolepsy is inability to maintain each behavioral state, which results in sleep/wakefulness fragmentation. When orexin-A was injected intracerebroventricularly into rats during the light period, it caused increased wakefulness time and decreased REM and non-REM sleep time [30]. In rats, Fos expression of orexin neurons is increased during the dark active period [31], and orexin level in cerebrospinal fluid also peaks during the dark period and decreases during the light rest period [32]. These observations suggest that orexin neurons are active during the active period and support wakefulness, and are inactive during the sleep period. The activities of the monoaminergic neurons in the brain stem are also synchronized and strongly associated with behavioral states: they fire tonically during wakefulness, less during non-REM sleep, and not at all during REM sleep [33]. This regulation might be at least in part by orexin neurons, which are also wake-active, because orexin neurons project to and excite histaminergic neurons in the TMN, noradrenergic neurons in the LC and serotoninergic neurons in the dorsal raphe (DR) [7–9], and the presence of OX1R in the LC and OX2R in TMN and both receptors in the DR has been confirmed [6]. Consistent with this hypothesis, isolated cells from these nuclei are all activated by orexins in vitro [30,34,35]. Orexins also have a strong direct excitatory effect on cholinergic neurons of the basal forebrain [36], which is hypothesized to play an important role in behavioral and electrocortical arousal [37]. These observations suggest that orexin neurons are active during the wake period, and exert an excitatory influence on the basal forebrain cholinergic neurons and monoaminergic neurons in the brain stem to maintain arousal. In addition, orexin neurons also appear to act on LDT/PPT cholinergic neurons, because orexin neurons project directly to the PPT/LDT nuclei and direct injection of orexin-A into the LDT of cats results in an increase in wakefulness and a decrease in REM sleep [38]. In addition, several reports showed that orexin induces long-lasting excitation of cholinergic neurons in the LDT [39]. However, a group of cholinergic neurons in the LDT/PPT, which are silent during wakefulness and active in the REM period, constitute a system to induce and maintain REM sleep [40]. Therefore, orexin neurons might not exert a direct excitatory influence on these cells during wakefulness. Orexin neurons might activate another type of cholinergic neurons in the PPT and LDT, which are active in wakefulness as well as the REM-sleep period. Recent work also shows that orexin inhibits cholinergic neurons in the PPT via activation of GABAergic local interneurons and GABAergic neurons in the substantia nigra pars reticulata [41]. These results suggest that hypothalamic orexin neurons affect the activity of LDT/PPT cholinergic neurons directly and/or indirectly to appropriately regulate the activity of these cells to control behavioral states.

Several reports showed that the effect of orexin on wakefulness is largely mediated by activation of the histaminergic system mediated through OX2R. In rats, i.c.v. injection of orexin during the light period potently increases the wake period, and this effect is markedly attenuated by the H1 antagonist, pyrilamine [35]. Furthermore, the effect of orexin-A on wakefulness in mice is almost completely absent in H1-receptor deficient mice [42]. Furthermore, OX2R knockout mice exhibit a narcoleptic phenotype, while OX1R knockout mice show only mild fragmentation of behavioral states [43]. Because OX2R is strongly expressed in the TMN, while OX1R is strongly expressed in the LC, the TMN seems to be an important effector site of orexin for sleep/wakefulness regulation. Orexin neurons also contain an additional neurotransmitter, dynorphin [14]. Dynorphin in orexin neurons might inhibit GABAergic input to TMN neurons, and thus act in concert with orexin to increase the excitability of these neurons [17].

However, several findings indicate that signaling through OX1R is also important for the regulation of wakefulness. As mentioned before, OX2R knockout mice exhibit characteristics of narcolepsy [25]. Interestingly, OX1R knockout mice do not have any overt behavioral abnormalities and exhibit only mild fragmentation of behavioral states [43]. However, the behavioral and electroencephalographic phenotype of OX2R knockout mice is less severe than that found in prepro-orexin knockout mice and double receptor knockout (OX1R- and OX2R-null) mice, which appear to have the same phenotype as prepro-orexin knockout mice. These observations suggest that OX1R also has additional effects on sleep/wakefulness regulation. These findings suggest that despite the lack of an overt OX1R phenotype,
loss of signaling through both receptor pathways is necessary for severe narcoleptic phenotype.

Willie et al. [25] classified, using behavioral, electrophysiological, and pharmacological criteria, two distinct classes of behavioral arrest exhibited by mice deficient in orexin-mediated signaling. Both OX2R and prepro-orexin knockout mice are similarly affected with behaviorally abnormal attacks of non-REM sleep (“sleep attacks”) and show similar degrees of disrupted wakefulness. In contrast, OX2R knockout mice are only mildly affected with cataplexy-like attacks of REM sleep, whereas orexin knockout mice are severely affected. Absence of OX2R eliminates orexin-evoked excitation of histaminergic neurons in the hypothalamus, which gate non-REM sleep/wake transition. While normal regulation of wake/non-REM sleep transition depends critically upon OX2R activation, the profound dysregulation of REM sleep control unique to the narcolepsy syndrome emerges from loss of signaling through both OX2R-dependent and OX1R-dependent pathways in mice.

Thus, orexin neurons may be active during wakefulness, helping sustain activity in monoaminergic arousal regions [31], which in turn send inhibitory input to the ventrolateral preoptic area (VLPO) sleep-active neurons, and thereby further maintain wakefulness [44]. In the absence of orexin, these arousal regions may have reduced or nonsynchronized activity, resulting in an inappropriately low threshold for transition into non-REM sleep. Narcoleptic mice also have fragmented non-REM sleep; orexin-deficient mice have more frequent transitions between all states, as has been noted in human narcolepsy. Therefore, orexin neurons might also be necessary for maintenance of non-REM sleep.

7. Regulation of orexin neuronal activity

Until recently, little was known about the factors that influence the activity of orexin neurons. Recent electrophysiological studies have identified several activators and inhibitors of orexin neurons. By recording from hypothalamic slices of transgenic mice expressing green fluorescent protein (GFP) only in orexin neurons, it was shown that agonists of ionotropic glutamate receptors (AMPA and NMDA) excite orexin neurons, whereas glutamate antagonists (AP-5, CNQX or NBQX) reduce their activity [45]. These results indicate that orexin neurons are tonically activated by glutamate. Although orexin has little direct effect on the activity of orexin neurons, it increases this glutamate signaling by acting on presynaptic terminals [45]. This mechanism may reinforce and coordinate the activity of orexin neurons in the LHA.

Several researchers have hypothesized that monoamines excite orexin neurons, forming positive feedback loops that would maintain wakefulness [21], but our electrophysiological studies showed just the opposite; both noradrenaline and serotonin hyperpolarize and inhibit GFP-expressing orexin neurons [45,46]. Histamine has no effect on orexin neurons. It seems strange that wake-active monoaminergic areas would inhibit wake-active orexin neurons. Therefore, we hypothesize that orexin neurons might be innervated by monoaminergic cells in regions...
other than the wake-active regions. To evaluate this hypothesis, precise retrograde mapping study of afferent neurons to orexin neurons is required.

8. Roles of orexins in the regulation of energy homeostasis

The altered energy homeostasis in human narcolepsy patients suggests roles of orexin in regulation of energy homeostasis [47,48]. The finding of decreased caloric intake [49] combined with an increased body mass index [47] suggests that narcolepsy patients have a feeding abnormality with reduced energy expenditure or low metabolic rate, and orexin neurons have a role in the regulation of energy homeostasis. Consistently, orexin neuron-ablated mice show hypophagia and late-onset obesity [24].

Electrophysiological studies on orexin neurons showed that, in addition to monoamines and acetylcholine, peripheral humoral factors related to energy metabolism also influence the activity of orexin neurons; activity of isolated orexin neurons is inhibited by glucose and leptin, and stimulated by ghrelin [50]. Consistently, orexin expression of normal and ob/ob mice correlates negatively with changes in blood glucose, leptin, and food intake. These findings are consistent with our original idea that orexins have a role in the regulation of feeding and energy homeostasis [2].

9. Orexin: a link between energy homeostasis and arousal

Proper maintenance of arousal during food search and intake of an animal is essential for its survival. Therefore, the two vital processes, feeding and sleep/wake behavior, have to be appropriately coordinated. When faced with a negative energy balance due to reduced food availability, mammals respond behaviorally with phases of increased wakefulness and locomotor activity that support food seeking [51–55]. The discovery that orexin neurons are regulated by peripheral metabolic cues suggests that orexin neurons might have important roles in the molecular and physiological basis of this phenomenon. During starvation, orexin neurons might be activated by low leptin and glucose levels, along with high ghrelin level. These mechanisms may directly modulate activity of orexin neurons according to appetite and body energy stores. Indeed, we found that transgenic mice, in which orexin neurons are ablated, fail to respond to fasting with increased wakefulness and activity [50].

These findings indicate that orexin neurons provide a crucial link between energy balance and arousal (Fig. 4). These properties might allow the orexin neurons to promote alertness in a hungry animal and maintain long periods of wakefulness throughout the day. These mechanisms may be important in maintenance of prolonged wakefulness during the active period, and in the regulation of energy homeostasis that helps to ensure survival in nature, but may counteract attempts to treat obesity by food restriction.

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