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Review

Orexin neuronal circuitry: Role in the regulation of sleep and wakefulness

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Abstract

Orexin A and orexin B were initially identified as endogenous ligands for two orphan G protein-coupled receptors [104]. They were initially recognized as regulators of feeding behavior in view of their exclusive production in the lateral hypothalamic area (LHA), a region known as the feeding center, and their pharmacological activity [104,30,49,107]. Subsequently, the finding that orexin deficiency causes narcolepsy in humans and animals suggested that these hypothalamic neuropeptides play a critical role in regulating sleep/wake cycle [22,46,71,95,117]. These peptides activate waking-active monoaminergic and cholinergic neurons in the hypothalamus/brain stem regions to maintain a long, consolidated awake period.

Recent studies on efferent and afferent systems of orexin neurons, and phenotypic characterization of genetically modified mice in the orexin system further suggested roles of orexin in the coordination of emotion, energy homeostasis, reward system, and arousal [3,80,106,137]. A link between the limbic system and orexin neurons might be important for increasing vigilance during emotional stimuli. Orexin neurons are also regulated by peripheral metabolic cues, including ghrelin, leptin, and glucose, suggesting that they might have important roles as a link between energy homeostasis and vigilance states [137]. Recent research has also implicated orexins in reward systems and the mechanisms of drug addiction [13,48,91]. These observations suggest that orexin neurons sense the outer and inner environment of the body, and maintain proper wakefulness of animals for survival. This review discusses the mechanism by which orexins maintain sleep/wakefulness states, and how this mechanism relates to other systems that regulate emotion, reward, and energy homeostasis.

Keywords: Orexin; Hypocretin; Sleep; Wakefulness; Arousal; Hypothalams; Emotion; Reward; Narcolepsy

1. History of orexin (hypocretin)

Perhaps the most intriguing and successful example of 'reverse pharmacological' approach relates to orexin. Much work has clearly suggested that the orexin system regulates sleep and wakefulness through interactions with systems that regulate emotion, reward, and energy homeostasis.

In 1998, identifications of orexin/hypocretin were reported by two independent groups utilizing completely different strategies. De lecea et al. utilized a molecular biological technique to isolate a series of cDNA clones that are expressed in the hypothalamus by subtractive hybridization [28]. Sakurai et al. used 'reverse-pharmacological' approach, ligand screening using recombinantly expressed orphan receptors [104]. Sakurai et al. showed that intracerebroventricular (ICV) administration of orexin A or orexin B in the lateral ventricle increased food intake in rats [104].

In 1999, two independent studies utilizing dog forward genetics and mouse reverse genetics, respectively, suggested the importance of orexin in the regulation of sleep/wakefulness. Lin et al. identified mutations in the *orexin-2 receptor* gene responsible for canine narcolepsy–cataplexy by positional cloning [71]. Chemelli et al. reported that *prepro-orexin* knockout mice exhibited a phenotype strikingly similar to human narcolepsy–cataplexy [22]. Human

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narcolepsy–cataplexy is a common sleep disorder characterized by excessive daytime sleepiness, premature transitions to REM sleep (sleep-onset REM) and cataplexy (sudden skeletal muscle weakness without impairment of consciousness).

Disruptions of the orexin system in human narcolepsy–cataplexy were confirmed in 2000. Nishino et al. found that orexin A (hypocretin-1) was undetectable in the cerebrospinal fluid (CSF) of up to 95% of narcolepsy–cataplexy patients [92]. Drastic reductions of *orexin* mRNA and immunoreactivity in postmortem brains of narcolepsy–cataplexy patients were also shown by Peyron et al. [95] and Thannickal et al. [117].

In 2003, Willie et al. demonstrated that distinct roles of each orexin receptor subtype in the regulation of sleep and wakefulness [127]. Yamanaka et al. indicated that orexin neurons are activated under the negative energy balance, and are necessary to increase arousal to look for meal [137].

In 2004, Mieda et al. showed that ectopic expression of a *prepro-orexin* transgene or administration of orexin A in the brain of orexin neuron-ablated mice suppressed the narcoleptic phenotype of these mice [81].

In 2005–2006, neuronal populations that send afferent projections to orexin neurons were identified. Sakurai et al. utilized the mice with a genetically encoded retrograde tracer [106], while Yoshida et al. mapped afferent neurons by combination of retrograde and anterograde tracers [141]. Single unit recordings from orexin neurons by Mileykovskiy et al. [85] and Lee et al. [68] indicated that orexin neurons have very low discharge levels in both REM and slow-wave sleep. In contrast, alert waking was associated with a high discharge rate. Important roles of the orexin neuronal system in reward processing and addiction were indicated by several reports [13,48,91,11].

In 2007, Brisbare-Roch et al. showed that a orally administered orexin antagonist (ACT-078573) that selectively blocks both orexin receptors at nanomolar concentrations is affective as a sleep inducer [14].

2. Orexin and orexin receptors

2.1. Identification of orexin (hypocretin)

Most neuropeptides work though G protein-coupled receptors (GPCRs). There are numerous (approximately 100–120) "orphan" GPCR genes in the human genome. We applied an approach, so called "reverse pharmacology", that aims to identify ligands for these 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. In this process, we identified orexin A and orexin B as endogenous ligands for two orphan GPCRs found as human expressed sequence tags (ESTs) [104]. This approach is now widely accepted as "reverse pharmacology", which has been successfully applied to identify several novel biologically active substances and their receptors [130].

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 Da, with an N-terminal pyroglutamyl residue and C-terminal amidation (Fig. 1a). The four Cys residues of orexin A formed two sets of intrachain disulfide bonds. 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 Da, which is 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, while 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. Other than mammalian species, the structures of fish, xenopus, and chicken orexin A and orexin B, which have also conserved structures as compared with mammalian sequences, have been elucidated (Fig. 1a) [58,93,111].

Both orexin A and orexin B are produced from a common 130-residue (rodent) or 131-residue (human) polypeptide, prepro-orexin. The human and mouse prepro-orexin sequences are 83% and 95% identical to the rat counterpart, respectively [105]. The majority of amino acid substitutions was found in the C-terminal part of the precursor, which appears unlikely to encode another bioactive peptide.

An mRNA encoding the same precursor peptide was independently isolated by de Lecea et al. as a hypothalamus-specific transcript [28]. They predicted that this transcript encodes two neuropeptides, named hypocretin-1 and -2. The names "hypocretin" and "orexin" are currently used as synonyms.

2.2. Prepro-orexin gene and promoter

The human *prepro-orexin* gene, which is located on chromosome 17q21, consists of two exons and one intron distributed over 1432 bp [105]. 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 122-bp 5′-non-coding region of exon 1, was reported to have the 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 [105]. This promoter is useful to examine the consequences of expression of exogenous molecules in

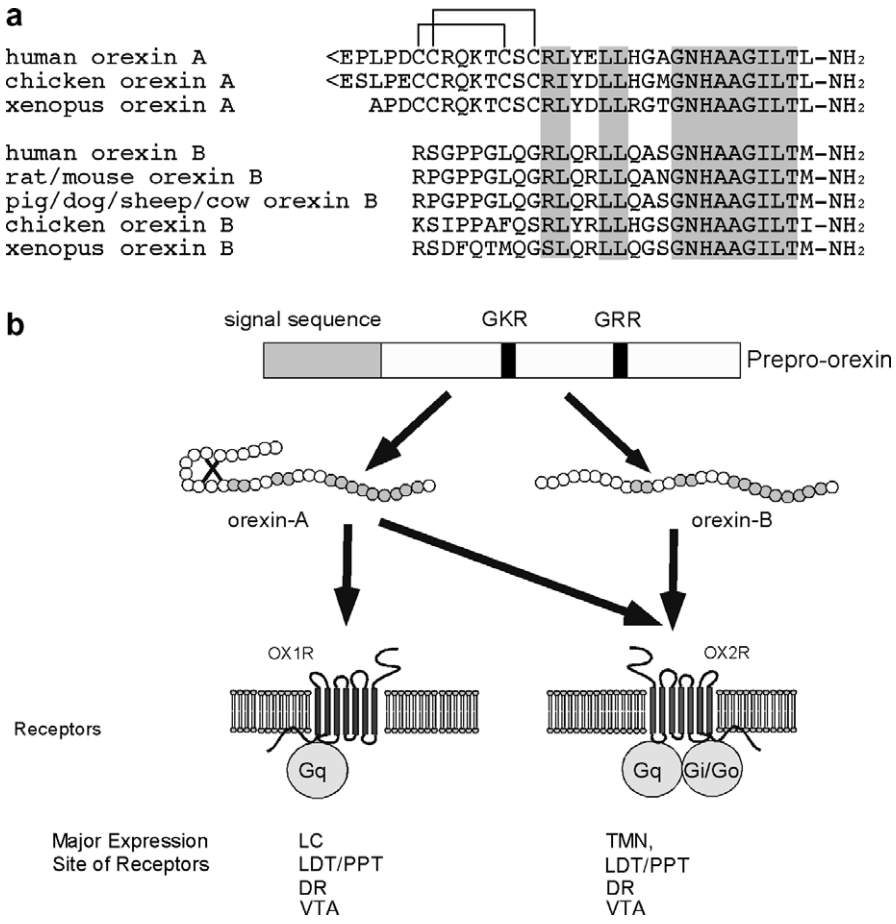

Fig. 1. Overview of orexin system. (a) Structures of mature orexin A and orexin B peptides. The topology of the two-intrachain disulfide bonds in orexin A is indicated above the sequence. Shadows indicate amino acid identity. Mammalian orexin A sequences thus far identified (human, rat, mouse, pig, dog, sheep, and cow) are all identical. (b) Schematic representation of orexin system. Orexin A and orexin 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. OX_1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX_2R couples to $G_{i/o}$ and/or G_q. OX_1R is abundantly expressed in the locus coeruleus (LC), while OX_2R is abundantly expressed in the tuberomammillary nucleus (TMN) and both receptors are expressed in laterodorsal/pedunculopontine tegmental nucleus (LDT/PPT), dorsal raphe nucleus (DR) and ventral tegmental area (VTA).

orexin neurons of transgenic mice, thereby manipulating the cellular environment in vivo. For example, this promoter was used to establish several transgenic lines, including orexin neuron-ablated mice and rats [46,9], mice in which orexin neurons specifically express green fluorescent protein [138] or calcium-sensitive fluorescent protein (yellow cameleon Yc2.1) [120], and mice with specific expression of cre recombinase in orexin neurons (our unpublished observation).

The regulation of expression of the *prepro-orexin* gene still remains unclear. *Prepro-orexin* mRNA was shown to be up-regulated under fasting conditions, indicating that these neurons somehow sense the animal's energy balance [104]. Several reports have shown that orexin neurons express leptin receptor- and STAT-3-like immunoreactivity, suggesting that orexin neurons are regulated by leptin [45]. Consistently, we found that continuous infusion of leptin into the third ventricle of mice for 2 weeks resulted in marked down-regulation of *prepro-orexin* mRNA [46]. Therefore, reduced leptin signaling may be a possible factor that up-regulates expression of *prepro-orexin* mRNA during starvation. *Prepro-orexin* level were also increased in hypoglycemic conditions, suggesting that expression of the *prepro-orexin* gene is also regulated by plasma glucose level [42]. These observations are consistent with our electrophysiological study of GFP-expressing orexin neurons in transgenic mice showing that orexin neurons are regulated by extracellular glucose concentration and leptin [137]. The physiological relevance of this mechanism will be discussed later.

2.3. 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. 1b) [104]. Among various classes of G protein-coupled receptors, OX_1R is structurally 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).

There is 64% amino acid identity between the deduced full-length human OX_1R and OX_2R sequences. Thus, these receptors are much more similar to each other than they are to other GPCRs. The amino acid identity between the human and rat homologues of each of these receptors is 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 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 nM. Human orexin B also acted as a specific agonist on CHO cells expressing OX_1R. However, human orexin B has significantly lower affinity compared to human OX_1R: the calculated IC_{50} in competitive binding assay was 250 nM for human orexin B, indicating two orders of magnitude lower affinity as 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 IC_{50} of 20 nM. Orexin A also had high affinity for this receptor with IC_{50} of 20 nM, which is similar to the value for orexin B, suggesting that OX_2R is a non-selective receptor for both orexin A and orexin B (Fig. 1b).

2.4. Distribution of orexin receptors

Although orexin receptors are expressed in a pattern consistent with orexin projections, mRNA for OX_1R and OX_2R were differentially distributed throughout the brain. Within the hypothalamus, a low level of OX_1R mRNA expression is observed in the dorsomedial hypothalamus (DMH), while a high 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), LHA, and most significantly, the tuberomammillary nucleus (TMN) [78]. 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 notably, the locus coeruleus (LC). OX_2R mRNA is expressed in the cerebral cortex, nucleus accumbens, subthalamic nucleus, paraventricular thalamic nuclei, anterior pretectal nucleus, and the raphe nuclei.

LC neurons exclusively express OX_1R, whereas TMN neurons exclusively express OX_2R. The raphe nuclei and ventral tegmental area (VTA) contain both receptor mRNAs. These observations suggest that strong interaction between orexin neurons and the monoaminergic systems and each receptor play a different role in arousal system (Fig. 3).

2.5. Signal transduction system of orexin receptors

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 rectifier potassium channels (GIRKs or Kir3 channels),

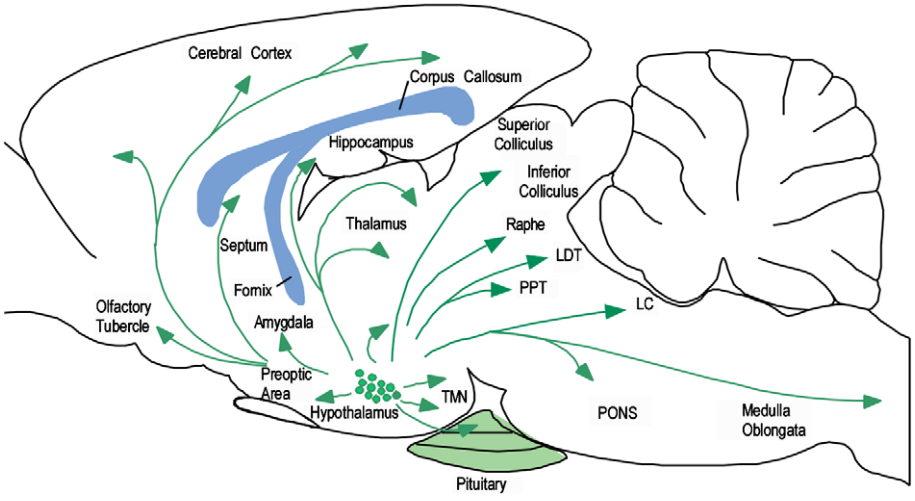

Fig. 2. Schematic drawing of sagittal section of rat brain, summarizing the organization of the orexin neuronal system. Orexin neurons are found only in the lateral hypothalamic area and project to the entire central nervous system. Abbreviations: 3V, third ventricle; TMN, tuberomammillary nucleus; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus.

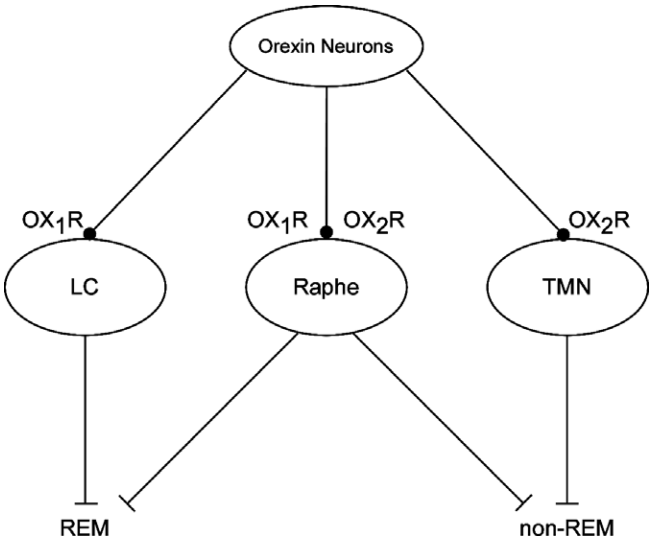

Fig. 3. Regulation of vigilance states by orexin neurons through two orexin receptors. Noradrenergic neurons in the LC express OX_1R, and histaminergic neurons in the TMN express OX_2R, while serotonergic neurons in the DR express both receptor subtypes. Orexin neurons in the LHA send excitatory projections to these monoaminergic neurons. Studies in KO mice suggest that activation of TMN histaminergic neurons via OX_2R is crucial for maintenance of arousal and gating of non-REM sleep, while both receptors are involved in inhibition of REM sleep.

there might be a substantial degree of selectivity in the coupling to one or other of these channels in neurons (Fig. 1b). 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, pertussis toxin (PTX) treatment did not show any effect on orexin-induced increase in $[Ca^{2+}]_i$. These results suggest that OX_1R does not couple to PTX-sensitive $G_{i/o}$ proteins [147]. 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, the orexin-induced increase in $[Ca^{2+}]_i$ was not affected by PTX treatment in OX_2R-expressing cells. These results indicate that OX_2R couples to PTX-sensitive G proteins, which were involved in the inhibition of adenylyl cyclase by orexin. These results 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.

In vivo studies have shown that orexins have an excitatory activity in monoaminergic neurons. For instance, noradrenergic cells of the LC [44], dopaminergic cells of the ventral tegmental area [89], and histaminergic cells from the TMN [136] have been shown to be activated by orexins. Because LC neurons exclusively express OX_1R, while 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. reported that orexin increases local glutamate signaling by facilitation of glutamate release from presynaptic terminals [69]. It is also possible that G_i-coupled OX_2R might inhibit neurotransmitter release in axonal terminals.

2.6. Structure–activity relationship of orexins and orexin receptor antagonists

Solution structure of human orexin A and orexin B contain two α-helices at C-terminal region [60,114,67]. Orexin A have two disulfide bonds at N-terminal half region and these induce a hydrophilic turn. Structure–activity analysis of truncated orexin A analogues at OX_1R reveal that the hydrophobic C-terminus of orexin A are critical for functional potency at OX_1R [26,64]. Especially, a minimum C-terminal sequence of 19 residues is required for a significant agonist effect and residues 15 through 25 may play an important conformational role in receptor interaction. 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 [7]. The ability of N- or C-terminally truncated analogs of orexin B to increase cytoplasmic Ca^{2+} level in cells showed that the absence of N-terminal residues has 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 results in moderate loss of activity, in the order of peptide length. In particular, deletion of the conserved sequence between orexin A and orexin B causes a profound loss of biological activity, and C-terminally truncated peptides were also inactive for both receptors. These findings suggest that the conserved sequence between orexin A and orexin B is important for the activity of both receptors.

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) decreases the activity, just as C-terminal truncation does. 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 (15–33), the C-terminal half of orexin A, and orexin B (10–28) have similar sequences; however, their selectivity to OX_1R and OX_2R is different. This finding indicates that the activity and the ligand/receptor selectivity are also related to the C-terminal half of the orexin sequence.

SB-334867 is a selective OX_1R antagonist [101]. Administration of SB-224867 (30 mg/kg, i.p.) induced reduction of food intake and active behaviors, while increasing resting. Moreover, SB-334867 (20–30 mg/kg, i.p.) blocked reward-seeking effect induced by orexin A [13,48,91]. Takai

et al. indicated that two regions in orexin A appear to resemble SB334867 [114]. First region is Tyr17 and His21 and second region is Pyr1 and Pro2. Since the C-terminal region (15–33) of orexin A is required for binding to OX_1R and activating it, similarity between SB-334867 and orexin A might be long on the selectivity for OX_1R but short on the activating it.

Recently, Brisbare-Roch, C. et al. reported a new orexin antagonist (ACT-078573) which specifically blocks both orexin receptors [14]. This drug is orally active and can across the blood–brain barrier. When the dual antagonist administered orally during the active period, in rats, dogs, and humans, it caused somnolence without cataplexy.

3. Orexin neuronal system

Orexin-producing neurons (orexin neurons) are located exclusively in the lateral posterior hypothalamic regions, including the perifornical area, lateral hypothalamus, and posterior hypothalamus. The lateral hypothalamus has classically been implicated in a wide variety of behavioral and homeostatic regulatory systems, and thus the localization of orexin-expressing neurons has generated hypotheses as to their physiological relevance [33,8].

Within the rodent brain, in the rostral to caudal plane, the orexin neuronal field extends from just caudal to the paraventricular hypothalamic nucleus to just rostral to the tuberomammillary nucleus. In the medial to lateral plane, while orexin neurons encroach medially through the dorsomedial nucleus as far as the third ventricle and laterally as far as the optic tracts, the majority of orexin neurons reside within the perifornical area.

These neurons are variable in size (cell body diameter of 15–40 μm) and shape (spherical, fusiform, and multipolar), and have been assumed to number around 3000 in the whole rat brain and 50,000 in the human brain. From these regions, these cells widely project to the entire neuroaxis, excluding the cerebellum [27,90,94] (Fig. 2). The densest staining of orexin-immunoreactive nerve endings in the brain was found in the paraventricular nucleus of the thalamus, arcuate nucleus, locus coeruleus, and tuberomammillary nucleus in rodent brain. Together with the tissue distribution of both orexin receptors, these observations suggest that these regions are major effector sites of orexins.

It appears that neurons expressing *prepro-orexin* are likely to contain both orexin A and orexin B peptides. Orexin peptides localize within secretory vesicles, implying that both orexin A and orexin B are co-released at terminals [27]. There is general agreement on immunohistochemical staining for prepro-orexin, orexin A, and orexin B, further implying that both orexin peptides are produced in orexin-containing neurons.

Melanin-concentrating hormone (MCH) neurons show very similar localization to orexin neurons in the LHA [10]. However, orexin and MCH neurons are distinct and independent neuronal populations [15,32]. Thus, the orexin- and MCH-expressing cells comprise two neurochemically unique neuronal populations in the lateral hypothalamus, both of which have been suggested to play a role in body weight homeostasis [33,97]. Notably, the orexin population is distinct from yet a third neuronal population within the lateral hypothalamus, those neurons that express neuronal nitric oxide synthase (nNOS) [24]. Orexins do not colocalize with cocaine and amphetamine-regulated transcript (CART) or nitric oxide synthase, either [24]. On the other hand, orexin colocalizes with dynorphin [23], galanin [100], neuronal activity-regulated pentraxin (NARP) [99], and glutamate [1]. Dynorphin suppresses GABAergic input and thus disinhibits histaminergic neurons in the TMN [34]. Therefore, co-localized dynorphin and orexin might synergistically activate TMN histaminergic neurons [23].

Many orexin neurons express vesicular glutamate transporters, suggesting that many if not all orexin neurons are glutamatergic [102,119]. In contrast, orexin neurons do not express GAD-67 mRNA, suggesting that orexin neurons are not GABAergic [102].

4. Input to orexin neurons

4.1. Anatomical analysis of neuronal input to orexin neurons

Until recently, little has been known about the synaptic input of orexin neurons. It has been challenging to study the neuronal afferents to these cells, because the cells are dispersed mediolaterally within the LHA. To address this point, we recently performed a retrograde tracing study using a genetically encoded retrograde tracer in mice [106]. We identified labeled cells in multiple specific brain regions, including the VLPO, medial and lateral preoptic areas, posterior/dorsomedial hypothalamus, and raphe nuclei. We also found many labeled neurons in regions associated with emotion including the amygdala, infralimbic cortex, shell region of the nucleus accumbens, the lateral septum, and the bed nucleus of the stria terminalis (BST).

Using a combination of antero- and retrograde tracers, Yoshida et al. mapped afferents of orexin neurons in rats [141]. They found abundant projections from the lateral septum, preoptic area, BST, and posterior hypothalamus. In addition, they also found that hypothalamic regions preferentially innervate orexin neurons in the medial and perifornical parts of the field, but most projections from the brainstem target the lateral part of the field.

4.2. Input from hypothalamus

Recent studies have established the role of the hypothalamus in regulating food intake and body weight. Especially, the lateral hypothalamic area (LHA) has long been considered essential in regulating food intake. Prior studies indicated that the LHA is innervated by several hypothalamic regions [20,25,29,118,122]. Some of these innervations might include projections to orexin neurons in the LHA. Indeed, input of orexin neurons from the hypothalamic

regions were identified by anatomical studies. Orexin neurons were showed by early studies to receive innervations from NPY-, agouti-related peptide-, and α-melanin-stimulating hormone-immunoreactive fibers which presumably come from the arcuate nucleus (Arc) [15,32].

4.3. Input from limbic system

Input coming from regions implicated in emotion might be important for regulation of orexin neuron activity upon emotional stimuli to evoke emotional arousal or fear-related responses, including elevation of sympathetic outflow. In fact, the importance of these inputs is readily apparent in the defense response, or "fight or flight" response: in an awake and freely moving condition, cardiovascular and locomotor responses to emotional stress in the resident–intruder paradigm as measured by telemetry in orexin knockout mice are diminished [59]. Similarly, air jet stress-induced elevations of blood pressure and heart rate were attenuated in conscious orexin/ataxin-3 mice, in which orexin neurons are ablated [144].

The neural input from the limbic system to orexin neurons might also be implicated in the pathophysiology of cataplexy, because strong, generally positive emotional stimuli are known to trigger cataplexy in human narcolepsy patients. This implies that orexin neurons may play a role in the physiological responses associated with emotions in humans. Local injection of orexin into the PPT strongly inhibited REM-related atonia in the cat [116]. Therefore, it is hypothesized that emotional stimuli increase orexin release in the PPT to prevent muscle atonia in wild-type animals.

The innervations from the limbic system might also be important to maintain activity of orexin neurons during the active period by conveying various emotional stimuli to orexin neurons.

The input to orexin neurons from the limbic system might also be involved in the regulation of feeding behavior, because some of the affective content of the perception of food is thought to be processed in the amygdala and limbic system. This information may be conveyed to orexin neurons. In fact, food perception often evokes cataplexy in narcoleptic dogs (food-elicited cataplexy) [98]. Food-elicited cataplexy in narcoleptic dogs, in which the orexin signaling system is disrupted, suggests that orexin signaling is physiologically activated upon the perception of food, and this system is necessary to evoke proper feeding behavior.

4.4. Input from preoptic areas

The preoptic area, especially the ventrolateral preoptic nucleus (VLPO), appears to play a critical role in NREM sleep initiation and maintenance. GABA and galanin are the primary inhibitory neurotransmitters of VLPO neurons [110]. VLPO sends out multiple inhibitory projections to neurons that release wake-promoting neurotransmitters, including histamine, noradrenaline, 5-HT, and acetylcholine [110,75]. Neurons in the VLPO fire at a rapid rate during sleep, with attenuation of firing during the awake period. Likewise, neurons in wake-promoting centers fire rapidly during wakefulness and are relatively quiescent during sleep, with the exception of cholinergic neurons, which are divided into two classes of neurons; one is active in both the awake and REM sleep period, and the other is active only in the REM sleep period [103].

Orexin neurons are strongly inhibited by both a $GABA_A$ agonist, muscimol, and a $GABA_B$ receptor agonist, baclofen [138,132]. Orexin neurons are also shown to be innervated by cells in the VLPO that also contain GABA [106,141]. These observations suggest that VLPO neurons send GABAergic inhibitory projections to orexin neurons. This pathway might be important to inhibit orexin neurons during sleep.

4.5. Input from suprachiasmatic nucleus

Although direct input to orexin neurons from the suprachiasmatic nucleus (SCN) appears to be sparse, orexin neurons receive abundant innervations from the BST, supraventricular zone and dorsomedial hypothalamus (DMH) [106,141], which receive input from the SCN. This suggests the possibility that orexin neurons receive circadian influences from the SCN via these regions [66].

4.6. Factors that influence activity of orexin neurons

Electrophysiological studies have identified several neurotransmitters and neuromodulators that activate or inhibit orexin neurons (Table 1). By recording from hypothalamic slices of transgenic mice expressing green

Table 1
Factors that influence the activity of orexin neurons

Excitation	Receptor involved
Glutamate	AMPA-R, NMDA-R mGluRs
Ghrelin	GHS-R
CCK	CCK-A
Neurotensin	ND
Vasopressin	V1a
Oxytocin	V1a
mAch (27%)	ND
Glucagon-like peptide-1	ND
CRF	CRFR1
ATP	P2X
H^+, CO_2	ND
Inhibition	
Glucose	unknown
GABA	$GABA_A$, $GABA_B$
Serotonin	5HT1A
Noradrenalin	α2
Dopamine	α2
Neuropeptide Y	Y1
Leptin	OB-R
Ach (muscarinic) (6%)	ND
Adenosine	A1

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, and NBQX) reduce their activity [138,69]. These results indicate that orexin neurons are tonically activated by glutamate.

In addition, several other factors have been shown to act on orexin neurons. Importantly, electrophysiological studies showed that dopamine, noradrenaline, and serotonin hyperpolarize and inhibit orexin neurons via α_2 and $5HT_{1A}$ receptors, respectively [138,70,87,139], while histamine has no effect on orexin neurons and a cholinergic agonist, carbachol, showed various responses [106,138]. These observations suggest that monoaminergic cells might send inhibitory, feedback projections to orexin neurons (Fig. 4).

Recently, a short 2 h period of total sleep deprivation was shown to change the action of noradrenaline on orexin neurons from excitation to inhibition. This mechanism might contribute to the growing sleepiness that accompanies sleep deprivation [43], although this phenomenon is not observed in mice [139].

Using transgenic mice in which orexin neurons specifically express yellow cameleon 2.1, we screened for factors that affect the activity of orexin neurons (a total of 21 peptides and six other factors were examined) and found that a sulfated octapeptide form of cholecystokinin (CCK-8S), neurotensin, oxytocin, and vasopressin activate orexin neurons [120], while GABA, glucose, 5-HT, noradrenaline, and leptin inhibit these cells. Other factors that reportedly influence the activity of orexin neurons include corticotrophin-releasing factor (CRF) [129], glucagon-like peptide 1 (GLP-1) [2], ATP [131], neuropeptide Y (NPY) [37], and adenosine [73] (Table 1).

A recent electrophysiological study demonstrated that orexin neurons were markedly affected by physiological fluctuations in acid and CO_2 levels [125]. These neurons show increased excitability in an acidic condition, whereas they show depressed excitability in an alkaline condition. These characteristics might possibly relate to stimulation of breathing and arousal in respiratory acidosis, because orexins pharmacologically stimulate breathing [142].

4.7. Regulation of orexin neurons by humoral factors

Changes in extracellular glucose concentration produce electrophysiological changes in isolated orexin neurons [137]. Increasing extracellular glucose concentrations induce striking hyperpolarization and cessation of action potentials in orexin neurons. Conversely, decreasing the glucose concentration induces depolarization and increases the frequency of action potentials in these same neurons [137,18]. Importantly, this mechanism is sufficiently sensi-

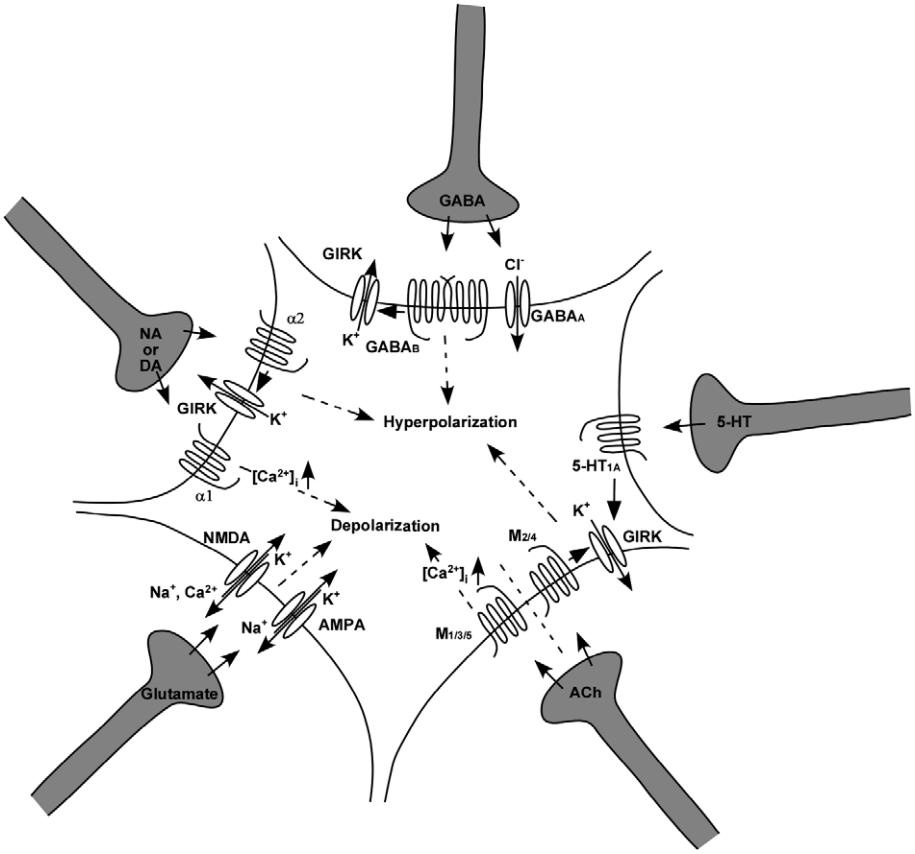

Fig. 4. Schematic drawing of afferent system of orexin neurons. 5-HT, serotonin; Ach, acetylcholine; NA, noradrenaline; DA, dopamine; GIRK, G protein-coupled inward rectifier potassium channel; $[Ca^{2+}]_i$, intracellular calcium concentration; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate.

tive to encode variations in glucose levels reflecting those occurring physiologically between normal meals.

A recent study demonstrated that inhibition of orexin neurons by glucose is mediated by tandem-pore K^+ (K_{2P}) channels [17]. These results reveal a novel energy-sensing pathway in neurons that regulate states of consciousness and energy balance [17].

The orexigenic peptide ghrelin activated 60% of dispersed orexin neurons when applied in superfused solution, with depolarization and an increase in action potential frequency [137]. In contrast, bath-application of leptin was found to robustly inhibit most of the orexin neurons examined, causing hyperpolarization and a decrease in firing rate. Notably, insulin exerted no direct effect on orexin neurons.

These findings show that peripheral humoral factors that are related to energy metabolism including glucose, leptin and ghrelin influence the activity of orexin neurons. Consistently, orexin expression of normal and *ob/ob* mice is negatively correlated with changes in blood glucose, leptin, and food intake [137]. These findings are consistent with the idea that orexins neurons act as a sensor of the nutritional status of the body [104].

Peripheral metabolic cues, including glucose, leptin, cholecystokinin, and ghrelin, might also influence the activity of orexin neurons via vagal afferents and the nucleus of the solitary tract (NTS), because it was reported that orexin neurons are stimulated by hypoglycemia at least partly via the NTS [19,124].

Then, what is the physiological relevance of the regulation of orexin neurons by factors that act as indicators of an animal's nutritional state? When faced with a negative energy balance due to reduced food availability, mammals respond behaviorally with phases of increased wakefulness and alertness that would presumably enhance the ability to find food in nature [56,21]. Orexin neuron-ablated mice fail to exhibit fasting-induced arousal [137], suggesting that orexin neurons are necessary to evoke adaptive behavioral arousal during fasting. During periods of nutritional depletion, orexin increases arousal, reinforcing food-seeking/feeding pathways. These mechanisms may also be important in the maintenance of prolonged wakefulness during the active period, and in the regulation of energy homeostasis, which helps to ensure survival in nature, but may counteract attempts to treat obesity by food restriction.

5. Mechanisms of actions

This section discusses the physiological roles of orexins, in relation to the output of orexin neurons, which might be important for stabilization of behavioral states.

5.1. Orexins activate waking-active monoaminergic neurons to stabilize wakefulness

When orexin A or orexin B was injected ICV into animals during the light period, it increased awake time and decreased REM and non-REM sleep time [44,12,96,123,133,38]. What are the mechanisms of this pharmacological effect of the orexin?

As already mentioned, the raphe nuclei, LC and TMN monoaminergic neurons express orexin receptors and are heavily innervated by orexin neurons. These observations suggest that these monoaminergic regions are major effector sites of orexins. Consistent with this hypothesis, isolated cells from these nuclei are all activated by orexins in vitro. For instance, noradrenergic cells of the LC [44], dopaminergic cells of the ventral tegmental area (VTA) [89], serotonergic cells of the dorsal raphe (DR) [74,16], and histaminergic cells of the TMN [136] have all been shown to be activated by orexins. These observations suggest that the activity of these monoaminergic neurons is at least partly regulated by orexins. Orexins also have a strong direct excitatory effect on cholinergic neurons of the basal forebrain [31], which is hypothesized to play an important role in arousal [4].

The activities of monoaminergic neurons in the brain stem and hypothalamus, including histaminergic cells in the TMN, noradrenergic cells in the LC, and serotonergic cells in the DR, are known to be synchronized and strongly associated with sleep/awake states: they fire tonically during an awake state, less during non-REM sleep, and not at all during REM sleep [121]. These observations suggest that orexin neurons are activated during the awake period, and exert an excitatory influence on these waking-active neurons to sustain activity of these neurons.

In addition, orexin neurons project directly to the laterodorsal tegmental/pedunculopontine tegmental nucleus (LDT/PPT) cholinergic neurons. Direct injection of orexin A into the LDT of cats results in an increase in awake time and a decrease in REM sleep time [133]. In addition, several reports have shown that orexin induces long-lasting excitation of cholinergic neurons in the LDT [113]. Recent work also showed that orexin A inhibits cholinergic neurons in the PPT via activation of GABAergic local interneurons and GABAergic neurons in the substantia nigra pars reticulata [115]. 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 REM sleep.

Some reports showed that the effect of orexin on wakefulness is largely mediated by activation of the TMN histaminergic system through OX_2R. In rats, ICV injection of orexin during the light period potently increases the wake period, and this effect is markedly attenuated by the H_1 antagonist, pyrilamine [136]. The pharmacological effect of orexin A on awake time in mice is almost completely absent in histamine H_1-receptor-deficient mice [54]. Furthermore, OX_2R knockout mice exhibit a narcoleptic phenotype, while OX_1R knockout mice show only mild fragmentation of the sleep–wake cycle [126]. Because OX_2R is abundantly expressed in the histaminergic TMN, while OX_1R is strongly expressed in the noradrenergic LC, the TMN seems to be an important effector site of orexin for sleep/wake regulation.

However, several findings indicate that signaling through OX_1R is also important for the proper regulation of vigilance states. As mentioned before, *OX_2R* knockout mice exhibit characteristics of narcolepsy [127], and *OX_1R* knockout mice do not have any overt behavioral abnormalities and exhibit only very mild fragmentation of the sleep–wake cycle [126]. However, interestingly, the behavioral and electroencephalographic phenotype of *OX_2R* knockout mice is less severe than that found in *prepro-orexin* knockout mice and double receptor knockout (*OX_1R*- and *OX_2R*-null) mice, which appear to have the same phenotype as *prepro-orexin* knockout mice. Importantly, both *OX_2R* knockout and *prepro-orexin* knockout mice are similarly affected by behaviorally abnormal attacks of non-REM sleep ("sleep attacks") and show a similar degree of disrupted wakefulness [127]. In contrast, *OX_2R* knockout mice are only mildly affected by cataplexy and direct transitions to REM sleep from an awake state, whereas orexin knockout mice and double receptor knockout mice are severely affected. These observations suggest that OX_1R also has additional effects on sleep–wake regulation (Fig. 3), and despite the lack of an overt OX_1R phenotype, loss of signaling through both receptor pathways is necessary for emergence of a complete narcoleptic phenotype (Fig. 3).

These observations suggest that inhibition of wake/non-REM sleep transition seems to critically depend upon OX_2R activation, while the profound dysregulation of REM sleep control unique to the narcolepsy syndrome emerges from loss of signaling through both OX_1R-dependent and OX_2R-dependent pathways (Table 2).

5.2. Regulations of feeding behavior and energy homeostasis

The altered energy homeostasis in human narcolepsy patients as well as mouse narcolepsy models suggests roles of orexin in the regulation of energy homeostasis [109,52] (Table 2). The finding of decreased caloric intake combined with an increased body mass index in narcolepsy patients, in which orexin neurons are missing, also suggests that they have a feeding abnormality with reduced energy expenditure or a low metabolic rate [109,62], and orexin neurons have a role in the regulation of energy homeostasis. Consistently, orexin neuron-ablated mice also show hypophagia and late-onset obesity, although the severity of the obese phenotype critically depends on their genetic background [46,47].

Supporting the physiological relevance of orexin in the control of feeding, ICV administration of anti-orexin antibody or an OX_1R-selective antagonist reduced food intake [134,50], and *prepro-orexin* knockout mice and transgenic mice lacking orexin neurons ate less than control wild-type mice [46,126,145]. Especially, energy expenditure in orexin neuron-deficient mice decreased upon awakening at a greater rate than in control mice [145]. These results may explain the paradox between the reduction of food intake and the increase of body weight. Moreover, an OX_1R selective antagonist reduced food intake and ameliorated obesity of leptin-deficient *ob/ob* mice [51], suggesting that leptin deficiency at least partly activates the orexin pathway to increase food intake.

Orexin neurons densely project to the arcuate nucleus [27,94,53,135], and Fos expression was induced in NPY neurons of the arcuate nucleus by ICV injection of orexin, suggesting that orexin-stimulated feeding may occur at least partly through NPY pathways [135]. Consistently, orexin neurons abundantly innervated NPY neurons in the arcuate nucleus, and the orexin A-induced increase in food intake was partly inhibited by prior administration of BIBO3340, an NPY-Y1 receptor antagonist, in a dose-dependent manner [135]. These experiments suggest that orexin-stimulated food intake is at least partially mediated by activation of NPY neurons. However, because NPY antagonist (which completely abolished NPY-induced feeding) only partially (50%) abolished orexin-induced feeding in rats, other pathways by which orexin induces feeding might exist. Such a pathway might include inhibition of proopiomelanocortin (POMC)-expressing neurons [88]. Indeed, orexin suppresses action potential firing and hyperpolarizes the membrane potential of ARC POMC neurons [77]. This effect is mediated by a reduction of excitatory input and an increase in inhibitory input.

Table 2
Phenotypes of rodent narcolepsy models produced by genetic engineering

	Sleep/wake state abnormality	Other phenotypes	Ref.
Prepro-orexin knockout	Cataplexy (+), sleep attacks (+) Sleep/wake fragmentation (severe)	Slight decrease in food intake, mild tendency for obesity (dependent on genetic background)	[22,47]
OX_1R knockout	Cataplexy (−), sleep attacks (+) Sleep/wake fragmentation [57]	ND	[126]
OX_2R knockout	Cataplexy (+) [57], sleep attacks (+) Sleep/wake fragmentation (severe)	ND	[126]
Orexin/ataxin-3 mouse	Cataplexy (+), sleep attacks (+) Sleep/wake fragmentation (severe)	Decrease in food intake, mild tendency for obesity (dependent on genetic background), lack of food-entrainable activity, lack of fasting-induced increase in wake time	[46,47]
Orexin/ataxin-3 rat	Cataplexy (+), sleep attacks (+) Sleep/wake fragmentation (severe)	ND	[9]

Complex pathways of orexin-mediated feeding behavior are also suggested by the finding that orexin-mediated feeding involves both leptin-sensitive and -insensitive pathways [146]. Leptin only partially inhibited the orexin A- or orexin B-induced increase of food intake, while both NPY- and galanin-induced feeding behavior were completely inhibited by preadministration of leptin, suggesting that NPY- and galanin-induced increases of feeding involve a leptin-sensitive pathway, while orexin-induced feeding involves both leptin-sensitive and -insensitive pathways.

As discussed later, orexin-mediated maintenance of consolidated wakefulness states might also be important in supporting feeding behavior, because proper maintenance of arousal during food searching and intake is essential for an animal's survival. For example, when faced with reduced food availability, animals adapt with a longer awake period, which disrupts the normal circadian pattern of activity [56,21]. As discussed later, we found that transgenic mice in which orexin neurons are ablated fail to respond to fasting with increased wakefulness and activity [137]. This suggests that orexin neurons have a critical role in maintenance of arousal during fasting. These mechanisms may modulate the activity of orexin neurons according to energy stores to maintain wakefulness.

The activity of orexin neurons also contributes to the promotion and maintenance of food anticipatory activity (FAA) [3,80]. Daily restricted feeding produces an anticipatory locomotor activity rhythm and entrains the peripheral molecular oscillator, which is independent of the central clock located in the suprachiasmatic nucleus (SCN). Restricted feeding was shown to shift the peak of Fos expression of orexin neurons from night to period of restricted feeding [3,80]. Formation of the FAA is severely impaired in orexin neuron-ablated, *orexin/ataxin-3* transgenic mice [3,80]. The expression of *mNpas2* mRNA, a transcription factor thought to be involved in regulation of the food-entrainable oscillator as well as *mPer1* and *mBmal1* mRNA, is reduced in *orexin/ataxin-3* mice. These observations suggest that orexin neurons convey an efferent signal from the putative food-entrainable oscillator or oscillators to increase wakefulness and locomotor activity.

Recently, a part of the dorsomedial hypothalamic nucleus (DMH) was shown to have robust oscillation of *mPer* expression only under restricted feeding [82]. The oscillation persisted for at least 2 days even when mice were given no food during the expected feeding period after the establishment of food-entrained behavioral rhythms. Gooley et al. also demonstrated that lesions in DMH neurons in rats blocked food entrainment of wakefulness, locomotor activity, and core body temperature [41]. Taken in conjunction with our recent finding that DMH neurons directly project to orexin neurons [106], which are essential for proper expression of food-entrained behavioral rhythms, these findings indicate that a link between DMH neurons and orexin neurons plays a key role as a central FEO in the feeding-mediated regulation of circadian behaviors. However, we should also noted that Landry et al. reported that DMH-ablated rats persisted with a food-entrained behavioral rhythms, with a significant attenuation of light–dark-entrained activity rhythms [63]. The authors mentioned that the difference is presumably related to the measure of behavior and possibly the configuration of the recording apparatus.

5.3. Influences on autonomic nervous system

Pharmacologically, ICV orexin injection increases blood pressure and heart rate [112], and these effects are abolished by administrations of an α or β blocker [5]. On the other hand, i.v.-injection of orexin A and orexin B failed to cause any cardiovascular effect in conscious rats [72] and rabbits [79]. Orexin-deficient mice show 10–15 mm Hg lower blood pressure than wild-type littermates [59,144]. These results suggest that orexins physiologically stimulate sympathetic outflow. Therefore, orexin deficiency might decrease sympathetic tone, which might result in decreased energy expenditure.

As suggested from the effects on sympathetic tone, although orexins stimulate feeding behavior, they do not slow the metabolic rate, as might be expected in a system geared for weight gain. Instead, orexins both increase food intake and increase the metabolic rate [76]. Because animals must be aware and active when they seek and eat food, this function might be important for feeding behavior. This suggests that the function of orexins might support reward-seeking behavior with an increase of vigilance, awareness, and sympathetic outflow.

5.4. Reward and stress system

Psychostimulants such as amphetamine and methylphenidate are used to treat narcolepsy patients. However, it is known that drug addiction hardly occurs in these patients, suggesting that the orexin system is necessary for establishing drug addiction. This notion is consistent with recent reports on animal models in which *orexin* knockout mice showed attenuated morphine dependence and withdrawal [91,40], and orexin neuron-ablated mice failed to show increased arousal and activity in food deprivation [137]. These reports indicate that the orexin system is strongly associated with reward-seeking behavior. Orexin neurons strongly innervate the ventral tegmental area (VTA) [89,36], and orexin directly activates VTA dopaminergic neurons [89,61]. ICV or local VTA infusion of orexin drives behavior motivated by either food or drug rewards [104,13,48]. On the other hand, an OX_1R antagonist completely abolished an olfactory cue-induced reinstatement of alcohol-seeking behavior in alcohol-preferring rats [65]. Recent work has also shown that orexin A input to the ventral tegmental area (VTA) potentiates *N*-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission via a PLC/PKC-dependent insertion of NMDARs in VTA dopamine neuron synapses in slice preparations [11]. Furthermore, intra-VTA microinjection of an OX_1R antagonist abolished a conditioned place preference for morphine [91]

or locomotor sensitization to cocaine [11]. These data indicate that orexin signaling plays an important role in the VTA in neural plasticity relevant to addiction. To elucidate specific environmental cues related to rewards, it appears that the orexin neuron should be activated. As discussed later, this might be due to input from the limbic system. Moreover, activation of this system can cause relapse of drug-seeking behavior that has disappeared, showing that this system takes part in the memory for stimulation related to the reward and in drug dependence relapse. This effect might be mediated by glutamate receptors in the VTA by generating the plasticity of synapses.

On the other hand, Boutrel et al. reported that ICV infusion of orexin A elevated the intracranial self-stimulation threshold, suggesting that orexin also negatively regulates the activity of brain reward circuitry [13]. They discussed that orexin-induced reinstatement of reward-seeking might relate to the stress response, because stress also produces reinstatement of reward-seeking behavior, and orexin neurons are activated upon stress [129]. Consistent with this interpretation, the selective OX_1R antagonist SB-334867 blocked footshock-induced reinstatement of previously extinguished cocaine-seeking behavior.

Relationship between orexin and stress response was earlier suggested by finding that orexin neurons are activated by corticotropin-releasing factor (CRF) through CRF-R1 [129,128]. After stressor stimuli, CRF stimulates the release of orexin to activate and maintain arousal associated with the stress response.

6. Roles of orexin neuronal system: mechanisms that maintain wakefulness

6.1. Reciprocal interaction of sleep/wake control circuit and orexin neurons

Orexin neurons send excitatory influences to monoaminergic neurons to maintain the activity of these cells. Recent electrophysiological and histological findings suggest that these monoaminergic neurons send inhibitory, feedback projections to orexin neurons [138,87,139]. This feedback circuitry might maintain the activity of monoaminergic neurons, because when small disturbances decrease the activity of monoaminergic cells, it results in a decrease in the inhibitory influence on orexin neurons. Therefore, orexin neurons are disinhibited and have an increased excitatory influence on monoaminergic cells to increase their activity. This feedback mechanism might maintain the awake period (Fig. 5).

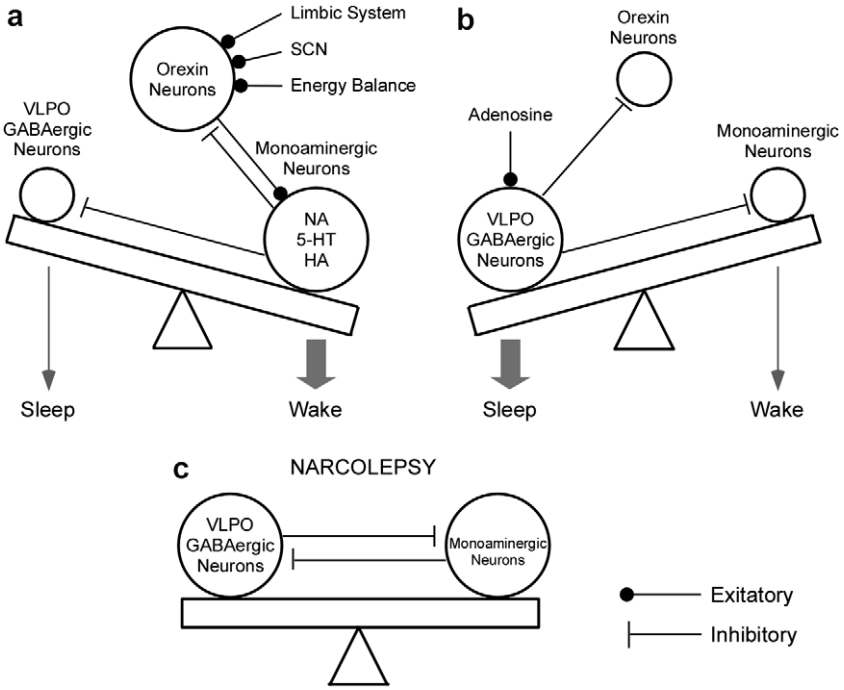

Fig. 5. Schematic summary of interactions between orexin neurons and monoaminergic wake center and VLPO sleep center during each behavioral state. (a) Awake period. Orexin neurons send excitatory influences to monoaminergic neurons, and monoaminergic neurons send inhibitory, feedback projections to orexin neurons. This system might maintain the activity of monoaminergic neurons. When a small disturbance decreases the activity of monoaminergic cells, it results in a decrease in the inhibitory influence on orexin neurons. Therefore, orexin neurons are disinhibited and have an increased excitatory influence on monoaminergic cells to maintain their activity. These monoaminergic cells send excitatory projections to the forebrain cortex, and send inhibitory projections to the VLPO sleep center. These mechanisms maintain awake states. Orexin neurons receive influences from the limbic system. Emotion provokes wakefulness by this pathway. (b) Sleep period. VLPO sleep-active neurons are activated and send inhibitory influences to monoaminergic neurons and orexin neurons to maintain sleep. (c) Narcolepsy. If orexin neurons are removed from this circuit, monoaminergic neurons and VLPO neurons set up a mutually inhibitory circuit, which can cause unwanted abrupt transitions between each state.

Sleep-active neurons in the POA send projections to descending pathways, that terminate within wake-promoting populations in the posterior hypothalamus and brain stem [75]. Current evidence suggests that this output originates in POA sleep-active GABAergic neurons. During sleep, VLPO sleep-active neurons are activated presumably by adenosine [86,6,55], and send inhibitory influences to monoaminergic neurons. Recent work suggests that these sleep-active neurons also send inhibitory projections to orexin neurons [106,141] (Fig. 5).

These circuits are important for proper regulation of wakefulness. If orexin neurons are removed from this system, these sleep-active neurons in the VLPO and monoaminergic neurons would set up a ''flip-flop'' circuit, a circuit containing mutually inhibitory elements [108], because monoaminergic neurons send inhibitory influences to VLPO sleep-active neurons [39]. In such a circuit, when either side begins to overcome the other, the switch abruptly turns into the alternative state, because when a small disturbance gives one side a sudden advantage, it can turn off the alternative state abruptly [108]. This mechanism explains the instability of sleep/waking states in orexin deficient animals.

6.2. Diurnal changes in orexinergic activity and changes across sleep–wake cycle

We examined the effect of constitutive expression of orexin in transgenic mice, using orexin cDNA driven by the CAG promoter to study the sleep/wakefulness abnormality as a consequence of constitutive activation of orexinergic systems. In these mice, orexin is expressed in a diffuse, ectopic pattern in the brain in an unregulated fashion [81]. These transgenic mice show fragmented non-REM sleep in the light period and incomplete REM sleep atonia with abnormal myoclonic activity during REM sleep (our unpublished results).

These findings suggest that orexin neurons should be appropriately turned off during sleep to maintain consolidated non-REM sleep and muscle atonia during REM sleep, while they should be activated during awake periods. In fact, in rats, Fos expression of orexin neurons is increased during the dark-active period in which awake states are dominant [35], and orexin level in cerebrospinal fluid also peaks during the dark period and decreases during the light period in which sleep states are dominant [140]. These observations suggest that orexin neurons are active during the awake period, and are inactive during the sleep period.

Recent in vivo recording studies further revealed changes of orexin neuronal activity across the sleep–wake cycle. Mileykovskiy et al. recorded activity of orexin neurons in unanesthetized, unrestrained rats [85]. They identified orexin neurons by antidromic and orthodromic activation from the VTA and LC, and recorded the activity of these cells. They found that orexin neurons are relatively inactive in a quiet awake state but are transiently activated during sensory stimulation. These cells are silent in non-REM sleep and tonic periods of REM sleep, with occasional burst discharge in phasic REM sleep.

On the other hand, Lee et al. recorded neurons in the LHA of head-fixed rats and then labeled them with neurobiotin to identify them by combination with immunohistochemical staining to be orexin neurons [68]. They found that orexin neurons fire during an active awake state, have decreased discharge during a quiet awake state, and virtually cease firing during REM and non-REM sleep. They increase firing before the end of REM sleep and thereby herald by several seconds the return of awake states. These observations suggest that these cells are activated during an awake state, while they are inhibited during sleep.

7. Orexin deficiency and narcolepsy–cataplexy

Narcolepsy is a sleep disorder characterized by a primary disorganization of behavioral states. This disorder affects approximately 1 in 2000 individuals in the United States [83]. Most cases of human narcolepsy start during adolescence (around 12–14 years old). The most important symptom of the disorder is excessive daytime sleepiness (an insurmountable urge to sleep), manifested particularly as attacks of falling asleep at inappropriate times (sleep attack). The latency for rapid eye movement (REM) sleep is markedly reduced in narcolepsy patients, and the existence of sleep-onset REM periods (where REM sleep is directly preceded by a wakefulness period) is one of the diagnostic criteria for narcolepsy. Nocturnal sleep is often disturbed by sleep fragmentation together with the occurrence of hypnagogic hallucinations, vivid dreaming, and sleep paralysis.

Narcolepsy patients often suffer from an attack called ''cataplexy'', which is characterized by sudden weakening of postural muscle tone, which can range from jaw dropping and speech slurring to complete bilateral collapse of the postural muscles. These attacks are often triggered by emotional stimuli. Consciousness is preserved during cataplexy.

Symptoms of narcolepsy are divided into two pathological phenomena (Table 3). One is an inability to maintain a long awake period, characterized by an abrupt transition to non-REM sleep (dysregulation of non-REM sleep onset). This symptom is clinically manifested as excessive daytime sleepiness or sleep attacks. Available therapy for these symptoms is medication using psychostimulating drugs, such as amphetamine, methyl phenidate, modafinil, and caffeine. Another is a pathological intrusion of REM sleep into wakefulness (dysregulation of REM sleep onset), which consists of cataplexy, hypnagogic hallucinations, and sleep paralysis. Available therapy for these symptoms are tricyclic antidepressants such as imipramine and selective serotonin reuptake inhibitors (SSRI) [143]. Sodium oxybate (r-hydroxybutylate: GHB) is a only drug effective for both symptoms.

Clues suggesting the involvement of orexin in narcolepsy first came from animal models; mice lacking either the

Table 3

Pathological phenomena	Clinical symptoms	Available therapy	Receptor involved
Inability to maintain wakefulness (dysregulation of wake/non-REM sleep gating)	Excessive daytime sleepiness (sleep attacks)	Methylphenidate, modafinil, and caffeine	OX_2R
Pathological intrusion of REM sleep into wakefulness (dysregulation of REM sleep onset)	Cataplexy, hypnagogic hallucinations, and sleep paralysis	Tricyclic antidepressants	Both OX_1R and OX_2R

orexin gene (*prepro-orexin* knockout mice) or orexin neurons (*orexin/ataxin-3* transgenic mice), as well as mice and dogs with null mutations in one of the orexin receptor genes (OX_2R), all show phenotypes remarkably similar to human narcolepsy [22,46,71,127] (Table 2). *Prepro-orexin* knockout mice, OX_1R/OX_2R double knockout mice and *orexin/ataxin-3* mice all show a very similar phenotype, characterized by behavioral arrest similar to cataplexy, occasional direct transition to REM sleep from wakefulness, and a highly fragmented sleep–wake cycle [22,46].

A postmortem study of human narcolepsy subjects has shown undetectable levels of orexin peptides in projection sites such as the cortex and pons, and an 80–100% reduction in the number of neurons containing detectable *prepro-orexin* mRNA or orexin-like immunoreactivity in the hypothalamus [95,117], supporting an earlier report showing an undetectable level of orexin A in cerebrospinal fluid (CSF) in most narcolepsy patients [92]. More recent studies show that approximately 90% of patients with narcolepsy show decreased orexin A levels in the cerebrospinal fluid [143,84].

These results suggest either a loss of orexin neurons or a lack of orexin production in these neurons, if still present. The cause of the specific loss or degradation of orexin neurons in narcolepsy is totally unknown thus far, but because of its strong association with certain HLA alleles [57], it is possible that narcolepsy may result from selective immune-mediated degeneration of orexin neurons. Regardless of the mechanism of the cell loss, deficiency of the orexin signaling pathway in narcolepsy suggests that this neuropeptide system has an important role in regulation of vigilance states, especially maintenance of long, consolidated awake periods.

The phenotype of narcolepsy is thus characterized by inability to maintain vigilance states, pathological intrusion of non-REM and/or REM sleep into wakefulness, and frequent transitions between each vigilance state. Thus, narcolepsy can be viewed as a behavioral state boundary disorder [108]. This suggests that orexins have highly important roles in maintenance and stabilization of a long, consolidated awake period, and inhibition of sleep.

8. Clinical implications

Since narcolepsy is a disorder of organization of the sleep–wake cycle resulting from an absence of orexin, replacement therapy using orexin receptor agonists may provide an effective treatment for narcolepsy. Indeed, our study showed that chronic overproduction of orexin peptides from an ectopically expressed transgene prevented the development of narcolepsy syndrome in orexin neuron-ablated (*orexin/ataxin-3*) mice [81]. Acute administration of orexin A also maintained wakefulness, suppressed sleep, and inhibited cataplectic attacks in narcoleptic mice [81]. Together, these findings provide strong evidence for a specific relationship between absence of orexin peptides in the brain and the development of narcolepsy syndrome. However, as mentioned previously, chronic overexpression of orexin in an unregulated fashion results in disruption of non-REM sleep. Therefore, if orexin agonists were available, a short half-life (<12 h) might be desirable.

On the other hand, orexin antagonists might be effective for inducing sleep and treating insomnia. Indeed, ACT-078573, dual OX_1/OX_2 receptor antagonist, induced a more physiological sleep pattern than previous hypnotic drugs [14]. The decreased CSF orexin A peptide level in most narcolepsy patients also suggests that measuring CSF orexin A might be a definitive diagnostic test [84]. Indeed, measurement of orexin A in CSF has been included in the diagnostic criteria for narcolepsy in the 2nd revision of the international classification of sleep disorder.

9. Conclusion

Symptoms of narcolepsy unequivocally show that orexins and orexin receptors play highly important roles in regulating sleep/awake states and the maintenance of arousal by regulating monoaminergic/cholinergic nuclei in the brain. At the same time, this system is also related to the limbic system, which regulates emotional responses, the reward system in the VTA, and hypothalamic mechanisms that stimulate feeding behavior. Orexin neurons in the LHA are anatomically well placed to link between the limbic system, energy homeostasis, and brain stem monoaminergic/cholinergic neurons.

The link between the limbic system and orexin neurons might be important for emotional arousal and sympathetic responses during emotional events. On the other hand, the responsiveness of orexin neurons to peripheral metabolic cues, leptin and glucose, suggests that these cells might act as a sensor for the metabolic status of animals. Recent evidence also suggests that orexins have critical roles in reward systems. These findings indicate that orexin neurons provide a crucial link between energy balance, emo-

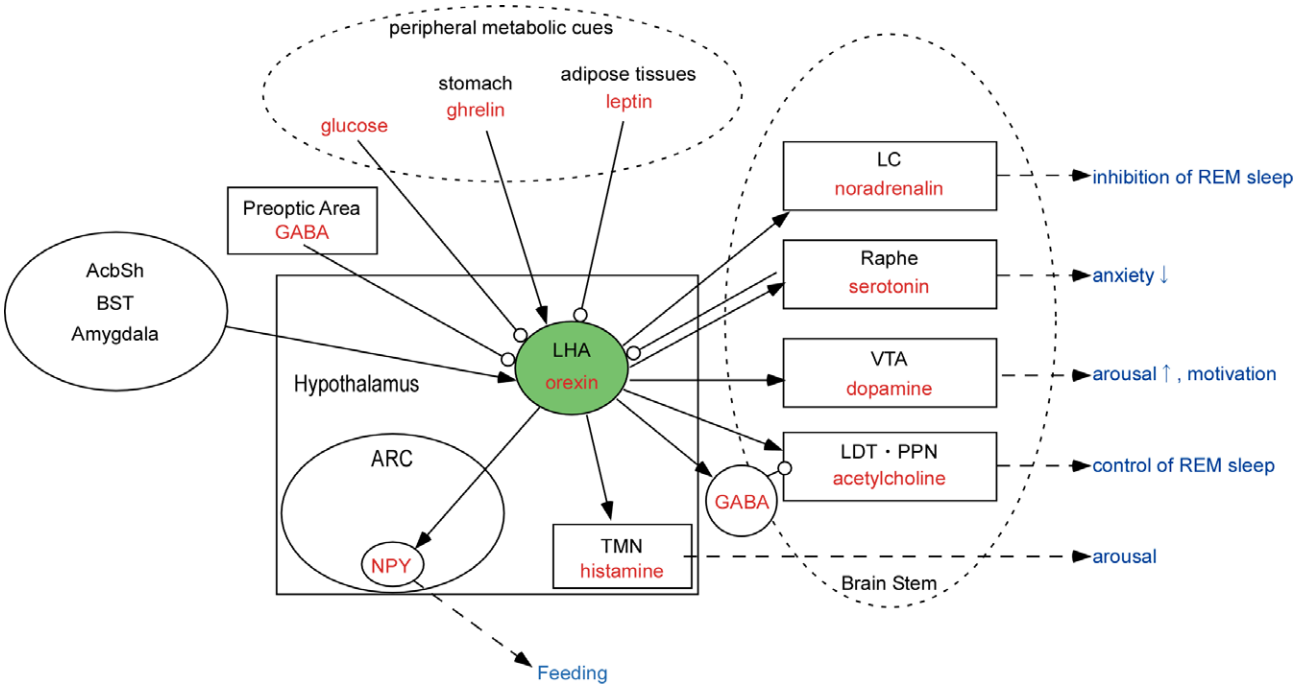

Fig. 6. Input and output of orexin neurons. Orexin neurons regulate the hypothalamic nuclei involved in feeding behavior. At the same time, they increase cortical arousal and promote wakefulness through the aminergic nuclei and other sleep-related nuclei. Stimulation of dopaminergic, limbic, and cholinergic centers by orexins can modulate reward systems, motor activity, and emotional arousal. Peripheral metabolic signals, leptin, ghrelin, and glucose, and circadian rhythms influence orexin neuronal activity to coordinate arousal and energy homeostasis. Input from the limbic system may be important to regulate the activity of orexin neurons upon emotional stimuli to evoke emotional arousal or fear-related responses. Input from the limbic system may also influence feeding behavior.

tion, reward systems and arousal, and act as a regulator that maintains proper vigilance states according to inner and outer environments of our bodies (Fig. 6).

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