The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness

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Abstract | Sleep and wakefulness are regulated to occur at appropriate times that are in accordance with our internal and external environments. Avoiding danger and finding food, which are life-essential activities that are regulated by emotion, reward and energy balance, require vigilance and therefore, by definition, wakefulness. The orexin (hypocretin) system regulates sleep and wakefulness through interactions with systems that regulate emotion, reward and energy homeostasis.

Narcolepsy
A neurological condition mostly characterized by excessive daytime sleepiness, uncontrollable sleep attacks and disorder of REM sleep.

Limbic system
A collection of cortical and subcortical structures important for processing memory and emotional information. Prominent structures include the hippocampus and amygdala.

Ghrelin
Stomach-derived orexigenic peptide.

Leptin
An adipocyte-derived protein hormone that has a key role in regulating energy intake and energy expenditure.

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The neuropeptides orexin A and orexin B (also known as hypocretin 1 and hypocretin 2), produced in hypothalamic neurons, are crucial regulators of sleep and wakefulness. These peptides activate wake-active monoaminergic and cholinergic neurons in the hypothalamus and brain stem to maintain a long, consolidated awake period, and it is this role in particular that will form the focus of this review.

Orexins were initially identified as endogenous ligands for two orphan G-protein-coupled receptors[1] (BOX 1). They were recognized as regulators of feeding behaviour, firstly because of their exclusive production in the lateral hypothalamic area (LHA), a region known as the feeding centre, and secondly owing to their pharmacological activity; intracerebroventricular (ICV) injection of orexins during the light period induces feeding behaviour in rats and mice[1–4]. Subsequently, the finding that an orexin deficiency causes narcolepsy in humans and animals indicated that these hypothalamic neuropeptides also have a crucial role in regulating sleep and wakefulness[5–9].

Recent studies of orexin-producing neurons' efferent and afferent systems, as well as phenotypic characterizations of mice with genetic alterations in the orexin system, have suggested further roles for orexin in the coordination of emotion, energy homeostasis, reward, drug addiction and arousal[10–17].

Orexin neurons receive abundant input from the limbic system[14,15], which might be important for increasing arousal during emotional stimuli. Orexin neurons are also regulated by peripheral metabolic cues, including ghrelin, leptin and glucose, indicating that orexin neurons might provide a link between energy homeostasis and vigilance states[11].

Together, these observations suggest that, broadly speaking, orexin neurons are involved in sensing the body's external and internal environments and regulate states of sleep and wakefulness accordingly, which is beneficial for survival. This review will discuss the mechanisms by which the orexin system maintains sleep and wakefulness, and how this mechanism relates to other systems that regulate emotion, reward and energy homeostasis.

Narcolepsy and orexins

The symptoms and pathophysiology of the sleep disorder narcolepsy, caused by an orexin deficiency[5–8] (BOX 2), provide insight into the physiological roles of orexin. Narcolepsy is characterized by the inability to maintain vigilance states, pathological intrusion of rapid eye movement sleep (REM sleep) and/or non-REM (NREM) sleep into wakefulness, and frequent transitions between states of sleep and wakefulness, which indicates that orexins have important roles in the maintenance and stabilization of sleep and wakefulness.

The first clues towards an involvement of the orexins in narcolepsy came from animal models; mice lacking the orexin gene and dogs with null mutations in the orexin receptor 2 (*OX_2R*) gene show phenotypes remarkably similar to humans with narcolepsy[7,8] (see Supplementary information S1 (table)). Mice lacking the orexin precursor prepro-orexin, orexin neuron-ablated (orexin/ataxin 3 transgenic) mice and *OX_1R*/*OX_2R* double knockout mice exhibit similar phenotypes that have strong parallels to the human condition. These are characterized by behavioural arrests that are similar to a condition called cataplexy (BOX 2), occasional direct transitions to REM sleep from wakefulness, and highly fragmented sleep-wake cycles[7,9], all of which are important elements of narcolepsy.

Rapid eye movement sleep
(REM sleep) The stage of sleep characterized by rapid movements of the eyes.

Cataplexy
An episodic condition featuring loss of muscle function, ranging from slight weakness (such as limpness at the neck or knees, sagging facial muscles or inability to speak clearly) to complete body collapse.

The link between orexin dysfunction and narcolepsy, especially when accompanied by cataplexy (narcolepsy–cataplexy), has since been supported by findings in human patients. A post-mortem study of human narcolepsy patients found that orexin peptides were undetectable in the cortex and pons, in which orexinergic projections are normally found (FIG. 1), and that there was an 80–100% reduction in the number of neurons containing detectable prepro-orexin mRNA or orexin-like immunoreactivity in the hypothalamus[5,6]. This supports earlier reports that orexin A was undetectable in the cerebrospinal fluid of narcolepsy patients[18]. Approximately 90% of patients with narcolepsy, especially those with narcolepsy–cataplexy, show decreased orexin A levels in the cerebrospinal fluid[19]. Therefore, a low cerebrospinal fluid level of orexin A is now one of the diagnostic criteria for narcolepsy–cataplexy, according to the 2nd edition of the international classification of sleep disorders[20].

A recent finding showing concomitant loss of dynorphin, neuronal activity-regulated pentraxin and orexin, which colocalize in orexin neurons, further indicates a loss of orexin neurons in narcolepsy–cataplexy[21]. The cause of the specific loss or degradation of orexin neurons in narcolepsy has been unknown so far, but because of its strong association with certain human leukocyte antigen (HLA) alleles[22] it is possible that narcolepsy could result from selective immune-mediated degeneration of orexin neurons, although no specific antibody against orexin neurons has been found in the serum of affected individuals. Regardless of the cause of the neuron loss, the orexin signalling deficiency in narcolepsy–cataplexy shows that this neuropeptide system has an important role in the regulation of sleep and wakefulness, especially in the maintenance of long, consolidated awake periods.

Box 1 | **Overview of the orexin/hypocretin system**

Orexin A and orexin B were identified by our group as endogenous ligands for two orphan G-protein-coupled receptors[1]. Orexin A and B are derived from a common precursor peptide, prepro-orexin. An mRNA encoding the same precursor peptide was independently isolated as a hypothalamus-specific transcript[98]. It was predicted that the transcript encoded a polypeptide precursor that is cleaved to form two neuropeptides, termed hypocretin 1 and hypocretin 2. To avoid confusion, the orexin nomenclature is used throughout this review, but it should be noted that the names 'orexin' and 'hypocretin' are currently used synonymously in many papers.

Orexins constitute a novel peptide family, with no significant homology with any previously described peptides[41]. Orexin A is a 33 amino acid peptide with an amino (N)-terminal pyroglutamyl residue, two intra-chain disulphide bonds and carboxy (C)-terminal amidation. This structure is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog and pig). Orexin B is a 28 amino acid, C-terminally amidated linear peptide. The C-terminal half of orexin B is very similar to that of orexin A, whereas the N-terminal half is more variable.

The actions of orexins are mediated by two receptors, named orexin 1 (OX_1R) and orexin 2 (OX_2R) receptors (also known as HCRTR1 and HCRTR2). OX_1R has one-order-of-magnitude greater affinity for orexin A than orexin B. By contrast, orexin A and orexin B bind OX_2R with similar affinities[1]. OX_1R is thought to transmit signals through the $G\alpha_{11}$ class of G protein, which results in the activation of phospholipase C with subsequent triggering of the phosphatidylinositol cascade and influx of extracellular Ca^{2+}, probably through transient receptor potential (TRP) channels. OX_2R is thought to be coupled to both $G\alpha_{11}$ and inhibitory G_i G proteins[99]. *OX_1R* and *OX_2R* mRNAs exhibit a markedly different and basically complementary distribution, indicating that these receptors have distinct physiological roles[27].

The orexin system's role in wakefulness

How do the orexins physiologically regulate sleep and wakefulness, and why does a lack of orexin signalling result in narcolepsy? In this section, I will discuss the mechanisms of action of these peptides at both the cellular and the systems level.

Orexins stabilize wakefulness. It has been shown that ICV injection of orexin A or orexin B in rats during the light (rest) period, the equivalent of night-time in humans, increases awake time and decreases REM and NREM sleep time[23]. By what mechanisms does the orexin system evoke this pharmacological effect?

Orexin neurons originate in the hypothalamus and are almost exclusively localized in the LHA and posterior hypothalamus[24–26]. These neurons are variable in size (the cell body diameter ranges from 15–40 μm) and shape (spherical, fusiform or multipolar), and have been assumed to number around 3000 in the rat brain, or 7000 in the human brain[25,26]. From these regions, orexin neurons project widely to the entire neuroaxis, excluding the cerebellum[24–26] (FIG. 1). The densest staining of orexin-immunoreactive nerve endings in the brain is found in the paraventricular nucleus of the thalamus, the arcuate nucleus and, most notably, the locus coeruleus (LC, containing noradrenergic neurons), dorsal raphe (DR, which contains serotonergic neurons) and tuberomammillary nucleus (TMN, containing histaminergic neurons)[5,24,25]. The distribution of mRNA for the orexin receptors is consistent with these projection sites; within the brain, *OX_1R* mRNA is most abundantly expressed in the LC, whereas *OX_2R* mRNA is highly expressed in the TMN[27]. Both regions are important for the maintenance of arousal[27]. The DR and ventral tegmental area (VTA) contain both *OX_1R* and *OX_2R* mRNA[27]. These observations indicate that these monoaminergic regions are important effector sites of orexins.

Consistent with this hypothesis, electrophysiological experiments using brain slice preparations or isolated cells have shown that cells of these nuclei are activated by orexins *in vitro*. Indeed, noradrenergic cells of the LC[23,28], dopaminergic cells of the VTA[29], serotonergic cells of the DR[30,31] and histaminergic cells of the TMN[32] have all been shown to be activated by orexins. Many of these monoaminergic neurons are implicated in increasing arousal and promoting wakefulness. The activity of monoaminergic neurons in the TMN, LC and DR is known to be synchronized and strongly associated with sleep and wakefulness: the neurons fire tonically during wakefulness, less during NREM sleep, and not at all during REM sleep[33]. These observations indicate that orexin-mediated arousal results from the activation of these wake-active monoaminergic neurons. Specifically, orexin neurons, activated during wakefulness, exert an excitatory influence on these wake-active neurons, thereby sustaining their activity.

Additional evidence for a role of orexin in wakefulness is provided by the strong, direct excitatory effect of orexins on cholinergic neurons in the basal forebrain[34], which are important for maintaining arousal[35].

Box 2 | **What is narcolepsy (narcolepsy–cataplexy)?**

Narcolepsy is a serious neurological disorder that affects approximately 1 in 2000 individuals in the United States[100]. Onset of the condition is usually during adolescence (around 12–14 years old). A cardinal symptom of the disorder is excessive daytime sleepiness (an insurmountable urge to sleep), which manifests itself primarily as the subject falling asleep at inappropriate times ('sleep attacks'). The latency of rapid eye movement (REM) sleep is notably reduced in narcolepsy patients, and the existence of 'sleep onset REM periods' (that is, REM sleep directly preceded by an awake period) is one of the diagnostic criteria for narcolepsy. Nocturnal sleep is often disturbed by sleep fragmentation combined with the occurrence of hypnagogic hallucinations, vivid dreaming and sleep paralysis, which usually occur when patients fall asleep. Narcolepsy patients often suffer from a condition called 'cataplexy', which is characterized by a sudden weakening of muscle tone, ranging from jaw dropping and speech slurring to complete bilateral collapse of the postural muscles. These attacks are triggered by emotional stimuli. Consciousness is preserved during cataplexy. Narcolepsy with cataplexy is sometimes referred as 'narcolepsy–cataplexy'.

Symptoms of narcolepsy–cataplexy can be divided into two pathological phenomena. One is an inability to maintain a long awake period, characterized by abrupt transition to non-REM (NREM) sleep (dysregulation of NREM sleep onset). This phenomenon manifests clinically as excessive daytime sleepiness or a sleep attack. Recent studies suggested that it largely results from lack of OX_2R activation[42]. Psychostimulant drugs, such as modafinil, methyl phenidate, amphetamine and caffeine are used to treat these symptoms. The other key phenomenon is the pathological intrusion of REM sleep into wakefulness (dysregulation of REM sleep onset); it is during these periods that the patient might experience cataplexy, hypnagogic hallucinations and sleep paralysis. Available therapy for this symptom consists of tricyclic antidepressants such as imipramine and selective serotonin reuptake inhibitors[101]. Lack of signalling from both receptors is critically associated with this symptom.

In addition, orexin neurons project directly to the laterodorsal tegmental/pedunculopontine tegmental nucleus (LDT/PPT) cholinergic neurons, some populations of which are implicated in the in the maintenance of wakefulness[36]. Other populations of LDT/PPT neurons are implicated in the regulation of REM sleep and muscle atonia during REM sleep[36]. Direct injection of orexin A into the LDT of cats results in an increased awake time and a decreased REM sleep time[37]. In addition, several reports have shown that orexin induces long-lasting excitation of cholinergic neurons in the LDT[38]. However, more recent work has shown that orexin A inhibits cholinergic neurons in the PPT through activation of GABA (γ-aminobutyric acid)-containing local interneurons and GABA-containing neurons in the substantia nigra pars reticulata[39]. These results indicate that hypothalamic orexin neurons affect the activity of LDT/PPT cholinergic neurons both directly and indirectly to regulate arousal and REM sleep. However, further studies are needed to understand the precise effects of orexins on LDT/PPT cholinergic neurons.

Orexin receptors in sleep and wakefulness. Some reports have indicated that the effect of orexin on wakefulness is largely mediated by activation of the histaminergic system through the OX_2R. In rats, ICV injection of orexin during the light period potently increases the duration of wakefulness, and this effect is markedly attenuated by the histamine H_1 receptor antagonist, pyrilamine[32]. This pharmacological effect of orexin A on awake time is almost completely absent in H_1-deficient mice[40]. Furthermore, whereas OX_1R-knockout mice show only a mild fragmentation of sleep and awake states[41], OX_2R-knockout mice exhibit a narcoleptic phenotype[42] (FIG. 2; Supplementary information S1 (table)). OX_2R is abundantly expressed in the histaminergic TMN, whereas OX_1R is highly expressed in the noradrenergic LC, indicating that the TMN might be an important effector site of orexin for the regulation of sleep and wakefulness.

However, one should not disregard the importance of OX_1R in the regulation of sleep and wakefulness. The behavioural and electroencephalographic phenotype of prepro-orexin-knockout mice and double-receptor knockout (OX_1R- and OX_2R-null) mice[41], which seem to have similar phenotypes[41], is more severe than that of OX_2R-knockout mice[42], supporting an important but less significant contribution of OX_1R (FIG. 2; Supplementary information S1 (table)). OX_2R-knockout and prepro-orexin-knockout mice are similarly affected by behaviourally abnormal attacks of NREM sleep (sleep attacks)[42], but OX_2R-knockout mice show a lower degree of disrupted wakefulness compared with double-receptor knockouts[42] (FIG. 2). In particular, OX_2R-knockout mice are only mildly affected by cataplexy and direct transitions to REM sleep from awake states[42], whereas prepro-orexin-knockout mice and double-receptor knockout mice are severely affected[7,41,42] (FIG. 2). These observations indicate that OX_1R has additional effects on sleep–wake regulation, especially the regulation of REM sleep. So, despite the lack of an overt $OX_1R^{-/-}$ phenotype, loss of signalling through both receptor pathways seems to be necessary for the emergence of a complete narcoleptic phenotype, indicating that both receptors are involved in the regulation of sleep and wakefulness.

Orexinergic activity in the sleep–wake cycle. In transgenic mice with constitutive activation of orexinergic tone (*CAG*/orexin mice), orexin is expressed in a diffuse, ectopic pattern in the brain in an unregulated fashion[43]. The mice exhibited abnormal sleep and wakefulness patterns, including fragmented NREM sleep in the light period and incomplete REM sleep atonia with abnormal myoclonic activity during REM sleep (T.S., unpublished observations). These results indicate that orexin neurons need to be switched off to maintain consolidated NREM sleep and the muscle atonia that accompanies REM sleep, but have to be activated during awake periods.

Orthodromic and antidromic activation
Neural stimulation in the same and the opposite direction of the physiological nerve conductance, respectively.

Consistent with this idea, *Fos* expression (a marker of neuronal activity) in orexin neurons in rats is increased during the dark, active period in which the awake state is dominant[44]. Moreover, orexin levels in cerebrospinal fluid peak during the dark period and decrease during the light period in which the sleep state is dominant[45]. Recent *in vivo* recording studies revealed further changes of orexin neuronal activity across the sleep–wake cycle. Mileykovskiy *et al.* recorded orthodromic and antidromic activity of VTA and LC to identify orexin neurons in unanaesthetized, unrestrained rats[46]. They found that orexin neurons were relatively inactive in quiet waking but were transiently activated during sensory stimulation. Furthermore, the neurons were silent during NREM sleep and tonic periods of REM sleep, with occasional burst discharges during phasic REM sleep.

Lee *et al.* also recorded from orexin neurons, identified using a combination of neurobiotin labelling and immunohistochemistry, in the LHA of head-fixed rats[47]. They found that the orexin neurons fired during active waking, decreased discharge during quiet waking, and virtually ceased firing during both REM and NREM sleep. Orexin neurons increased firing before the end of REM sleep and thereby heralded by several seconds the return of the awake state. Although the numbers of cells examined are too small to provide a complete picture of orexin neurons' activity across the sleep–wake cycle, these seminal studies provide the strongest evidence that these cells are activated during wakefulness, and inhibited during sleep.

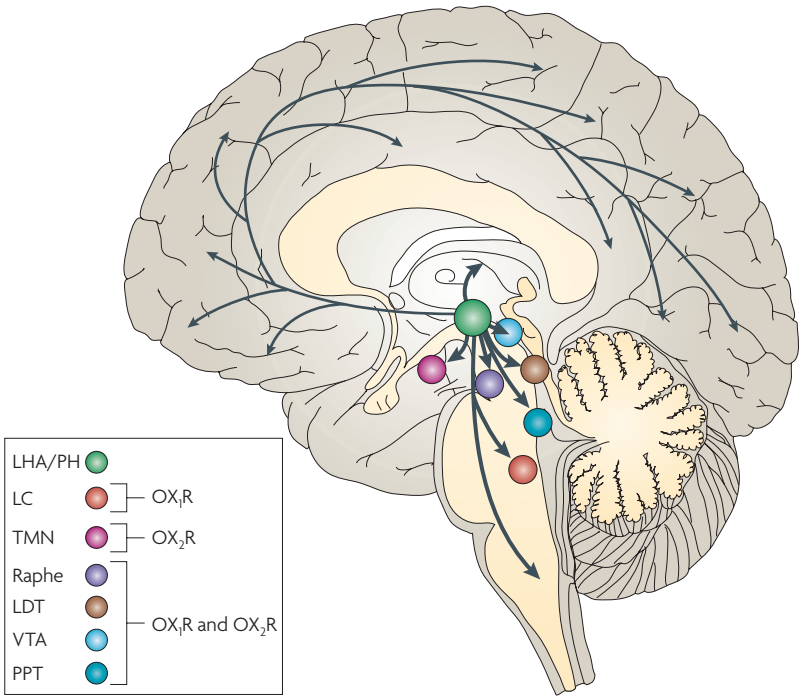

Figure 1 | **Schematic drawing showing main projections of orexin neurons.** This figure summarizes predicted orexinergic projections in the human brain. Please note that distributions of orexin fibres and receptors (OX_1R, OX_2R) are predicted from the results of studies on rodent brains, as it is on rodents that most histological studies on the orexin system have been carried out. Circles show regions with strong receptor expression and dense orexinergic projections. Orexin neurons originating in the lateral hypothalamic area (LHA) and posterior hypothalamus (PH) regulate sleep and wakefulness and the maintenance of arousal by sending excitatory projections to the entire CNS, excluding the cerebellum, with particularly dense projections to monoaminergic and cholinergic nuclei in the brain stem and hypothalamic regions[24–32,38], including the locus coeruleus (LC, containing noradrenaline), tuberomammillary nucleus (TMN, containing histamine), raphe nuclei (Raphe, containing serotonin) and laterodorsal/pedunclopontine tegmental nuclei (LDT/PPT), containing acetylcholine). Orexin neurons also have links with the reward system through the ventral tegmental area (VTA, containing dopamine) and with the hypothalamic nuclei that stimulate feeding behaviour. Anatomical image adapted, with permission, from REF. 108 © (1996) Appleton & Lange.

Other functions of orexins

Feeding behaviour and energy homeostasis. Narcolepsy patients have a decreased caloric intake but an increased body mass index, indicating that the abnormality that gives rise to narcolepsy has links to a reduced energy expenditure or a low metabolic rate[48,49]. Orexin neurons have been shown to have a role in the regulation of energy homeostasis. For example, orexin neuron-ablated mice exhibit hypophagia and late-onset obesity, although the extent to which this is the case critically depends on the genetic backgrounds of the mice[9,50].

Supporting the physiological relevance of orexin in the control of feeding, ICV administration of an anti-orexin antibody or an OX_1R-selective antagonist reduced food intake[3,51], and prepro-orexin-knockout mice and transgenic mice lacking orexin neurons ate less than control wild-type mice[9,41]. Moreover, an OX_1R-selective antagonist reduced food intake and ameliorated obesity in leptin-deficient *ob*/*ob* mice[2].

Consistent with the dense projection of orexin neurons to the arcuate nucleus[24,26,52], several studies have suggested that the increased food intake following orexin A administration is at least partly mediated by the activation of neuropeptide Y neurons in the arcuate nucleus[52,53]. Other events involved in orexin-induced feeding behaviour include the inhibition of pro-opiomelanocortin neurons in the arcuate nucleus, which are thought to have an important role in leptin-mediated inhibition of food intake[53]. Recent reports also showed that infusions of orexin A into the shell of the nucleus accumbens (NAc) increase feeding behaviour[54]. In addition, infusions of the $GABA_A$ receptor agonist muscimol into the NAc shell strongly induced food intake and simultaneously increased *Fos* expression specifically in orexin neurons[55]. These findings indicate that reciprocal interactions between the orexin and limbic systems have a role in the regulation of feeding.

Orexin-mediated maintenance of consolidated wakefulness might also be important in feeding behaviour, because 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[11,56,57]. This response is absent in transgenic mice with ablated orexin neurons[11], indicating that these neurons are crucial for evoking adaptive maintenance of arousal during fasting. In other words, if energy stores are low, the activity of orexin neurons could be modulated to maintain wakefulness, allowing more time to search for food.

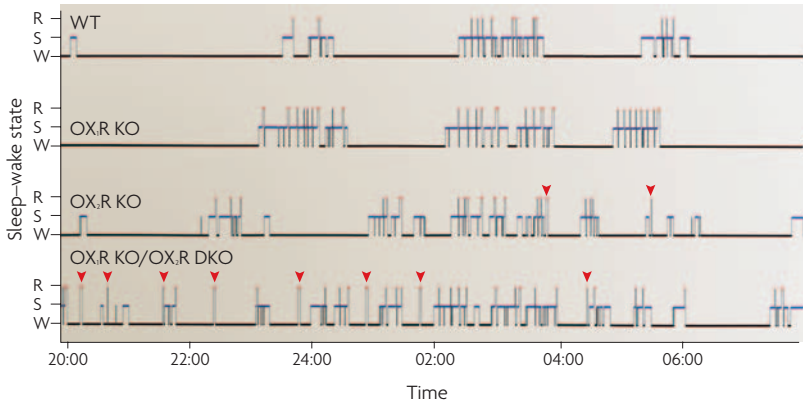

Figure 2 | **Sleep state abnormalities in orexin receptor-knockout mice.** Typical representative 12 hour dark period (20:00–08:00) hypnograms for wild-type (WT), OX_1R-knockout (OX_1R KO), OX_2R-knockout (OX_2R KO) and double-receptor knockout mice (OX_1R/OX_2R DKO), all on a C57B/6J background, are shown. The different levels above the baseline indicate states of sleep and wakefulness (R, rapid eye movement (REM) sleep; S, non-REM (NREM) sleep; W, awake) of the mouse at the time. Episodes of direct transition from wakefulness to REM sleep are shown by red arrowheads. Note the greater awake/NREM sleep episode fragmentation and reduced duration of wakefulness in the hypnograms of OX_2R-knockout and double-receptor knockout mice compared with wild-type and OX_1R-knockout mice. Episodes of direct transition from wakefulness to REM sleep were not observed in OX_1R-knockout mice, and barely in OX_2R-knockout mice, whereas they were frequently observed in double-receptor knockout mice. Hypnograms were obtained by simultaneous electroencephalography (EEG) and electromyography (EMG) recording for 4 weeks (N = 18–40).

The activity of orexin neurons also contributes to the promotion and maintenance of food anticipatory activity (FAA)[12,13]. Daily restricted feeding produces an anticipatory locomotor activity rhythm and entrains a molecular oscillator that is independent of the central clock, which is located in the suprachiasmatic nucleus (SCN). Restricted feeding shifts the peak of *Fos* expression in orexin neurons from night to the period during which feeding was restricted, indicating that orexin neurons are activated when animals need to be awake and seek food[12,13]. The establishment of FAA was severely impaired in orexin/ataxin 3 transgenic mice, in which orexin neurons are ablated[12,13]. The transgenic mice also showed reduced expression of mRNA for murine period 1 (*mPer1*), brain and muscle arnt-like protein 1 (*Bmal1*) and neuronal PAS domain protein 2 (*Npas2*), a transcription factor thought to be involved in regulating the food-entrainable oscillator. These observations indicate that orexin neurons convey an efferent signal from a 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 a robust oscillation of *mPer* gene expression only under restricted feeding[58]. The oscillation persisted for at least 2 days, even when mice were given no food during the expected feeding period after the establishment of the FAA. It has also been demonstrated that lesions in the DMH in rats blocked food entrainment of wakefulness, locomotor activity and core body temperature[59]. Taken in conjunction with recent findings that DMH neurons project to orexin neurons[14,15], these results indicate that the connection between the DMH and orexin neurons has a key role, as a central food-entrainable oscillator, in the feeding-mediated regulation of circadian behaviours.

Orexin and the autonomic nervous system. Several studies have clearly shown that orexins have a role in regulating autonomic function. It has been demonstrated that ICV orexin injections increase blood pressure and heart rate, and that these effects are abolished by the administration of drugs that block α- or β-adrenoceptors[60]. Moreover, blood pressure in orexin-deficient mice is 10–15 mmHg lower than in wild-type littermates[61,62]. These results indicate that orexins physiologically stimulate sympathetic outflow and provide a possible explanation for the increased body mass index observed in conditions of low orexin: orexin deficiency might decrease sympathetic tone, which could result in decreased energy expenditure. As might be expected in a system geared for weight gain, orexins do not slow the metabolic rate. Instead, they increase both food intake and metabolic rate[63]. Because animals must be vigilant and active when they seek and eat food, an orexin-induced increase in sympathetic nerve activity might be important for feeding behaviour.

As discussed later, the orexin-mediated increase in sympathetic tone could also be involved in the mechanisms by which the limbic system modulates the sympathetic outflow responding to emotional stimuli[61,62].

Orexin and the reward system. Anatomically, orexin neurons are well-positioned to alter reward functioning. Orexin neurons project to reward-associated brain regions, including the NAc and VTA, and orexin directly activates VTA dopaminergic neurons through OX_1R[29] (FIG. 3). This indicates a possible role for orexins in reward function and motivation, consistent with previous studies implicating orexins in feeding. In fact, the activation of orexin neurons was shown to be strongly linked to preferences for cues associated with drug and food rewards[16]. Dopaminergic neurons that originate in the VTA and project into the forebrain, particularly the NAc, have classically been identified as the 'reward pathway'. Drugs of abuse stimulate this pathway. ICV or local VTA infusions of orexin have been shown to reinstate drug-seeking or food-seeking behaviour in rodents[10,16]. Conversely, the subcutaneous morphine (μ-opioid receptor agonist)-induced place preference and hyperlocomotion observed in wild-type mice were abolished in mice that lacked the prepro-orexin gene[17], and injections of an OX_1R antagonist into the VTA block the development of morphine-conditioned place preference[17]. These observations indicate the strong functional interaction between orexinergic pathways and the dopaminergic system.

Recent work has provided interesting insights into the cellular and molecular mechanisms underlying these effects by showing that orexin A input to the VTA potentiates NMDAR (*N*-methyl-D-aspartate receptor)-mediated neurotransmission through a protein kinase C-dependent insertion of NMDARs in VTA dopamine neuron synapses in slice preparations[64].

Food anticipatory activity
(FAA). Behavioural activation induced by restricted access to food; a manifestation of the food-entrained oscillator.

mPer1
The *PER1* gene is a core clock factor that has an essential role in generating circadian rhythms. *mPer1* is the mouse counterpart of the human *PER1* gene.

Bmal1
Bmal1 (brain and muscle arnt-like protein 1) is a putative clock gene which encodes a basic helix-loop-helix-PAS transcription factor.

Furthermore, *in vivo* administration of an OX_1R antagonist blocks locomotor sensitization to cocaine and occludes cocaine-induced potentiation of excitatory currents in VTA dopamine neurons[64]. These results suggest an important role for orexin signalling in the VTA in the neural plasticity associated with reward, and indicate that orexins also contribute to cocaine-induced psychomotor sensitization and reward-seeking. These findings highlight the key role of orexin in the mechanisms of reward and drug addiction. Consistently, prepro-orexin-knockout mice are less susceptible than wild-type animals to developing morphine dependence, as measured by physical withdrawal responses[65]. Interestingly, some narcolepsy patients with daytime sleepiness who were treated with amphetamine-like stimulants and/or sodium oxybate (γ-hydroxybutyrate, also known as GHB) for a long time rarely developed drug abuse[66].

Orexin and the stress response. Orexin influences neuroendocrine function, and thereby affects arousal and the stress response. For example, ICV injection of orexin stimulates the hypothalamic–pituitary–adrenal (HPA) axis[67] and decreases prolactin secretion[23]. ICV administration of orexin A strongly activates corticotropin releasing factor (CRF)-expressing neurons in the periventricular hypothalamic nucleus (PVN) and the central nucleus of the amygdala (CeA)[68]. The link between the CRF system and orexin neurons is reciprocal[69], and might maintain wakefulness during stressful events.

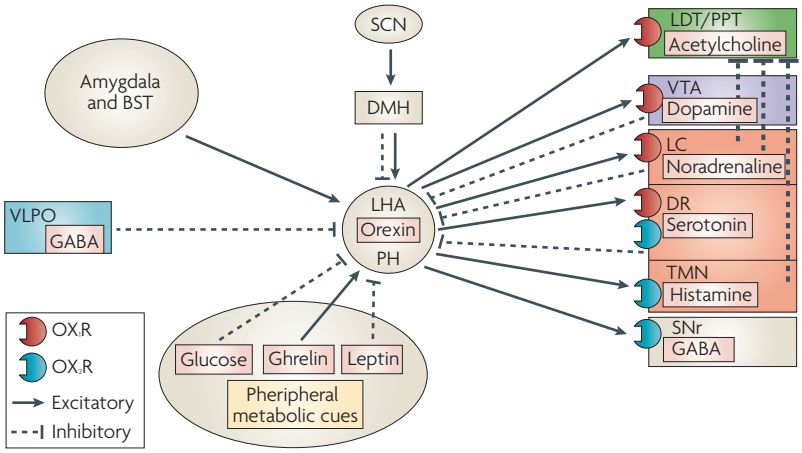

Figure 3 | **Interactions of orexin neurons with other brain regions implicated in sleep and wakefulness.** Orexin neurons in the lateral hypothalamic area (LHA) and posterior hypothalamus (PH) are anatomically well placed to provide a link between the limbic system, systems involved in energy homeostasis and monoaminergic and cholinergic neurons in the brain stem. Solid arrows show excitatory projections, and broken lines inhibitory ones. Wake-active regions, sleep-active regions and REM-active regions are shown by red, blue and green boxes, respectively. Orexin neurons promote wakefulness through the monoaminergic nuclei that are wake-active. Stimulation of dopaminergic centres by orexins can modulate reward systems (purple). Peripheral metabolic signals such as leptin, ghrelin and glucose influence orexin neuronal activity to coordinate arousal and energy homeostasis. The nucleus suprachiasmaticus (SCN), the central body clock, sends signals to orexin neurons via the dorsomedial hypothalamus (DMH). The DMH acts as a food-entrainable ossilator, and influences orexin neuronal activity. Input from the limbic system (amygdala and bed nucleus of the stria terminalis (BST)) might regulate the activity of orexin neurons upon emotional stimuli to evoke emotional arousal or fear-related responses[61,62]. VLPO, ventrolateral preoptic area; DR, dorsal raphe; GABA, γ-aminobutyric acid; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; SNr, substantia nigra pars reticulata; TMN, tuberomammillary nucleus.

Mechanisms regulating orexin neuron activity

To establish the physiological relevance of orexins, understanding the system that regulates the activity of orexin neurons is key. This section discusses several factors and systems involved in this regulation.

Neurotransmitters and neuromodulators. Electrophysiological studies have identified several neurotransmitters and neuromodulators that activate or inhibit the activity of orexin neurons (TABLE 1). By recording from hypothalamic slices of transgenic mice that express green fluorescent protein (GFP) selectively in orexin neurons, it was shown that agonists of ionotropic glutamate receptors (AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA) excite orexin neurons, whereas glutamate antagonists (AP5 (D(–)-2-amino-5-phosphonovaleric acid), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) or NBQX (6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione)) reduce their activity[70,71]. These results indicate that orexin neurons are tonically activated by glutamatergic neurons.

In addition, several other neurotransmitters have been shown to influence the activity of orexin neurons. Importantly, both noradrenaline and serotonin (5-hydroxytryptamine, 5-HT) hyperpolarize and inhibit GFP-expressing orexin neurons through the activation of G-protein-regulated inwardly rectifying K^+ (GIRK or Kir3) channels by α_2-adrenoceptors and $5\text{-}HT_{1A}$ receptors, respectively[70–72]. The cholinergic agonist carbachol activates 27% and inhibits 6% of orexin neurons[14,71], whereas histamine seems to have no effect on orexin neurons. These observations indicate that serotonin and noradrenaline neurons might send inhibitory feedback projections to orexin neurons. Furthermore, although orexin neurons do not express functional dopamine receptors, dopamine can inhibit orexin neurons by acting on α_2-adrenoceptors[71,72].

A recent study showed that a short 2 hour period of total sleep deprivation changed the action of noradrenaline on orexin neurons from excitation to inhibition. This mechanism might contribute to the growing sleepiness that accompanies sleep deprivation[73], although this phenomenon was not observed in mice[72].

Using transgenic mice in which orexin neurons specifically express a genetically encoded intracellular calcium indicator (Yellow Cameleon, Yc2.1), we screened for factors that affect the activity of orexin neurons and found that a sulphated octapeptide form of cholecystokinin (CCK-8S), as well as neurotensin, oxytocin and vasopressin activate orexin neurons[74], whereas GABA, glucose, serotonin, noradrenaline and leptin inhibit them (TABLE 1). Finally, a recent paper described how

Table 1 | **Factors that influence the activity of orexin neurons**

Factor	Receptor involved	References
Excitatory		
Glutamate	AMPAR, NMDAR, mGluRs	11,70
Ghrelin	GHSR	71
Cholecystokinin	CCK-A	74
Neurotensin	ND	74
Vasopressin	V1a	74
Oxytocin	V1a	74
Glucagon-like peptide 1	ND	105
CRF	CRFR1	69
mACh (effect in 27% of orexin neurons)	M3	14
ATP	P2X	106
Inhibitory		
Glucose	Unknown	11
GABA	$GABA_A$, $GABA_B$	11,70,89
Serotonin	$5\text{-}HT_{1A}$	71,91
Noradrenaline	α_2	71,72
Dopamine	α_2	71
Neuropeptide Y	Y_1	107
Leptin	OB-R	11
mACh (effect in 6% of orexin neurons)	ND	14,71
Adenosine	A_1	75

α_2, α_2 adrenergic receptor; $5\text{-}HT_{1A}$, 5-hydroxytryptamine receptor 1A; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; CCK-A, cholecystokinin receptor A; CRFR1, corticotropin-releasing factor receptor 1; GABA, γ-aminobutyric acid; GHSR, growth-hormone secretagogue receptor; mACh, muscarinic acetylcholine; mGluRs, metabotropic glutamate receptors; ND, not determined; NMDAR, *N*-methyl-D-aspartate receptor; OB-R, leptin receptor; P2X, purinocepter.

adenosine inhibits orexin neurons via the adenosine A_1 receptor[75]. This mechanism might relate to the sleep-promoting effect of adenosine[75].

Humoral factors. Metabolic signals also contribute to the regulation of orexin neuron activity: decreasing the extracellular glucose concentration produced depolarization and increased the frequency of action potentials in orexin neurons, whereas increasing it induced marked hyperpolarization and cessation of action potentials in the same neurons[11,76]. Importantly, this mechanism is sufficiently sensitive to encode variations in glucose levels reflecting those occurring physiologically between normal meals[11,76].

A recent study demonstrated that the inhibition of orexin neurons by glucose is mediated by tandem-pore K^+ (K_{2P}) channels[77]. Glucose seemed to act at an extracellular site on orexin neurons, as it inhibited orexin neurons only when applied extracellularly[77]. An undetermined intracellular messenger that was not ATP, Ca^{2+} or glucose itself transmitted this information to the channels[77]. These results reveal an unexpected energy-sensing pathway in neurons that regulates states of wakefulness and energy balance[77].

Ghrelin applied in a superfused solution activated 60% of dispersed orexin neurons, with depolarization and an increase in action potential frequency[11]. By contrast, bath-application of leptin was found to robustly inhibit most of the orexin neurons examined, causing hyperpolarization and a decrease in firing rate[11]. Notably, insulin exerted no direct effect on orexin neurons[11].

The above findings show that peripheral humoral factors that are related to energy metabolism influence the activity of orexin neurons. In addition, orexin expression in wild-type and *ob*/*ob* mice is negatively correlated with changes in blood glucose, leptin and food intake[11]. This is consistent with the idea that orexin neurons act as sensors of the nutritional status of the body[1,11,41].

Orexin neurons have been shown to be stimulated by hypoglycemia at least partly via the nucleus of the solitary tract (NTS)[78,79], indicating that peripheral metabolic cues might also influence the activity of orexin neurons indirectly through vagal afferents and the NTS.

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 behaviourally with phases of increased wakefulness and alertness that presumably enhance the ability to find food[56,57]. Orexin neuron-ablated mice fail to exhibit this fasting-induced arousal[11], indicating that orexin neurons are necessary for evoking adaptive behavioural arousal during fasting. So, nutritional depletion-induced metabolic cues activate orexin neurons, and orexin increases arousal, thereby reinforcing food-seeking/feeding pathways. These mechanisms might also be important in the maintenance of prolonged wakefulness during the active period; in the regulation of energy homeostasis that helps to ensure survival; and, interestingly, might hinder attempts to treat obesity by food restriction. This might also explain why orexin receptor antagonists decrease food intake[3].

Neuronal input. Until recently, very little was known about the synaptic input into hypothalamic orexin neurons, and this seems to be largely because of the challenges associated with the cells being dispersed mediolaterally within the LHA. In mice with a genetically encoded retrograde tracer, the neuronal populations that send afferent innervations to orexin neurons were mapped[14]. Labelled cells were identified in multiple brain regions, including the basal forebrain cholinergic neurons, GABA-containing neurons in the ventrolateral preoptic nucleus (VLPO), neurons in the posterior/dorsomedial hypothalamus and serotonergic neurons in the raphe nuclei. Labelled neurons were also found in regions associated with emotion including the amygdala, infralimbic cortex, NAc shell, lateral septum and the bed nucleus of the stria terminalis (BST).

By combining antero- and retrograde tracers, a study mapped afferents of orexin neurons in rats and found that hypothalamic orexin neurons received abundant projections from the lateral septum, preoptic area, BST and posterior hypothalamus[15]. In addition, it was found that hypothalamic regions preferentially innervated

Box 3 | **The extended amygdala and emotion**

A key component of the neural circuitry of emotion in animals is the amygdala and its related regions (extended amygdala), which consist of a well-defined subcortical nuclear group that in vertebrates is a centre for emotional responses, including fear[102–104]. The amygdala receives many kinds of sensory information directly from the periphery, or via the thalamus and cortex. For example, sensory stimuli that predict an aversive outcome will change neural transmission in the amygdala to produce the somatic, autonomic and endocrine signs of fear, as well as increased attention and arousal to that stimulus. Consolidation of emotional memory involves lateral and basolateral parts of the amygdala, where the association between incoming sensory stimuli leads to potentiation of synaptic transmission. These parts project to the central amygdala (CeA), which in turn sends efferents to the hypothalamus and brain stem that trigger the expression of emotions including arousal, autonomic and endocrine responses. The regions closely associated with the amygdala are also important for fear learning, and include the bed nucleus of the stria terminalis (BST). Orexin neurons have been shown to receive innervations from these regions[14,15], indicating that these cells have a role in the emergence of emotional responses, such as increased arousal and sympathetic outflow during fearful events.

the medial and perifornical parts of the orexin neuron field, but most projections from the brainstem targeted the lateral part of the field, indicating a functional dichotomy of orexin neurons.

However, tracing studies might not show all input to orexin neurons. Monoaminergic and peptidergic systems sometimes use 'volume transmission', which includes short- (but larger than the synaptic cleft, that is, roughly 20 nm) and long-distance diffusion of signals through the extracellular and cerebrospinal fluid[80]. Therefore, although tracing studies showed that projections of noradrenergic and dopaminergic neurons to orexin neurons are sparse, it is important not to disregard the effects that these factors might have on orexin neurons (TABLE 1).

Some studies have revealed that orexin neurons show apposition from peptidergic fibres, including neuropeptide Y, pro-opiomelanocortin and galanin-like peptide fibres[81,82]. Again, these data must be interpreted carefully; such chemically defined 'apposition' does not necessarily mean that the nerve terminals functionally synapse onto orexin neurons.

Finally, not only synaptic receptors contribute to regulating neuronal activity. Extrasynaptic receptors can sense ambient ligands such as CCK, leptin and glucose, which can act as a neuromodulators on orexin neurons[11,74] (TABLE 1).

Interactions with other neuronal systems

Orexin neurons interact with multiple neuronal systems (FIG. 3). These interactions provide a key to understanding the physiological roles of orexin neurons.

Input from the limbic system. Arousal resulting from emotional stimuli or fear-related responses increases sympathetic outflow. Orexin neurons receive input from the limbic system[14,15,69], indicating a role for this system in the regulation of orexin neuron activity (BOX 3). Indeed, the importance of this connection is readily apparent in the defence, or 'fight or flight', response: mice tested in a resident–intruder paradigm show cardiovascular and locomotor responses to the emotional stress evoked by this test, but these responses are diminished in prepro-orexin-knockout mice[83]. Similarly, air-jet stress-induced elevations of blood pressure and heart rate were attenuated in conscious orexin/ataxin 3 transgenic mice, in which orexin neurons are ablated[62].

Limbic inputs to orexin neurons include the CRF neurons that originate in the amygdala[69]. They activate orexin neurons through the CRF-R1 receptor[69]. The reciprocal link between the CRF system and orexin neurons might maintain wakefulness during stressful events. Indeed, activation of orexin neurons by foot shock stress is severely impaired in *CRF-R1*-deficient mice, indicating that such activation is mediated by CRF[69].

The neural input from the limbic system to orexin neurons might be implicated in the pathophysiology of cataplexy, because strong, generally positive emotional stimuli are known to trigger cataplexy in narcolepsy–cataplexy patients. A local injection of orexin into the PPT strongly inhibited REM-related atonia in cats[39]. Cholinergic neurons in the LDT/PPT are implicated in REM-related atonia[84], and the same pathway is implicated in cataplexy. Therefore, emotional stimuli might increase orexin release in the PPT to prevent muscle atonia in wild type animals. Projections to orexin neurons from the limbic system might also be important for maintaining orexin neuron activity during the active period by conveying various emotional stimuli to orexin neurons (FIG. 3).

The limbic input to orexin neurons might also be involved in the regulation of feeding behaviour, because some of the affective content of the perception of food is thought to be processed in the amygdala and limbic system[85], and this information might be passed on to orexin neurons. Food perception often evokes cataplexy in narcoleptic dogs[86], indicating that orexin signalling is physiologically activated on perception of food, and that this system is necessary to evoke proper feeding behaviour.

Input from preoptic areas. The preoptic area, especially the VLPO, seems to have a crucial role in NREM sleep initiation and maintenance. Neurons in the VLPO fire at a rapid rate during sleep, with attenuation of firing during wakefulness. GABA and galanin are the primary inhibitory neurotransmitters of the VLPO[87], which sends out multiple inhibitory projections to the LC, TMN and DR[87,88].

Orexin neurons, which are innervated by GABA-containing cells in the VLPO[14,15], are strongly inhibited by both the $GABA_A$ agonist muscimol and the $GABA_B$ receptor agonist bacrofen[11,89], indicating that the VLPO might be a source of GABA-containing inhibitory projections to orexin neurons. This pathway might be important for turning off orexin neurons during sleep (FIGS 3,4).

Input from the SCN. Although direct input to orexin neurons from the SCN seems to be sparse, orexin neurons receive abundant innervations from the BST, supraventricular zone and DMH[14,15], all of which receive input from the SCN. This indicates that orexin neurons might receive circadian influences indirectly from the SCN via these regions[90].

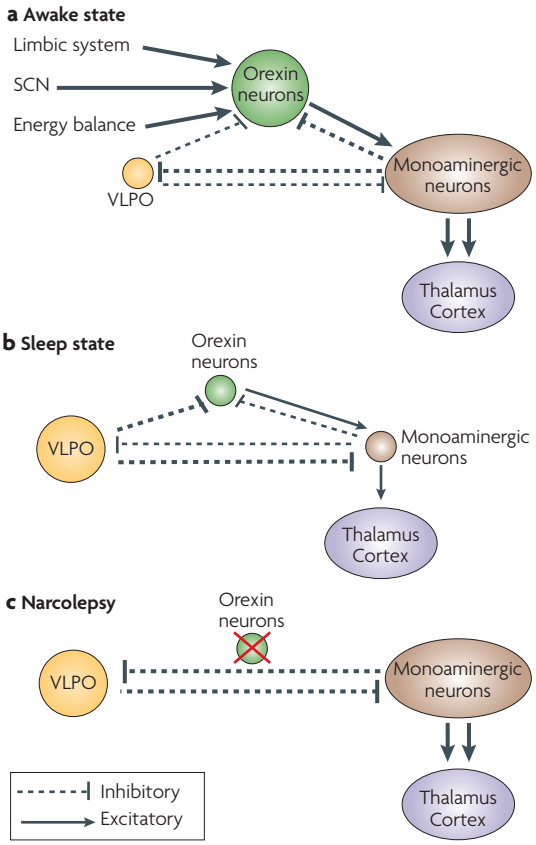

Figure 4 | **Mechanisms by which the orexin system stabilizes sleep and wakefulness.** The figures represent functional interactions between orexin neurons, monoaminergic wake-active centres and the ventrolateral preoptic area (VLPO) sleep-active centre during various states of sleep and wakefulness. Solid arrows show excitatory input, and broken lines inhibitory input. The thickness of arrows and lines represents the relative strength of excitatory and inhibitory input, respectively. Circle sizes represent relative activities of each region. **a** | Awake state. Orexin neurons send excitatory influences to monoaminergic neurons, which send inhibitory feedback projections to orexin neurons. This system might maintain the activity of monoaminergic neurons. A slight decrease in input to the monoaminergic neurons results in decreased inhibitory influence to orexin neurons. Orexin neurons, therefore, are disinhibited and increase excitatory influence to monoaminergic cells to maintain their activity. These monoaminergic cells send excitatory projections to the thalamus and cerebral cortex, and send inhibitory projections to the VLPO sleep centre. These mechanisms maintain wakefulness states. **b** | Sleep state. VLPO sleep-active neurons are activated and send inhibitory projections to monoaminergic neurons and orexin neurons to maintain sleep. **c** | Narcolepsy. If orexin neurons are removed, monoaminergic neurons and VLPO neurons set up a mutually inhibitory circuit, which can cause unwanted and abrupt transitions between the states. Activity in one of the competing sides shuts down inhibitory inputs from the other side, and therefore disinhibits its own action. So, when either side begins to overcome the other, the switch abruptly turns into the alternative state.

Mechanisms that stabilize sleep and wakefulness

Orexin neurons are an important component of the neural circuits that regulate sleep and wakefulness. How, then, do these neurons stabilize sleep and wakefulness through these circuits?

As previously discussed, a feedback loop between orexin neurons and monoaminergic neurons in the brain stem including the LC and DR[71,72,91] might maintain the activity of monoaminergic neurons. Decreases in monoaminergic neuron activity will decrease the inhibitory influence on orexin neurons. This disinhibition of orexin neurons then increases the excitatory influence on monoaminergic cells, thereby increasing their activity (FIG. 4).

Sleep-active, GABA-containing neurons in the VLPO send descending projections that terminate within wake-promoting populations in the TMN, LC and DR[88]. During sleep, VLPO sleep-active neurons are thought to be activated by sleep substances such as adenosine[92–94], and send inhibitory influences to monoaminergic neurons in the brain stem and hypothalamus. As discussed above, the sleep-active neurons also send inhibitory projections to hypothalamic orexin neurons[15,95].

These circuits are important for the regulation of wakefulness. If orexin neurons are removed from this system, as is the case in narcolepsy–cataplexy, the sleep-active neurons in the VLPO and monoaminergic neurons exhibit a 'flip-flop' property, owing to the mutual inhibition between these two neuronal elements[96]: monoaminergic neurons send inhibitory influences to VLPO sleep-active neurons and vice versa[97]. In such a circuit, when activity on either side begins to overcome the other, the system will flip into one of two possible extremes, because when a small perturbation gives one side a sudden 'advantage', it will turn off the alternative side abruptly[96]. A circuit of this type is thought to underlie the pathology of narcolepsy (FIG. 4).

Sleep modulation using the orexin system

Because narcolepsy–cataplexy is a disorder of sleep–wake cycle organization resulting from the absence of orexin, it is perhaps logical to consider that replacement therapy using orexin receptor agonists could provide an effective treatment for this disorder. Indications that this might be successful came from a study which showed that chronic overproduction of orexin peptides from an ectopically expressed transgene prevented the development of a narcolepsy syndrome in orexin neuron-ablated (orexin/ataxin 3 transgenic) mice[43]. Acute ICV administration of orexin A also maintained wakefulness, suppressed sleep and inhibited cataplectic attacks in orexin/ataxin 3 mice[43]. In fact, ICV administration of orexin A had stronger arousal effects in orexin/ataxin 3 transgenic mice than in wild-type controls[43]. The greater effectiveness might not have resulted from increased expression of orexin receptors[43]. Rather, in the orexin/ataxin 3 mice, monoaminergic neurons in the brain stem became more sensitive to various stimuli (T.S., unpublished observations). This mechanism might explain why narcoleptics cannot maintain long, consolidated NREM sleep periods.

The effectiveness of ICV-administered orexin in animals with a narcoleptic phenotype indicates that orexin receptor agonists would be of potential value for treating narcolepsy. However, as mentioned above, chronic overexpression of orexin in an unregulated fashion results in disruption of NREM sleep, and therefore it will be beneficial for therapeutically relevant orexin agonists to have a short half-life (< 12 hours). Conversely, orexin antagonists might be effective as a sleep-inducing drug.

Conclusion and perspectives

The symptoms and the cellular and systems-level bases of narcolepsy–cataplexy unequivocally show that orexins and orexin receptors are important regulators of sleep and wakefulness and of arousal maintenance by regulating monoaminergic and cholinergic nuclei in the brain. Orexin neurons receive afferents from multiple neuronal systems, and send excitatory signals to monoaminergic and cholinergic nuclei in the brain stem. Interactions with these systems indicate physiological functions of orexin neurons, as discussed in this review. These functions should be further explored in future studies using conditional knockouts of receptors that are expressed in orexin neurons. A more precise understanding of the mechanisms that regulate orexin neurons might provide further insights into how the systems that regulate emotion, energy homeostasis and reward interact with the mechanism that regulates sleep and wakefulness.

Note added in proof

A recent paper showed that a new, potent, orally available dual orexin receptor antagonist, ACT-078573, which blocks both OX_1R and OX_2R, effectively promotes sleep in rats, dogs and humans[109].

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