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## Discovery of Orexin (Hypocretin)

Neuropeptides orexin-A and orexin-B (hypocretin-1 and hypocretin-2, respectively) were initially reported in 1998 independently by two laboratories. Sakurai et al. identified these peptides as endogenous ligands for two orphan G-protein-coupled receptors (GPCR) [1]; GPCRs for which endogenous ligands are unknown are referred to as “orphan” GPCRs. Since intracerebroventricular (ICV) injection of these peptides in rats acutely stimulated food consumption, they were named orexin-A and orexin-B after the Greek word *orexis*, meaning “appetite.” Orexin-A and orexin-B are produced by cleavage of prepro-orexin, a single precursor polypeptide. Mammalian orexin-A is a 33-amino-acid peptide with two intrachain disulfide bonds that undergo pyroglutamylation and amidation at its N- and C-terminals, respectively, while orexin-B is a 28-amino-acid linear peptide that undergoes C-terminal amidation (Fig. 2.1a).

De Lecea et al. previously identified 38 rat mRNAs selectively expressed within the hypothalamus. They found that one of those mRNAs,

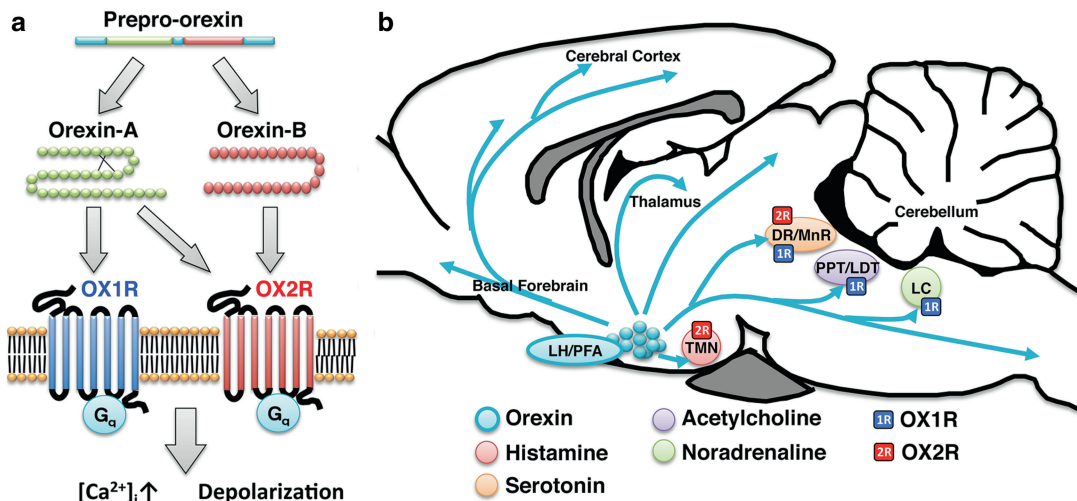
the clone 35, was expressed exclusively by a bilaterally symmetric structure within the posterior lateral hypothalamus [2]. The gene from which this clone derived encoded a polypeptide identical to prepro-orexin and named the putative mature peptides hypocretin-1 (orexin-A) and hypocretin-2 (orexin-B). Although the initial estimated structures of hypocretin-1 and hypocretin-2 were not the same as those of orexin-A and orexin-B, the terms “orexin” and “hypocretin” are currently used as synonyms in many papers.

The actions of orexins are mediated by two G-protein-coupled receptors, named orexin 1 (OX1R) and orexin 2 (OX2R) receptors (also known as HCRT1 and HCRT2) [1] (Fig. 2.1a). OX1R has a one-order higher affinity for orexin-A than for orexin-B, while OX2R binds orexin-A and orexin-B with similar affinities. Both receptors are coupled to the  $G_{q/11}$  subclass of G-proteins and have caused strong excitatory effects on neurons examined thus far [3], except in one study that reported the direct inhibitory action of orexin receptors on suprachiasmatic nucleus (SCN) neurons at night [4]. When over-expressed, OX2R has also been reported to couple to  $G_{i/o}$  in a neuronal cell line, suggesting that OX2R could exert inhibitory action in some neurons [5].

Neurons expressing orexins (orexin neurons) are distributed within an area consisting of three contiguous hypothalamic regions: the lateral hypothalamus (LH), perifornical area (PFA),

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**Fig. 2.1** The orexin system. **(a)** Orexin and orexin receptors. Orexin-A and orexin-B are derived from prepro-orexin, a common precursor peptide. The actions of orexins are mediated by two G-protein-coupled receptors: the OX1R and OX2R receptors. OX1R is selective for orexin-A, whereas OX2R shows similar affinities for both orexin-A and orexin-B. Both receptors are coupled to the G<sub>q/11</sub> subclass of G-proteins and cause strong excitatory effects on neurons. **(b)** Schematic drawing showing main projections of orexin neurons, through which the former may promote wakefulness. Circles show regions with strong receptor expression and dense orexinergic projections. Orexin neurons originating in the lateral hypotha-

lamic (LH) and perifornical (PFA) areas regulate sleep and wakefulness and the maintenance of arousal by sending excitatory projections to the entire central nervous system, excluding the cerebellum, with particularly dense projections to monoaminergic and cholinergic nuclei in the brainstem and hypothalamic regions, including the locus coeruleus (LC), which contains noradrenaline; the tuberomammillary nucleus (TMN), which contains histamine; raphe nuclei (raphe), which contain serotonin; and pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT), which contain acetylcholine. OX1R and OX2R show differential distributions in these brainstem cholinergic/monoaminergic neurons

and dorsomedial hypothalamic nucleus (DMH) (Fig. 2.1b) [1, 2, 6–8]. The number of these neurons has been estimated to be from 3000 to 4000 in rat and 70,000 in human brains [9, 10]. In contrast to the restricted localization of their cell bodies, orexin neurons send projections throughout the central nervous system (CNS), including the cerebral cortex, limbic system (such as the amygdala, bed nucleus of stria terminalis [BST], and hippocampus), hypothalamus (such as the arcuate nucleus [ARC] and tuberomammillary nucleus [TMN]), and brain stem area (such as the central gray, locus coeruleus [LC], and raphe nuclei) [6–8, 11]. Consistent with the broad projections of orexin neurons, OX1R and OX2R show partly overlapping but distinct distributions of their mRNA throughout the CNS [12, 13]. Concerning the nuclei implicated in the regulation of sleep and wakefulness, the LC, laterodorsal tegmental nucleus (LDT), and pedun-

culopontine tegmental nucleus (PPT) mainly express *OX1R* mRNA, while the TMN almost exclusively expresses *OX2R* mRNA (Fig. 2.1b).

### Disruption of the Orexin System Causes Narcolepsy–Cataplexy

Human narcolepsy is a debilitating neurological disease that affects approximately 1 in 2000 individuals in the United States [14–16]. Onset of the condition is usually during adolescence (approximately 12–14 years old). A cardinal symptom of the disorder is excessive daytime sleepiness (EDS, an insurmountable urge to sleep), which manifests itself primarily when the subject falls asleep at inappropriate times (“sleep attacks”). When normal individuals fall asleep, a certain period of non-rapid eye movement (NREM) sleep (approximately 90 min) precedes rapid eye move-

ment (REM) sleep. However, the latency of REM sleep is markedly reduced in narcolepsy patients. REM sleep is sometimes observed immediately after wakefulness (sleep-onset REM period, or SOREMP). Nocturnal sleep is also fragmented in patients and often accompanied by hypnagogic hallucinations, vivid dreaming, and sleep paralysis, which usually occur near sleep onset. Narcolepsy patients often suffer from a condition called “cataplexy,” which is characterized by a sudden weakening of muscle tone (muscle atonia), ranging from jaw dropping and speech slurring to complete bilateral collapse of the postural muscles. These attacks are often triggered by emotional stimuli such as laughter, excitement, and pleasure. Consciousness is preserved during cataplexy. Around 10 % of narcolepsy patients do not suffer from cataplexy, although they experience excessive daytime sleepiness. Therefore, narcolepsy with cataplexy is sometimes referred to as “narcolepsy–cataplexy” to stress the occurrence of cataplexy. *The International Classification of Sleep Disorders, Third Edition* (ICSD-3) classifies narcolepsy as either type 1 or type 2. Type 1 narcolepsy (narcolepsy with cataplexy) is defined as EDS that persists for at least 3 months, accompanied with at least two of the following: clear-cut cataplexy, a positive result on the Multiple Sleep Latency Test (MSLT, mean sleep latency is shorter than 8 min and two or more SOREMPs), or low levels of orexin in CSF. Type 2 narcolepsy (narcolepsy without cataplexy) is diagnosed as EDS that, in the presence of normal levels of orexin, persists for at least 3 months and scores a positive result on the MSLT.

The symptoms of narcolepsy can be divided into two independent pathological phenomena [17, 18]. One is the inability to maintain a consolidated awake period, characterized by abrupt transitions from wakefulness to NREM sleep (i.e., dysregulation of NREM sleep onset). This phenomenon manifests clinically as excessive daytime sleepiness or sleep attacks. The other phenomenon is the pathological intrusion of REM sleep or REM atonia into wakefulness or at sleep onset (i.e., dysregulation of REM sleep onset). It is during these periods that patients may experience cataplexy, hypnagogic hallucinations, and sleep paralysis.

Soon after the discovery of orexins, two independent studies using forward genetics with canines and reverse genetics with mice, respectively, elucidated a causal linkage between disruption of orexin signaling and narcolepsy–cataplexy. For decades, a Stanford University group has established and maintained canine breeds with autosomal recessive inheritance of a narcolepsy syndrome [19]. This canine model of narcolepsy displays emotionally triggered cataplexy, fragmented sleep patterns, excessive daytime sleepiness, and a higher frequency of SOREMP. In 1999, Lin et al. identified functionally null mutations in the *OX2R* gene responsible for canine narcolepsy by positional cloning [20].

Around the same time, Chemelli et al. reported that prepro-orexin knockout mice (*orexin*<sup>−/−</sup>) exhibit a phenotype strikingly similar to human narcolepsy [21]. They exhibit frequent sudden collapses during the dark phase, the portion of the circadian rhythm during which there is the most time awake and spent in activity. These attacks resemble human cataplexy attacks. Electroencephalogram/electromyogram (EEG/EMG) recordings correlated these attacks with direct transitions from wakefulness to REM sleep, suggesting that they are homologous to cataplexy. Quantitative sleep state parameters in *orexin*<sup>−/−</sup> mice revealed significantly decreased waking time, increased NREM and REM sleep time, decreased REM sleep latency, and, most importantly, a markedly decreased duration of waking episodes during the dark phase (i.e., inability to maintain a long awake period). Consistent with a presumed critical role of orexin in the regulation of sleep and wakefulness, orexin-immunoreactive nerve terminals were observed on neurons implicated in arousal regulation, including LC noradrenergic neurons, raphe serotonergic neurons, TMN histaminergic neurons, and PPT/LDT and basal forebrain cholinergic neurons. Subsequently, orexin receptor subtypes turned out to be expressed in these regions with different expression patterns, implying their differential role in the regulation of sleep and wakefulness (Fig. 2.1b) [12, 13].

Shortly afterward, disruptions of the orexin system in human narcolepsy were confirmed.

In contrast to normal control individuals, approximately 90 % of narcolepsy with cataplexy patients have low or undetectable levels of orexin neuropeptides in the cerebrospinal fluid (CSF) (<110 pg/mL) [22, 23]. Drastic reductions of *orexin* mRNA and immunoreactivity in postmortem brains of narcoleptic patients were also shown [24, 10]. A recent finding revealing the concomitant loss of dynorphin, neuronal activity-regulated pentraxin, and orexin, all of which colocalize in orexin neurons, strongly indicates a selective loss of orexin neurons in narcolepsy, instead of the selective inhibition of *orexin* gene expression [25]. Because narcolepsy is closely associated with *HLA-DQB1\*06:02*, polymorphisms in the T-cell receptor  $\alpha$  and *P2RY11* genes, and the pandemic anti-H1N1 vaccination, narcolepsy is likely to be caused by a selective autoimmune degeneration of orexin neurons [14, 26–29]. An increasing number of patients with a milder form of typical narcolepsy (type 2 narcolepsy), which involves EDS and SOREMPs yet without cataplexy, are being recognized [16]. In contrast to narrowly defined narcolepsy by the presence of cataplexy, most people (>75 %) diagnosed with narcolepsy without cataplexy have normal CSF orexin-A concentrations [22].

In addition to the evidence described above, the selective degeneration of orexin neurons has been demonstrated to cause narcolepsy with cataplexy in mice and rats [30–32], while sporadic canine narcolepsy has been associated with substantially decreased concentrations of orexin-A (hypocretin-1) in the CSF and brain [33]. Collectively, these studies established that the disruption of the orexin system causes narcolepsy–cataplexy.

Importantly, narcoleptic symptoms of animal models can be prevented by the replacement of orexins. Chronic overproduction of orexin peptides from an ectopically expressed transgene prevented the development of narcolepsy syndrome in orexin neuron-ablated mice [34]. Furthermore, acute ICV administration of orexin-A maintained wakefulness, suppressed sleep, and inhibited cataplectic attacks in these mice [34]. These results indicate that orexin neuron-ablated mice retain the ability to respond to orexin neuro-

peptides and that spatially targeted secretion of orexin is unnecessary in preventing narcoleptic symptoms. A similar result was also obtained by Fujiki et al., who demonstrated that orexin-A administered intravenously in an extremely high dose induced a very brief antiepileptic effect in an orexin-deficient narcoleptic canine [35]. Unfortunately, constitutive production of orexin peptides from a prepro-orexin transgene in mice caused fragmentation of NREM sleep episodes in the light period, when mice spend the most time asleep [34, 36]. These results indicate that orexin neurons should be turned on and switched off to maintain consolidated wakefulness and NREM sleep, respectively. Thus, orexin receptor agonists with half-lives of several hours (<12 h) would be of potential value for treating human narcolepsy–cataplexy. Such agonists might also be useful in the treatment of other conditions of excessive daytime sleepiness in humans.

Conversely, orexin receptor antagonists might be useful as safe hypnotics. For instance, suvorexant (Belsomra, Merck), an orally available antagonist of OX<sub>1</sub>R and OX<sub>2</sub>R, has been reported to increase subjective and objective electrophysiological signs of sleep in humans [37], approved for sale by the US Food and Drug Administration (FDA), and is now available in US and Japan.

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## The Regulation of Sleep and Wakefulness by Orexin Peptides

Sleep and wakefulness are controlled by a complex network of neurotransmitters and neuromodulators [38, 39]. Monoaminergic neurons, including LC noradrenergic, dorsal and median raphe (DR and MnR) serotonergic, and TMN histaminergic neurons, project diffusely to the cerebral cortex, thalamus, and brainstem, as well as are thought to promote arousal. They are active during wakefulness, reduce their firing rates during NREM sleep, and nearly cease discharge during REM sleep. By contrast, GABA/galaninergic neurons in the preoptic area (POA) of the hypothalamus, including lateral, median, and ventrolateral preoptic nuclei, are active during sleep, especially

during NREM sleep, and considered to be a sleep center. POA neurons and monoaminergic neurons are thought to reciprocally inhibit each other [38].

Orexin neurons send their projections densely to nuclei involved in the regulation of sleep and wakefulness, including LC noradrenergic neurons, DR/MnR serotonergic neurons, TMN histaminergic neurons, and cholinergic neurons in the pontine (PPT/LDT) and basal forebrain (BF) (Fig. 2.1b) [8, 21]. In accordance with the innervation, neurons in these nuclei express OX1R and/or OX2R in different combinations [12, 13]. ICV administration of orexin-A in rodents reduces REM and NREM sleep, as well as increases wakefulness [13, 40]. Furthermore, optogenetic excitation of orexin neurons results in reduced latencies to wakefulness from either NREM or REM sleep [41], while optogenetic silencing of these neurons induces NREM sleep in mice [42]. Similarly, pharmacogenetic modulation of orexin neurons using designer receptors exclusively activated by designer drugs (DREADD) alters states of sleep and wakefulness [43]. The application of orexin-A directly into the LC [44], TMN [45], BF cholinergic area [46, 47], and LDT [48] has also been reported to increase wakefulness. In vitro slice electrophysiology studies have shown that orexin-A and orexin-B increase firing rates of monoaminergic neurons in the LC [49, 50], DR [51, 52], TMN [53–55], and cholinergic neurons in the BF and LDT [56, 57]. These observations suggest that orexin neurons stabilize wakefulness by regulating these monoaminergic and cholinergic neurons. In addition, orexin neurons activate themselves directly and indirectly via local glutamatergic neurons, forming positive feedback circuits that may stabilize the activity of the orexin neuron network [58, 59].

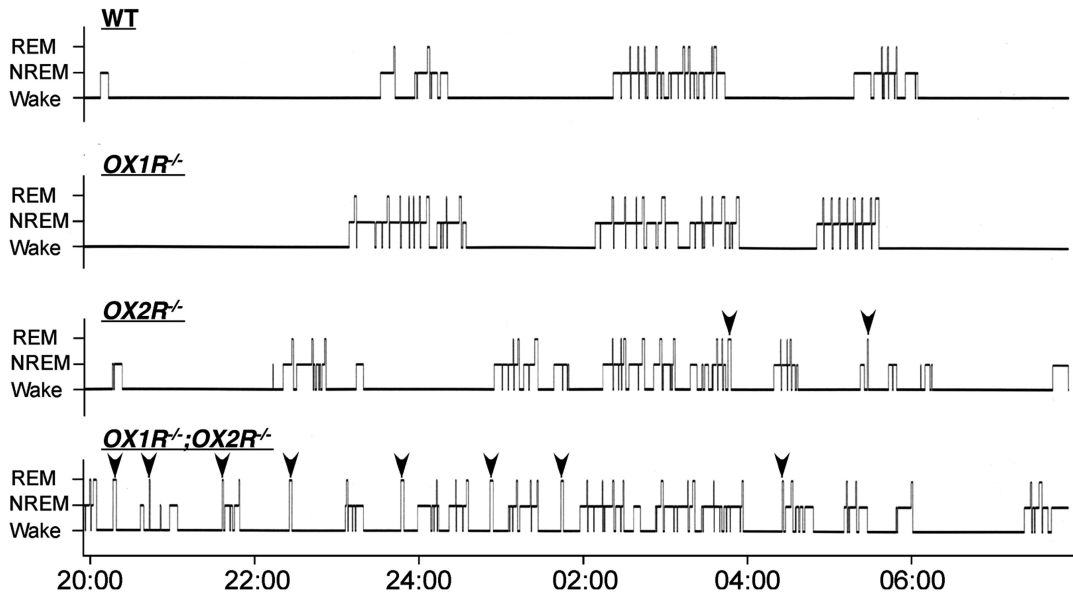
Considering symptoms of narcolepsy, orexin neurons are expected to be active during wakefulness and to be silent during sleep, as observed in wake-active monoaminergic neurons. In vivo single-unit recordings have confirmed this wake-active firing pattern of orexin neurons [60–62]. Importantly, firing rates of orexin neurons are much higher during active waking with movement than in quiet waking, suggesting that these

cells are activated during emotional and sensorimotor conditions similar to those that trigger cataplexy in narcoleptic animals. Indeed, extracellular orexin level is linked to emotion and social interaction in humans [63].

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### Differential Involvement of Deficient OX1R- and OX2R-Mediated Pathways in the Pathophysiology of Narcolepsy

The fact that functionally null mutations in the *OX2R* gene were found in two independent lines of familial narcoleptic canines suggests that OX2R may play a critical role in the regulation of sleep and wakefulness [20]. Studies of orexin receptor-deficient mice (*OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice) further elucidated the differential roles of OX1R and OX2R in detail (Fig. 2.2). First, *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice demonstrate narcoleptic phenotype nearly similar to that in *orexin*<sup>-/-</sup> mice, implying that these two receptors are sufficient to mediate the regulation of sleep and wakefulness by orexins [18, 64]. The deletion of *OX1R* produces no measurable effect on states of sleep and wakefulness in the baseline condition [18, 64]. However, *OX2R*<sup>-/-</sup> mice have clear characteristics of narcolepsy, although their behavioral and EEG phenotypes are less severe than that found in *orexin*<sup>-/-</sup> mice [18, 65]. In infrared videophotographic studies, during the dark phase, *OX2R*<sup>-/-</sup> mice showed abrupt cataplexy-like behavioral arrests, and the frequency of such arrests was far less in *orexin*<sup>-/-</sup> mice (31-fold lower frequency in *OX2R*<sup>-/-</sup> mice than in *orexin*<sup>-/-</sup> mice). By contrast, *OX2R*<sup>-/-</sup> mice showed a distinct variety of behavioral arrests with more gradual onsets (gradual arrests). Moreover, *orexin*<sup>-/-</sup> mice also exhibited gradual arrests with a frequency similar to *OX2R*<sup>-/-</sup> mice, in addition to plenty of abrupt arrests. A detailed characterization of behavioral, pharmacological, and electrophysiological features of *orexin*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice defined abrupt and gradual arrests as the presumptive mouse correlates of cataplexy and sleep attacks in human narcolepsy, respectively [65].



**Fig. 2.2** Sleep state abnormalities in orexin receptor knockout mice. Representative 12-h dark period (20:00–08:00) hypnograms for wild-type (WT), *OX1R*<sup>-/-</sup>, *OX2R*<sup>-/-</sup>, and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice, all on a C57BL/6J background, are shown. The different levels above the baseline indicate states of sleep and wakefulness (e.g., REM, NREM, and wakefulness) of mice at the time. Episodes of direct transition from wakefulness to REM sleep are shown by arrows. Note

the greater awake and NREM sleep episode fragmentation and reduced duration of wakefulness in the hypnograms of *OX2R*<sup>-/-</sup> and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice compared with WT and *OX1R*<sup>-/-</sup> mice. Episodes of direct transition from wakefulness to REM sleep were not observed in *OX1R*<sup>-/-</sup> mice and were hardly observed in *OX2R*<sup>-/-</sup> mice, though they were frequently observed in *OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice (modified from [18])

In addition to gradual behavioral arrests, *OX2R*<sup>-/-</sup> mice exhibit fragmentation of wakefulness, another sign of sleepiness, to an extent similar to that of *orexin*<sup>-/-</sup> mice (Fig. 2.2) [65]. These results of reverse genetic studies with mice suggest that the normal regulation of wakefulness and NREM sleep transitions depends critically on OX2R activation, whereas the profound dysregulation of REM sleep control unique to narcolepsy emerges from loss of signaling through both OX1R- and OX2R-dependent pathways.

The substantially lower frequency of cataplexy in *OX2R*<sup>-/-</sup> mice than in *orexin*<sup>-/-</sup> mice appears to be inconsistent with the fact that mutations of the *OX2R* gene are solely responsible for an inherited canine model of narcolepsy, which demonstrates a frequent occurrence of cataplexy as well as excessive sleepiness [20]. This circumstance may result from species difference (e.g., the precise expression patterns of two orexin receptors) and/or selection bias. However, even

in canines, the absence of orexin peptides may cause severe narcoleptic symptoms as compared to *OX2R* mutation. Early studies of narcoleptic Dobermans and Labradors found that these canines were 30- to 80-fold less severely affected with cataplexy than poodles with sporadic narcolepsy, which manifested in literally hundreds of attacks per day [66], an effect previously attributed solely to differences in breed and breed size.

In an experiment complementary to behavioral studies and baseline sleep/wakefulness recordings of *OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice, the arousal effects of ICV orexin-A administration were compared between wild-type, *OX1R*<sup>-/-</sup>, and *OX2R*<sup>-/-</sup> mice [13]. The effects of orexin-A on wakefulness and NREM sleep were significantly attenuated in both knockout mice as compared to wild-type mice, with substantially larger attenuation in *OX2R*<sup>-/-</sup> than in *OX1R*<sup>-/-</sup> mice. These results suggest that, although the OX2R-mediated pathway plays a pivotal role in the promotion of

wakefulness, OX1R also plays additional roles in promoting arousal.

By contrast, the suppression of REM sleep via orexin-A administration was slightly and similarly attenuated in both *OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice, which suggests a comparable contribution of the two receptors to REM sleep suppression [13]. The supplementary role of OX1R in the suppression of NREM sleep is consistent with the fact that *OX2R*<sup>-/-</sup> mice on a C57BL/6J genetic background show less fragmented wakefulness than *orexin*<sup>-/-</sup> mice and *OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice [18, 67, 68] but show similarly fragmented wakefulness on a C57BL/6J-129/SvEv-mixed background, as described above [65], which suggests that OX1R is indispensable for the maintenance of wakefulness in the absence of OX2R.

### Effector Neural Circuits That Stabilize Wakefulness Downstream to Orexin Neurons

Although the application of exogenous orexins has been shown to excite many types of neurons [3], neurons activated by the pharmacological application of exogenous orexin may not necessarily be essential to the endogenous mechanisms by which orexin neurons regulate sleep and wakefulness in a physiological condition. Thus, neurons directly downstream to orexin neurons in physiological conditions (i.e., neurons influenced by endogenous orexins that mediate their wake-promoting and REM-suppressing effects) have remained uncertain. Several reports have suggested that histaminergic neurons in the TMN play an important role in the arousal-promoting effect of orexin, which is supported by the facts that the effect of ICV orexin-A administration is both markedly attenuated by the histamine H1 receptor antagonist pyrilamine [55] and is absent in *H1 histamine receptor* knockout mice [45]. Accordingly, the TMN abundantly expresses OX2R [12, 13], the subtype whose absence causes the narcoleptic phenotype in mice and canines [20, 65]. Mochizuki et al. produced a mouse model in which a *loxP*-flanked gene cassette disrupted the production of OX2R, though

normal OX2R expression could be restored by Cre recombinase [67]. They showed that targeted Cre expression (i.e., focal restoration of OX2R expression) in the TMN and adjacent regions rescued the fragmentation of wakefulness in their mouse model, which further suggest that the orexin signaling mediated by OX2R in the TMN (and possibly its surrounding area in the posterior hypothalamus) is sufficient to prevent sleepiness caused by systemic OX2R deficiency.

However, this hypothesis remains controversial. Mice lacking both OX1R and histamine H1 receptors demonstrate no abnormality in sleep or wakefulness [64]. Moreover, a recent optogenetic study showed that orexin-mediated sleep-to-wakefulness transitions do not depend on histamine [69].

Recently, in order to identify neurons directly activated by endogenous orexins and that mediate their wake-stabilizing effect in a natural context, we searched for monoaminergic and cholinergic nuclei in which the focal rescue of orexin receptor expression in *OX1R*<sup>-/-</sup>; *OX2R*<sup>-/-</sup> mice by recombinant AAV vectors ameliorates their narcoleptic phenotype [68]. The targeted restoration of orexin receptor expression in the DR and LC of these mice differentially inhibited cataplexy-like episodes and the fragmentation of wakefulness (i.e., sleepiness), respectively. The suppression of cataplexy-like episodes correlated with the number of serotonergic neurons restored with orexin receptor expression in the DR, while the consolidation of fragmented wakefulness correlated with the number of noradrenergic neurons restored in the LC. Furthermore, the pharmacogenetic activation of these neurons using DREADD technology ameliorated narcolepsy in mice that lacked orexin neurons. These results suggest that DR serotonergic and LC noradrenergic neurons may play differential roles in the regulation of sleep and wakefulness by orexin neurons.

The suppression of cataplexy-like episodes by DR serotonergic neurons, but not by LC noradrenergic neurons, was quite unexpected [68], since LC noradrenergic neurons have been considered to be a candidate to prevent cataplexy, according to various pharmacological and

electrophysiological studies. For example, cataplexy in humans and canines is strongly suppressed by drugs that increase noradrenergic tone and is worsened by drugs that block noradrenergic signaling [19, 70]. In addition, LC neurons cease firing during cataplexy in canines [71]. Nevertheless, our abovementioned observations have never conflicted with the importance of the noradrenergic system in the pathophysiology of cataplexy, yet simply indicate that the sole regulation of LC noradrenergic neurons by endogenous orexins is not sufficient to suppress cataplexy in narcoleptic mice. It is also likely that non-LC noradrenergic neurons play an important role in the suppression of cataplexy by the pharmacological augmentation of systemic noradrenergic tone.

As described earlier, the disruption of both OX1R- and OX2R-mediated pathways is required for the frequent occurrence of cataplexy [18]. This fact is consistent with the contribution of orexin signaling in DR serotonergic neurons since most DR serotonergic neurons express both OX1R and OX2R [13]. DR serotonergic neurons greatly reduce firing rates during cataplexy in canines [72]. These neurons, as well as LC noradrenergic neurons, have also been implicated in the suppression of REM sleep by inhibiting REM-on cholinergic neurons in the PPT/LDT and/or by activating REM-off GABAergic neurons in the ventrolateral periaqueductal gray (vlPAG) and adjacent lateral pontine tegmentum (LPT), also known as dorsal deep mesencephalic reticular nuclei (dDpMe) [39, 73]. Indeed, we observed dense projections of DR serotonergic neurons to these brain areas, as well as to the amygdala [68], which suggests that DR serotonergic neurons may coordinately control multiple brain regions involved in the regulation of REM sleep and emotion.

Restoration of orexin receptor expression in the LC noradrenergic neurons significantly consolidated wakefulness to an extent comparable to that in *OX2R*<sup>-/-</sup> mice [68]. As described above, the fragmentation is less severe in *OX2R*<sup>-/-</sup> mice than in *orexin*<sup>-/-</sup> mice and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice on the same C57BL/6J genetic background [18, 67], which suggests that OX1R plays an important role in the maintenance of wakefulness in the

absence of OX2R [13]. In conjunction with the fact that LC noradrenergic neurons exclusively express OX1R in wild-type mice [13], these neurons are likely to be responsible for the contribution of OX1R to the maintenance of wakefulness, while another OX2R-mediated mechanism, most likely mediated by TMN histaminergic neurons, is required for the normal regulation of wakefulness duration. Recent optogenetic studies have demonstrated a causal relationship between the firing of LC noradrenergic neurons and transitions from sleep to wakefulness [74] as well as showed that the inhibition of these neurons blocked the arousal effects of the stimulation of orexin neurons [75], which further supports the importance of the orexinergic regulation of LC noradrenergic neurons in the consolidation of wakefulness.

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### Links among Emotion, Narcolepsy, and Orexins

As mentioned previously, cataplexy is most often triggered by positive emotions. The amygdala, which is important for processing emotions, may be a structure relevant to this characteristic of the pathophysiology of cataplexy [76]. Moreover, the amygdala and orexin neurons form reciprocal connections [8, 77, 78]. A recent study demonstrated that levels of orexin-A in the amygdala of humans are maximized during positive emotion, social interaction, and anger [63]. The amygdala sends inhibitory projections to the brainstem monoaminergic nuclei and to regions in the pons that suppress REM sleep and atonia [79], as well as non-GABAergic projections to REM-on neurons of the sublaterodorsal nucleus, which indirectly inhibits motor neurons [80]. In addition, many neurons in the amygdala of freely behaving narcoleptic canines increase activity during cataplexy [81]. A study using single-photon emission CT (SPECT) indicated hyperperfusion in several brain areas, including the right amygdala, during human cataplexy [82]. Humorous pictures reportedly also elicit enhanced amygdala response in patients [83]. Two studies have reported abnormal amygdala responses to emotional stimuli in

people with narcolepsy, with increased amygdala response to positive rewards and decreased amygdala response to aversive stimuli [84, 85]. Finally, amygdala lesions significantly reduce cataplexy in *orexin*<sup>-/-</sup> mice [79]. Altogether, positive emotions may trigger the weakening of muscle tone through the amygdala, which is antagonized by orexin neurons in healthy people, by enhancing the activity of neural circuits that inhibit atonia and by reducing the activity of the amygdala [76].

Regarding the roles of orexins in the function of the amygdala, two recent studies reported the importance of the orexinergic activation of the noradrenergic pathway from the LC to the amygdala in the formation of fear memory [86, 87]. These results are consistent with studies in human narcolepsy patients, who are impaired in acquiring a conditioned threat response and show reduced amygdala activity as compared to controls when exposed to aversively conditioned stimuli [85, 88].

## Conclusions

Identification of the orexin system has allowed a huge step forward in understanding the pathophysiology of narcolepsy as well as in understanding the physiology of the normal regulation of sleep and wakefulness. Future studies using multiple approaches with the orexin system and its afferent and efferent pathways promise to further elucidate the whole picture of neural mechanisms underlying the physiology and pathophysiology of sleep. By targeting the orexin receptors or by replacing orexin expression, novel therapies for narcolepsy are also expected to become available in the near future.

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