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The Roles of Orexins in Sleep/Wake Regulation

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ABSTRACT

Orexin A and orexin B are hypothalamic neuropeptides initially identified as endogenous ligands for two orphan G-protein coupled receptors (GPCRs). A deficiency of orexin signaling results in the sleep disorder narcolepsy-cataplexy in humans, dogs, and rodents, a sleep disorder characterized by excessive daytime sleepiness and cataplexy. Multiple approaches, including molecular genetic, electrophysiological, pharmacological, and neuroanatomical studies have suggested that orexins play critical roles in the maintenance of wakefulness by regulating the function of monoaminergic and cholinergic neurons that are implicated in the regulation of wakefulness. Here, I review recent advances in the understanding of how orexins regulate sleep/wakefulness and prevent narcolepsy.

KEY WORDS

Orexin, GPCR, sleep/wakefulness, narcolepsy, monoamine, efferent pathway

1. Discovery of orexin peptides

Orexin A and orexin B were initially identified as endogenous ligands for two orphan G-protein coupled receptors (GPCR) (Sakurai et al., 1998): GPCRs for which endogenous ligands are unknown are referred to as “orphan” GPCRs. Molecular cloning studies showed that both orexin A and orexin B are derived from a common single precursor peptide, prepro-orexin. 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. An mRNA encoding the same precursor peptide of hypocretin 1 (corresponding to orexin A) and hypocretin 2 (orexin B) was independently identified by de Lecea et al. as a hypothalamus-specific transcript (de Lecea et al., 1998). Orexin A and orexin B constitute a novel distinct peptide family. Orexin A is a 33-amino acid peptide with an N-terminal pyroglutamyl residue, two intrachain disulfide bonds, and C-terminal amidation, while orexin B is a 28-amino acid, C-terminally amidated linear peptide (Sakurai et al., 1998). 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 (OX1R) and orexin 2 (OX2R) receptors (also known as HCRTR1 and HCRTR2) (de Lecea et al., 1998; Sakurai et al., 1998). OX1R shows a higher affinity for orexin A than orexin B by one-order, while OX2R binds orexin A and orexin B with similar affinities. Both receptors are G_q-coupled and caused strong excitatory effects on neurons examined thus far (Sakurai and Mieda, 2011), except in one study that reported the direct inhibitory action of orexin receptors on surprachiasmatic nucleus (SCN) neurons at night (Belle et al., 2014).

Neurons expressing orexins (orexin neurons) are distributed within the perifornical lateral hypothalamus and send projections throughout the brain and spinal cord (Date et al., 1999; de Lecea et al., 1998; Nambu et al., 1999; Peyron et al., 1998; Sakurai et al., 1998; van den Pol, 1999). The number of these neurons has been estimated to be around 3,000 ~ 4,000 in rat and 70,000 in human brains (Kilduff and Peyron, 2000; Thannickal et al., 2000). Consistent with the broad projections of orexin neurons, *Ox1r* and *Ox2r* show partly overlapping but distinct distributions of their mRNA throughout the CNS (Lu et al., 2000; Marcus et al., 2001; Trivedi et al., 1998).

2. Disruption of the orexin system causes narcolepsy

Degenerative loss of orexin neurons in humans is associated with narcolepsy (type I narcolepsy: narcolepsy with cataplexy) (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000), a debilitating neurological disorder, providing a unique perspective on the mechanisms of sleep/wakefulness control (Sakurai and Mieda, 2011; Zeitzer et al., 2006). A series of discoveries leading to this conclusion started with findings by two animal studies: (i) functionally null mutations in the *Ox2r* gene were found to be responsible for familial canine narcolepsy (Lin et al., 1999), and (ii) *orexin* knockout mice (*orexin*$^{-/-}$) were shown to exhibit a phenotype strikingly similar to human narcolepsy (Chemelli et al., 1999).

Human narcolepsy affects approximately 1 in 2,000 individuals in the United States (Mignot, 1998; Sakurai and Mieda, 2011; Zeitzer et al., 2006). The syndrome consists of excessive daytime sleepiness that often results in sleep attacks (sudden onset of non–rapid eye movement [NREM] sleep), cataplexy (sudden bilateral skeletal muscle weakening triggered by emotions, without consciousness impairment), hypnagogic

hallucinations, and sleep paralysis. These symptoms can be divided into two independent pathological phenomena (Dauvilliers et al., 2007). The first is dysregulation of NREM sleep onset: the inability to maintain a consolidated awake period, characterized by abrupt transitions from wakefulness to NREM sleep. This phenomenon manifests clinically as excessive daytime sleepiness or sleep attacks. The second pathological phenomenon is dysregulation of REM sleep onset: the pathological intrusion of REM sleep or REM atonia into wakefulness or at sleep onset. It is during these periods that patients may experience cataplexy, hypnagogic hallucinations, and sleep paralysis.

Similarly, *orexin*$^{-/-}$ mice display a phenotype strikingly similar to human narcolepsy, with a markedly decreased duration of wakefulness episodes during the dark phase (i.e., inability to maintain a long wakeful period, or sleepiness), abrupt behavioral arrests with muscle atonia (i.e., potential cataplexy), which manifest as direct transitions from wakefulness to REM sleep in electroencephalogram/electromyogram (EEG/EMG) recordings, decreased REM sleep latency, and increased REM sleep time during the dark phase (Chemelli et al., 1999). Thus, orexins are likely to stabilize and maintain wakefulness episodes.

Narcolepsy patients lose their orexin neurons likely due to autoimmune reaction, and mutations of *prepro-orexin* or orexin receptor genes are very rare in human narcolepsy (Crocker et al., 2005; Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000). In addition to orexins, orexin neurons contain other co-transmitters, such as glutamate, dynorphin, and neurotensin (Chou et al., 2001; Furutani et al., 2013; Rosin et al., 2003). Therefore, loss of these co-transmitters may also contribute to the symptoms of human narcolepsy. However, transgenic mice in

which orexin neurons are ablated postnatally by overexpression of a neurotoxic form of ataxin-3 (*orexin-ataxin3* mice) showed essentially the same phenotype of sleep/wake regulation as *orexin*$^{-/-}$ mice, except for reduced amplitude of the circadian rhythm of REM sleep (Hara et al., 2001; Kantor et al., 2009). By contrast, mice with conditional and more complete ablation of orexin neurons demonstrated much more frequent cataplexy and less fragmented wakefulness than *orexin-ataxin3* mice (Tabuchi et al., 2014). Another study worth noting demonstrated that chronic overexpression of orexin peptides throughout the brain and ICV administration of orexin A prevented narcolepsy (Mieda et al., 2004). Collectively, although loss of orexin peptides is likely to contribute most of the symptoms of human narcolepsy, involvement of other factors expressed in orexin neurons cannot be excluded.

3. Regulation of sleep/wakefulness by orexin neurons

3.1 Neural Mechanism of Sleep/Wake Regulation

Monoaminergic neurons, including locus coeruleus (LC) noradrenergic, dorsal and median raphe nuclei (DRN, MRN) serotonergic, and tuberomammillary nucleus (TMN) histaminergic neurons, project diffusely to the cerebral cortex, basal forebrain, thalamus, hypothalamus, and brainstem, and are thought to promote arousal (Sakurai and Mieda, 2011; Saper et al., 2010). They are highly active during wakefulness, while they reduce their activities during NREM sleep and almost cease discharge during REM sleep (Aston-Jones and Bloom, 1981; McGinty and Harper, 1976; Steininger et al., 1999; Takahashi et al., 2010; Takahashi et al., 2006; Trulson and Jacobs, 1979). Other important wake-promoting signals are cholinergic fibers from the basal forebrain (BF) and the brainstem pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT).

PPT/LDT cholinergic neurons also interconnect with key forebrain targets. Their activity is highest during wakefulness and REM sleep. The BF neurons other than cholinergic ones are also implicated in sleep/wake regulation (Anaclet et al., 2015; Xu et al., 2015; Zant et al., 2016). Cholinergic, glutamatergic, and parvalbumin (PV)-expressing GABAergic neurons are wake/REM-active, are interconnected, and promote wakefulness (Xu et al., 2015). Cholinergic and PV GABAergic neurons also project to the cerebral cortex.

In contrast to those neurons implicated in the promotion of wakefulness, GABA/galaninergic neurons in the ventrolateral preoptic area (VLPO) and median preoptic area (MnPO) are active during sleep, especially during NREM sleep, and are considered to be a sleep center (Saper et al., 2010). The chemogenetic activation of preoptic area indeed increased sleep (Saito et al., 2013; Zhang et al., 2015). Somatostatin (SOM)-expressing GABAergic neurons in the BF are also sleep-active, inhibit three other types of BF neurons, and promote sleep (Xu et al., 2015). Importantly, VLPO/MnPO neurons and monoaminergic neurons reciprocally inhibit each other.

This section briefly summarized the regulatory network of sleep/wakefulness that has been demonstrated to interact with orexin neurons, as discussed in the next section. Excellent reviews on the known neural substrates of sleep/wakefulness regulation are available (e.g. Saper et al., 2010; Weber and Dan, 2016; as well as reviews in this special issue of Neurosci. Res., Miyazaki et al., 2017; Oishi and Lazarus, 2017; Ono and Yamanaka, 2017).

3.2 Orexin Neurons in Regulatory Circuit of Sleep/Wake

Orexin neurons send their projections densely to nuclei involved in sleep/wakefulness

regulation, including the LC noradrenergic, raphe serotonergic, TMN histaminergic, PPT/LDT and BF cholinergic neurons (Chemelli et al., 1999; Horvath et al., 1999; Peyron et al., 1998; Yamanaka et al., 2002). In accordance with the innervation, neurons in these nuclei express OX1R and/or OX2R in different combinations (Marcus et al., 2001; Mieda et al., 2011). ICV administration of orexin A in rodents reduces REM and NREM sleep, and increases wakefulness (Hagan et al., 1999; Mieda et al., 2011). Furthermore, optogenetic excitation of orexin neurons results in increases in the probability of an awakening event during both NREM and REM sleep (Adamantidis et al., 2007). The application of orexin A directly into the LC (Bourgin et al., 2000), TMN (Huang et al., 2001), BF (Espana et al., 2001; Thakkar et al., 2001), and LDT (Xi et al., 2001) 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 (Horvath et al., 1999; van den Pol et al., 2002), DRN (Brown et al., 2001; Liu et al., 2002), TMN (Bayer et al., 2001; Eriksson et al., 2001; Yamanaka et al., 2002), and cholinergic neurons in the BF and LDT (Burlet et al., 2002; Eggermann et al., 2001). These observations suggest that orexin neurons stabilize wakefulness by regulating these monoaminergic and cholinergic neurons.

At the same time, orexin neurons receive projections from nuclei involved in sleep/wake regulation. GABAergic neurons in the preoptic area, including the VLPO, densely innervate orexin neurons (Sakurai et al., 2005; Yoshida et al., 2006). Orexin neurons are strongly inhibited by both the $GABA_A$ receptor agonist muscimol and the $GABA_B$ receptor agonist baclofen (Li et al., 2002; Xie et al., 2006; Yamanaka et al., 2003a). Optogenetic stimulation of POA fibers indeed inhibits orexin neuronal activity (Saito et al., 2013). Orexin neurons are also innervated by BF cholinergic neurons (Li et

al., 2002; Sakurai et al., 2005; Xie et al., 2006). Carbacol, an agonist at muscarinic receptors, activates a subset of orexin neurons (Sakurai et al., 2005; Yamanaka et al., 2003b). Thus, orexin neurons are inhibited by sleep-promoting neurons and activated by wake-promoting BF neurons: this regulation of orexin neurons is consistent with their proposed function to stabilize wakefulness. In contrast, wake-active serotonergic neurons in the MRN send inhibitory projections to orexin neurons (Li et al., 2002; Muraki et al., 2004; Sakurai et al., 2005). Noradrenergic neurons also have inhibitory effects on orexin neurons (Li et al., 2002; Yamanaka et al., 2006; Yamanaka et al., 2003b). These negative feedback mechanisms may also be important for the fine adjustment of orexin neuronal activity to stabilize wakefulness (Tabuchi et al., 2013). Histamine has little effect on orexin neurons (Yamanaka et al., 2003b). Interestingly, a short two-hour period of total sleep deprivation was reported to change the action of noradrenaline on orexin neurons from excitation to inhibition in rats (Grivel et al., 2005). This mechanism may contribute to the growing sleepiness that accompanies sleep deprivation, although this phenomenon was not observed in mice (Yamanaka et al., 2006).

Local feedback circuits may also play important roles in the regulation of orexin neurons. 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 (Li et al., 2002; Yamanaka et al., 2010). On the other hand, orexins also activate local GABAergic input to orexin neurons. Genetic disruption of this input was reported to produce marked sleep/wake abnormality (Matsuki et al., 2009).

3.3 Vigilance state-dependent firing of orexin neurons

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. Fos expression (an indicator of neuronal activity) in orexin neurons in rats is higher during the dark phase (active period) than during the light phase (rest period) (Estabrooke et al., 2001). Moreover, orexin level in CSF peaks during the dark period and decreases during the light period (Yoshida et al., 2001). In vivo single-unit recordings have confirmed such a wake-active firing pattern of orexin neurons (Lee et al., 2005; Mileykovskiy et al., 2005; Takahashi et al., 2008). Essentially, orexin neurons fired most actively during active waking, such as exploring, grooming, and eating, showed decreased discharge during quiet waking, were virtually silent during NREM sleep, and were almost silent but exhibited occasional firing during REM sleep. During the transition from sleep to wakefulness, orexin neurons fired prior to the onset of EEG activation, the EEG sign of wakefulness (Lee et al., 2005; Takahashi et al., 2008). In addition, they responded with a short latency to an arousing sound stimulus given during sleep, causing EEG activation (Mileykovskiy et al., 2005; Takahashi et al., 2008). These characteristics of orexin neurons clearly contrast with those of histaminergic neurons, which display waking-specific discharge, indicating that orexin neurons are not simply wake-active and are activated during emotional and sensorimotor conditions similar to those that trigger cataplexy in narcoleptic animals (Saper et al., 2010; Takahashi et al., 2005). This idea was further supported by the measurements of orexin release in the amygdala in narcoleptic human (Blouin et al., 2013).

Recent fiber photometry recordings revealed that orexin neurons are rapidly activated by stress, such as application of an air-puff to tail, exposure to novelty, and

immobility, as well as by nociception (Gonzalez et al., 2016; Inutsuka et al., 2016). These results are consistent with the fact that stress and nociception increase arousal.

3.4 Causal relationship between the activity of orexin neurons and sleep/wakefulness

Optogenetic and chemogenetic studies directly demonstrated the causal relationship between the activity of orexin neurons and sleep/wakefulness. Selective stimulation of these neurons via channelrhodopsin-2 (ChR2) increased the probability of transitions to wakefulness from either NREM or REM sleep (Adamantidis et al., 2007), while selective silencing of these neurons induced and increased NREM sleep in free-moving mice (Tsunematsu et al., 2011; Tsunematsu et al., 2013). Similarly, experiments using excitatory and inhibitory DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) demonstrated that activation of orexinergic tone results in increased wakefulness time, while inhibition of these neurons results in reduced wakefulness (Sasaki et al., 2011).

3.5 Synaptic input organization of orexin neurons

Orexin neurons have been demonstrated to have unorthodox synaptic input organization (Horvath and Gao, 2005). They are controlled primarily by excitatory synaptic inputs, which outnumber inhibitory synapses by a ratio of 10:1. This organization is in sharp contrast to the fact that neuronal cell bodies in the central nervous system are either dominated by inhibitory inputs (long-projective neurons), or have an approximate ratio of excitatory to inhibitory inputs of 1:1. Such a unique input organization of orexin neurons may be a necessary element for the maintenance of a low threshold for arousal

and alertness. On the other hand, this circuitry may also be an underlying cause of insomnia. Furthermore, synaptic strength at glutamatergic synapses on neurons can be potentiated by prolonged wakefulness or fasting (Horvath and Gao, 2005; Rao et al., 2007). These may suggest that synaptic potentiation in orexin neurons may be required to maintain prolonged wakefulness in animals and that the development of synaptic plasticity in orexin neurons may provide a mechanism through which the arousal threshold is regulated to determine the behavioral state of animals.

3.6 Co-transmitters of orexin neurons

In addition to orexins, orexin neurons contain other co-transmitters, such as glutamate, dynorphin, and neurotensin (Chou et al., 2001; Furutani et al., 2013; Rosin et al., 2003). Upon stimulation of orexin neurons, orexins caused sustained activation of TMN histaminergic neurons, while glutamate caused rapid, transient activation of these neurons. Therefore, orexins and glutamate are likely to translate distinct features of activity of orexin neurons into parallel, nonredundant control signals for downstream effectors (Schone et al., 2014; Schone et al., 2012).

Orexin and another co-transmitter, dynorphin, are packaged in the same synaptic vesicles within the hypothalamus. Nevertheless, orexin excites and dynorphin inhibits dopamine neurons of the ventral tegmental area. Orexin facilitates reward by attenuating the anti-reward effects of dynorphin, which is co-released with orexin (Muschamp et al., 2014).

As discussed in Section 2, narcoleptic phenotypes may be different between mice lacking orexins and mice lacking orexin neurons (Kantor et al., 2009; Tabuchi et al., 2014), which may suggest some roles of those co-transmitters of orexin neurons in

the symptoms of narcolepsy.

4. Differential regulation of sleep/wakefulness by orexin receptors

4.1 Spatial pattern of orexin receptor expression

In clear contrast to the restricted localization of *orexin* mRNA expression exclusively in neurons distributed within the lateral hypothalamus (LH) and perifornical area (PFA) (de Lecea et al., 1998; Sakurai et al., 1998), *Ox1r* and *Ox2r* mRNA show wide distributions within the brain with partly overlapping but complementary distributions (Lu et al., 2000; Marcus et al., 2001; Mieda et al., 2011; Trivedi et al., 1998). This is consistent with the fact that orexin neurons project to almost all brain areas, with especially dense ones to monoaminergic and cholinergic nuclei of the brainstem and hypothalamus, which play important roles in the regulation of sleep/wakefulness states (Chemelli et al., 1999; Nambu et al., 1999; Peyron et al., 1998). These nuclei express OX1R and OX2R in differential manners (Marcus et al., 2001; Mieda et al., 2011). Histaminergic neurons in the TMN exclusively express *Ox2r*, while noradrenergic neurons in the LC exclusively express *Ox1r*. In the DRN and MRN, serotonergic neurons express *Ox1r* and/or *Ox2r*. In the LDT and PPT, cholinergic neurons express *Ox1r* but not *Ox2r* mRNA. Intriguingly, there are many GABAergic neurons expressing orexin receptors which are intermingled with these monoaminergic and cholinergic neurons (Mieda et al., 2011).

4.2 Genetic dissection of sleep/wakefulness regulation by orexin receptors

The fact that functionally null mutations in the *Ox2r* gene were responsible for two independent lines of familial narcoleptic canines indicates OX2R-mediated pathway as

a critical signaling in the regulation of sleep and wakefulness (Lin et al., 1999). Studies of orexin receptor-deficient mice (*Ox1r* $^{-/-}$ and *Ox2r* $^{-/-}$ mice) have provided a deeper insight into the differential roles of OX1R and OX2R (Sakurai, 2007). First, *Ox1r* $^{-/-}$*;Ox2r* $^{-/-}$ mice demonstrate a narcoleptic phenotype nearly similar to that found in *orexin*$^{-/-}$ mice (Hondo et al., 2010; Sakurai, 2007), confirming that orexinergic regulation of sleep/wakefulness is mediated by these two receptors. *Ox1r* $^{-/-}$ mice show little abnormality in the states of sleep and wakefulness in the baseline condition (Hondo et al., 2010; Sakurai, 2007). In contrast, *Ox2r* $^{-/-}$ mice have clear narcoleptic symptoms, although their phenotype is milder as compared to that found in *orexin*$^{-/-}$ mice (Mochizuki et al., 2011; Sakurai, 2007; Willie et al., 2003). During the dark phase, *Ox2r* $^{-/-}$ mice did show abrupt cataplexy-like behavioral arrests, which correlated with the occurrence of direct transitions from wakefulness to REM sleep in EEG/EMG recordings. However, the frequency of such arrests was far less compared to *orexin*$^{-/-}$ mice (31-fold lower frequency in *Ox2r* $^{-/-}$ mice than in *orexin*$^{-/-}$ mice). Importantly, close observation of the behavior of *Ox2r* $^{-/-}$ mice during the dark phase using infrared videophotography uncovered a distinct variety of behavioral arrests with more gradual onsets (gradual arrests). Moreover, such gradual arrests turned out to also manifest in *orexin*$^{-/-}$ mice with a frequency similar to *Ox2r* $^{-/-}$ mice, in addition to plenty of abrupt arrests. Abrupt and gradual arrests have been characterized as the presumptive mouse correlates of cataplexy and sleep attacks in human narcolepsy, respectively, according to their behavioral, pharmacological, and electrophysiological features (Willie et al., 2003). Consistent with this observation, wakefulness episodes of *Ox2r* $^{-/-}$ mice are fragmented to an extent similar to those of *orexin*$^{-/-}$ mice, indicating that these two strains of mouse suffer from comparable sleepiness. In contrast, *Ox2r* $^{-/-}$ mice show normal REM sleep

latency and normal amounts of REM sleep, which are profoundly shortened and increased, respectively, in both *orexin*$^{-/-}$ mice and *Ox1r*$^{-/-}$*;Ox2r*$^{-/-}$ mice (Sakurai, 2007; Willie et al., 2003).

Collectively, mouse reverse genetic studies 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 a loss of signaling through both OX1R- and OX2R-dependent pathways.

The conclusion in the previous paragraphs has been further confirmed by a complementary experiment, i.e., comparing the arousal effects of ICV orexin A administration between wild-type, *Ox1r*$^{-/-}$, and *Ox2r*$^{-/-}$ mice (Mieda et al., 2011). The effects of orexin A on wakefulness and NREM sleep were significantly attenuated in both *Ox1r*$^{-/-}$ and *Ox2r*$^{-/-}$ mice as compared to wild-type mice, with substantially larger attenuation in *Ox2r*$^{-/-}$ than in *Ox1r*$^{-/-}$ mice, suggesting the pivotal role of OX2R and the additional role of OX1R in the promotion of wakefulness. By contrast, the suppression of REM sleep via orexin A administration was marginally and similarly attenuated in both *Ox1r*$^{-/-}$ and *Ox2r*$^{-/-}$ mice, suggesting a comparable contribution of the two receptors to REM sleep suppression. The supplementary role of OX1R in the suppression of NREM sleep is consistent with the fact that *Ox2r*$^{-/-}$ mice with a C57BL/6J, but not C57BL/6J-129/SvEv-mixed, genetic background show less fragmented wakefulness than *orexin*$^{-/-}$ mice and *Ox1r*$^{-/-}$*;Ox2r*$^{-/-}$ mice (Hasegawa et al., 2014; Mochizuki et al., 2011; Sakurai, 2007; Willie et al., 2003), which suggests that OX1R is indispensable for the maintenance of wakefulness in the absence of OX2R.

4.3 Comparison between the studies using mice and dogs

The conclusion drawn from mouse genetics apparently contradicts the fact that an inherited canine model of narcolepsy, which demonstrates a frequent occurrence of cataplexy as well as excessive sleepiness, is attributable solely to mutations of the *Ox2r* gene (Lin et al., 1999). Species differences (e.g., the precise expression patterns of the two orexin receptors) and/or selection bias may explain such an inconsistency. It should be noted that, even in canines, the absence of orexin peptides may cause severe narcoleptic symptoms as compared to *Ox2r* mutation. Early studies reported that narcoleptic Dobermans and Labradors with *Ox2r* mutations were much less severely affected with cataplexy than poodles with sporadic narcolepsy, which were supposed to lack orexin peptides (Baker et al., 1982).

5. Neuronal pathways through which orexin neurons promote wakefulness and prevent narcolepsy

5.1 TMN histaminergic neurons as a candidate downstream pathway of orexin neurons for the maintenance of wakefulness episodes

The application of exogenous orexins has been shown to excite many types of neurons (Sakurai and Mieda, 2011) (also see above). As discussed earlier, monoaminergic and cholinergic nuclei of the hypothalamus and brainstem involved in the regulation of sleep and wakefulness especially receive dense projections of orexin neurons, express orexin receptors, and are activated by the application of orexin peptides in slice preparations. Furthermore, the administration of orexin A directly into monoaminergic and cholinergic nuclei has also been reported to increase wakefulness (Sakurai and Mieda, 2011). However, 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 from orexin neurons in physiological conditions (i.e., the site and subtype of orexin receptors that mediate the wake-promoting and REM-gating effects by endogenous orexins) have remained incompletely understood.

Histaminergic neurons in the TMN, which express OX2R exclusively, are good candidates for such downstream neurons contributing to the arousal effect of orexin. The wake-promoting effect of ICV orexin-A administration is both markedly attenuated by the histamine H1 receptor (H1R) antagonist pyrilamine (Yamanaka et al., 2002) and is absent in *H1r*$^{-/-}$ mice (Huang et al., 2001). In addition, Mochizuki et al. produced OX2R-deficient mice by inserting a *loxP*-flanked transcription-disrupter (TD) gene cassette into the *Ox2r* gene, in which normal OX2R expression could be restored by Cre recombinase-mediated excision of TD cassette (Mochizuki et al., 2011). Using such an elegant genetic model, they showed that focal restoration of OX2R expression in the TMN and adjacent regions completely reversed the fragmentation of wakefulness episodes observed in their OX2R-deficient mice.

However, this hypothesis remains controversial. Mice lacking both OX1R and H1R demonstrate no abnormality in sleep or wakefulness, which contradicts the idea that a H1R-mediated histaminergic pathway is the principal downstream component of OX2R-mediated orexinergic signaling (Hondo et al., 2010). Moreover, a recent study showed that increased probability of sleep-to-wakefulness transitions by optogenetic activation of orexin neurons does not depend on histamine (Carter et al., 2009).

5.2 DRN serotonergic and LC noradrenergic neurons play differential roles in orexin neuron–dependent regulation of sleep/wakefulness

Recently, Hasegawa and colleagues searched for monoaminergic and cholinergic nuclei of the brainstem and hypothalamus in which the focal restoration of orexin receptor expression by recombinant AAV vectors ameliorates narcoleptic phenotype of *Ox1r* $^{-/-}$*;Ox2r* $^{-/-}$ mice (Hasegawa et al., 2014). If the regional restoration of orexin receptors in a certain brain region suppresses narcoleptic symptoms in these mice, that particular region can at least be regarded as one of the important downstream targets of orexin neurons. The targeted restoration of orexin receptor expression in the DRN and LC of these mice differentially inhibited cataplexy and the fragmentation of wakefulness (i.e., sleepiness), respectively. The suppression of cataplexy correlated with the number of serotonergic neurons restored with orexin receptor expression in the DRN, while the consolidation of fragmented wakefulness correlated with the number of noradrenergic neurons restored in the LC. Furthermore, the chemogenetic activation of these neurons using DREADD technology ameliorated narcolepsy in mice that lacked orexin neurons. These results suggest that DRN serotonergic and LC noradrenergic neurons play differential roles in the regulation of sleep and wakefulness by orexin neurons (Fig. 1).

The suppression of cataplexy by DRN serotonergic neurons, but not by LC noradrenergic neurons, was an unexpected result (Hasegawa et al., 2014) because previous pharmacological and electrophysiological studies suggested that LC noradrenergic neurons are good candidates for downstream neurons to prevent cataplexy. For example, drugs that increase noradrenergic tone strongly suppress cataplexy in humans and canines, while blocking the noradrenergic signaling increases the frequency of cataplexy (Hirai and Nishino, 2011; Nishino and Mignot, 1997). In addition, LC neurons cease firing during cataplexy in canines (Wu et al., 1999). Nevertheless, the observations by Hasegawa and colleagues never deny the clinical importance of

enhancing noradrenergic systems for preventing cataplexy, yet simply indicate that the sole regulation of LC noradrenergic neurons by endogenous orexins is not sufficient to suppress cataplexy in narcoleptic mice. Non-LC noradrenergic neurons may also play an important role in the suppression of cataplexy by the pharmacological augmentation of systemic noradrenergic tone, which may be independent of the orexinergic regulation.

The contribution of orexin signaling in DRN serotonergic neurons in the suppression of cataplexy fits with the observations that these neurons express both *Ox1r* and *Ox2r* (Mieda et al., 2011) and that the disruption of both OX1R- and OX2R-mediated pathways is required for the frequent occurrence of cataplexy, as described earlier (Fig. 1A) (Sakurai, 2007). DRN serotonergic neurons greatly reduce firing rates during cataplexy in canines (Wu et al., 2004). In addition, these neurons, as well as LC noradrenergic neurons, have 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) (Luppi et al., 2011). Indeed, DRN serotonergic neurons send dense projections to these brain areas, as well as to the amygdala (Hasegawa et al., 2014), which suggests that DRN serotonergic neurons may coordinately control multiple brain regions involved in the regulation of REM sleep and emotion.

As described above, the fragmentation of wakefulness is less severe in *OX2R*$^{-/-}$ mice than in *orexin*$^{-/-}$ mice and *Ox1r* $^{-/-}$*;Ox2r* $^{-/-}$ mice with C57BL/6J genetic background (Mochizuki et al. 2011; Sakurai 2007), suggesting that OX1R plays an important role in the maintenance of wakefulness in the absence of OX2R (Mieda et al.

2011). Indeed, restoration of *OX1R* expression in the LC noradrenergic neurons of *Ox1r* $^{-/-}$*;Ox2r* $^{-/-}$ mice stabilized wakefulness episodes to an extent comparable to those in *Ox2r* $^{-/-}$ mice (Hasegawa et al. 2014). Considering the fact that LC noradrenergic neurons exclusively express *Ox1r* in wild-type mice (Mieda et al. 2011), these neurons may 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 further required for fully maintained wakefulness as in normal mice (Fig. 1B). Recent optogenetic studies have provided support for the importance of the orexinergic regulation of LC noradrenergic neurons in the consolidation of wakefulness. For instance, there is a causal relationship between the firing of LC noradrenergic neurons and transitions from sleep to wakefulness (Carter et al. 2010). Moreover, the optogenetic inactivation of these neurons prevents the arousal effects of the optogenetic stimulation of orexin neurons (Carter et al. 2012).

6. Pharmacological dissection of sleep/wakefulness regulation by orexin receptors

A series of non-selective (dual) antagonists for orexin receptors (DORAs), as well as subtype-selective antagonists (SORAs), have been developed. On the one hand, these drugs are drawing people's attention as novel medications for insomnia and other diseases (Mieda and Sakurai, 2013; Sakurai, 2014; Scammell and Winrow, 2011). On the other hand, they are also useful for studying the roles of each subtype in the regulation of sleep/wakefulness.

To a large extent, results obtained by pharmacological studies utilizing DORAs and SORAs are consistent with those derived from genetic studies described in the previous sections (Table 1). Selective blockade of OX2R efficiently increases

NREM sleep and shortens NREM sleep latency (Betschart et al., 2013; Bonaventure et al., 2015a; Dugovic et al., 2009; Dugovic et al., 2014; Etori et al., 2014; Gotter et al., 2016; Gozzi et al., 2011; Kuduk et al., 2015; Letavic et al., 2015). Blockade of both OX1R and OX2R does increase NREM sleep, but also causes a disproportionally large increase in REM sleep (Brisbare-Roch et al., 2007; Cox et al., 2010; Dugovic et al., 2009; Dugovic et al., 2014; Etori et al., 2014; Hoyer et al., 2013; Winrow et al., 2012; Yoshida et al., 2015). Except one study showing increases in REM and NREM sleep with SB-334867 (Morairty et al., 2012), the selective blockade of OX1R alone does not cause any statistically significant effects on baseline sleep/wakefulness (Bonaventure et al., 2015b; Dugovic et al., 2009; Dugovic et al., 2014; Gozzi et al., 2011; Steiner et al., 2013). However, OX1R blockade decreased NREM sleep latency after stress-induced arousal (Bonaventure et al., 2015b). Thus, OX2R is the principal regulator of wakefulness/NREM sleep transition, while both OX1R- and OX2R-mediated pathways are critical for gating REM sleep. Dugovic et al. further demonstrated that OX1R blockade counteracted the initiation and prolongation of the OX2R-SORA-induced NREM sleep possibly by increasing dopamine release (Dugovic et al., 2009; Dugovic et al., 2014). This result suggests that systemic inhibition of OX1R signaling by the administration of OX1R-SORA or by knocking out *Ox1r* gene alters activities in the brain regions that are different from the regulatory circuit of baseline sleep/wakefulness, including monoaminergic neurons, and secondarily reduce sleep. Such effects may mask a role of OX1R in the regulation of baseline sleep/wakefulness, which may explain the apparent discrepancy between the conclusion derived from pharmacological and knockout studies and that from the orexin receptor-restoration study (Hasegawa et al., 2014).

Administration of DORAs seldom induces cataplexy in normal animals, although there is a report that less than half of rats treated with high doses of SB-649868 demonstrated direct transitions from wakefulness to REM sleep (Dugovic et al., 2014). Therefore, as compared to the induction of NREM and REM sleep, nearly complete and/or chronic absence of both OX1R- and OX2R-mediated pathways may be needed for cataplexy to occur. This notion is consistent with the observation that the degeneration of more than 95% of orexin neurons is required for the occurrence of cataplexy in mice, whose frequency subsequently increases along with further degeneration (Tabuchi et al., 2014).

7. Integration of external and internal environmental information by orexin neurons to regulate wakefulness

In addition to sleep/wakefulness regulation, early descriptions of the projection patterns of orexin neurons had already suggested their involvement in a wide range of other physiological functions, such as feeding, autonomic regulation, and neuroendocrine regulation (Date et al., 1999; Peyron et al., 1998). Consistently, the central administration of orexin causes a wide variety of effects (Sakurai, 2007). Furthermore, systematical mappings of afferents to orexin neurons demonstrated that orexin neurons receive innervation from nuclei and regions involved in the regulation of feeding, autonomic and endocrine systems, reward, and emotion (Gonzalez et al., 2016; Sakurai et al., 2005; Yoshida et al., 2006). The activity of orexin neurons are also directly regulated by humoral factors that reflect the metabolic state of the body, including ghrelin, glucose, leptin, amino acids, and pH level (Burdakov et al., 2005; Karnani et al., 2011; Williams et al., 2008; Williams et al., 2007; Yamanaka et al., 2003a). Therefore,

orexin neurons are well-placed to act as a hub that links information about the internal and external environments of an animal to vigilance levels and internal bodily functions that support various motivated behaviors (Sakurai, 2014).

Recent studies using genetically modified mice and orexin receptor antagonists has suggested that orexins are involved in the regulation of motivated behaviors, such as feeding, drinking, reward seeking, and emotional behavior. Orexins also regulate autonomic and endocrine systems, which accompany motivated behaviors. Orexinergic regulation of those behaviors was thoroughly reviewed previously (Mahler et al., 2014; Sakurai, 2014). Therefore, not only do orexin neurons contribute to sleep/wake regulation by stabilizing the activity of wake-promoting neural circuits, they are involved in sensing the body's external and internal environments, and regulate states of sleep and wakefulness accordingly, which is beneficial for survival. They further coordinate autonomic tone and hormonal balance with arousal to maintain homeostasis or homeodynamics. The reciprocal interactions between orexin neurons and multiple neuronal systems raise the possibility that orexin neurons function as an interface between multiple regulatory systems including feeding, reward, emotional, circadian, autonomic, and endocrine systems.

For instance, when faced with a negative energy balance due to reduced food availability, mammals respond behaviorally with phases of increased wakefulness and alertness, which presumably enhances their ability to find food. However, orexin neuron-ablated mice are incapable of this fasting-induced arousal, indicating that orexin neurons are necessary for evoking adaptive behavioral arousal during fasting (Yamanaka et al., 2003a). The ability of orexin neurons to sense humoral metabolic cues, as well as innervations from regions that control metabolism and feeding, including the

hypothalamic arcuate nucleus, ventromedial hypothalamic nucleus, and nucleus of the solitary tract (NTS), are likely to enable orexin neurons to control arousal related to the peripheral energy balance (Mieda and Sakurai, 2009; Sakurai, 2007; Yamanaka et al., 2003a). At the same time, orexin neurons also regulate the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Coordinated increases of sympathetic and HPA tone in response to fasting-induced arousal directed by orexin neurons may further help animals to execute adaptive behavior along with autonomic and neuroendocrine responses.

8. Conclusions

Symptoms of type I narcolepsy (narcolepsy with cataplexy) unequivocally reveal that orexins and orexin receptors play critical roles in the regulation of sleep/wake states. Orexin neurons are likely to stabilize wakefulness episodes by promoting wake-active monoaminergic neurons. In addition, the existence of reciprocal connections between the orexin system and multiple neuronal systems indicates that orexin neurons provide crucial links between multiple brain functions, such as energy homeostasis, the reward processing, emotion, and arousal. Future studies utilizing multiple approaches, such as molecular genetics, receptor subtype-selective pharmacology, optogenetics and chemogenetics, would lead to further understanding of the integrative physiology orchestrated by the orexin system.

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FIGURE LEGENDS

Figure 1. Proposed model for OX1R- and OX2R-mediated pathways in suppressing narcoleptic symptoms. (**A**) Prevention of cataplexy. Serotonergic neurons in the DRN mediate most of the anti-cataplectic effect of orexin neurons via both OX1R and OX2R, while other OX2R-mediated pathways may exist. (**B**) Consolidation of wakefulness episodes. LC noradrenergic neurons function as the OX1R-mediated pathway to stabilize wakefulness, while another OX2R-mediated pathway, possibly by TMN histaminergic neurons, is further required for the normal maintenance of wakefulness episodes. Modified from (Hasegawa et al., 2014)

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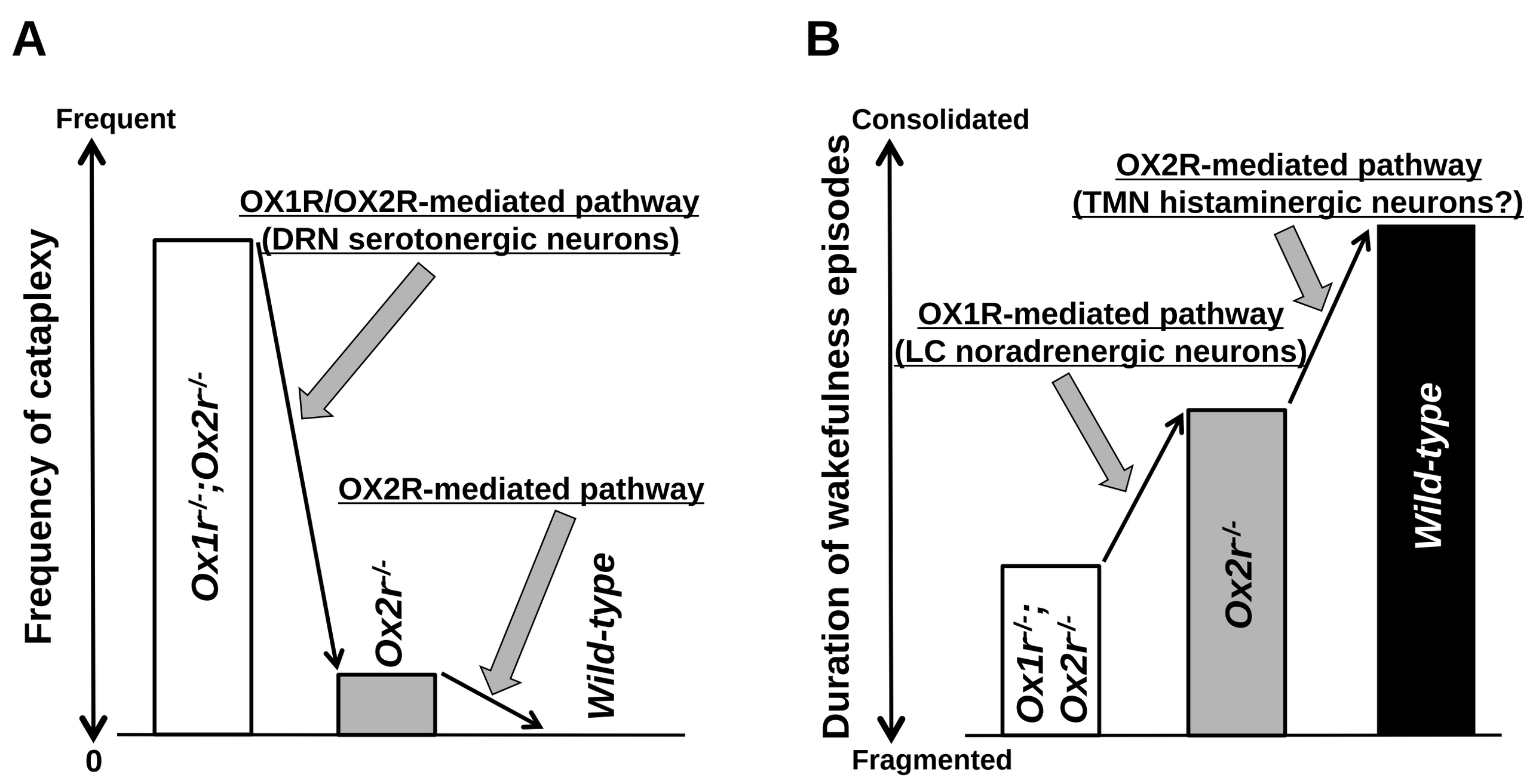
A
Frequent
Frequency of cataplexy
0
OX1R/OX2R-mediated pathway
(DRN serotonergic neurons)
OX2R-mediated pathway
Ox1r-/-;Ox2r-/-
Ox2r-/-
Wild-type
B
Consolidated
Duration of wakefulness episodes
Fragmented
OX2R-mediated pathway
(TMN histaminergic neurons?)
OX1R-mediated pathway
(LC noradrenergic neurons)
Ox1r-/-;
Ox2r-/-
Ox2r-/-
Wild-type

Table 1. Summary of results obtained by pharmacological studies using DORAs and SORAs

	DORA	NREM	REM/ NREM	2-SORA	NREM	REM/ NREM	1-SORA	NREM	REM/ NREM	1-SORA & 2-SORA	NREM	REM/ NREM
Brisbare-Roch et al, 2007	Almorexant (ACT-078573)	↑	Yes									
Dugovic et al., 2009	Almorexant (ACT-078573)	↑	Yes	JNJ-10397049	↑	No	SB-408124	→	No	JNJ-10397049 & SB-408124	→	No
Cox et al, 2010	Suvorexant (MK-4303)	↑	Yes									
Gozzi et al., 2011				JNJ-10307149	↑	No	GSK-1059865	→[a]	No			
Morairty et al., 2012	Almorexant (ACT-078573)	↑	No	EMPA	↑	No	SB-334867	↑	No			
Winrow et al, 2012	DORA22	↑	Yes									
Winrow et al, 2012	Filorexant (MK-6096)	↑	Yes									
Betschart et al., 2013	Suvorexant (MK-4303)	↑	Yes	IPSU	↑	No						
Steiner et al., 2013							ACT-335827	→	No			
Dugovic et al., 2014	SB-649868[b]	↑	Yes	JNJ-10397049	↑	No	GSK-1059865	→	No	JNJ-10397049 & GSK-1059865	→	Yes
Etori et al., 2014	Suvorexant (MK-4303)	→	Yes	C1m	↑	No						
Bonaventure et al., 2015a				JNJ-42847922	↑	No						
Bonaventure et al., 2015b							Compound 56	→	No	*Ox2r*$^{-/-}$ & Compound 56	→	Yes
Kuduk et al., 2015				MK-8133	↑	No						
Letavic et al., 2015				JNJ-42847922	↑	No						
Yoshida et al., 2015	E2006	↑	Yes									
Gotter et al., 2016				MK-1064	↑	No/Yes[c]						

NREM, time spent in NREM sleep after drug administration; REM/NREM, disproportionally large increase in REM sleep; DORA, non-selective orexin receptor antagonist; 1-SORA, selective OX1R antagonist; 2-SORA, selective OX2R antagonist; 1-SORA & 2-SORA, co-administration of 1-SORA and 2-SORA. In Bonaventire et al., (2015b), Compound 56 was administered in *Ox2r*$^{-/-}$ mice. [a]NREM sleep tended to increase in the first hour after administration; [b]Scarce episodes of direct transitions from wakefulness to REM sleep were observed; [c]Results were different between species.