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## **Physiological roles of orexin receptors on sleep/wakefulness regulation**

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## **Abstract**

Hypothalamic neuropeptides orexin-A and orexin-B play critical roles in the regulation of sleep/wakefulness, as well as in a variety of physiological functions including emotion, reward and energy homeostasis. The effects of orexin peptides are mediated by two receptors, orexin 1 (OX1R) and orexin 2 (OX2R) receptors. These receptors show differential expression patterns depending on brain regions and neuron types, suggesting their differential roles. Here, we review the current understanding of the physiological roles of each orexin receptor subtype, focusing on the regulation of sleep/wakefulness.

## Orexin receptors

The actions of orexins are mediated by two G-protein-coupled receptors, orexin 1 (OX1R) and orexin 2 (OX2R) receptors (Sakurai et al. 1998). 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 (Mieda et al. 2013), except in one study that reported the direct inhibitory action of orexin receptors on surrachiasmatic nucleus (SCN) neurons at night (Belle et al. 2014). When overexpressed, 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 (Zhu et al. 2003).

In clear contrast to the restricted localization of *orexin* mRNA expression exclusively in neurons distributed within the lateral hypothalamus (LH) and perifornical area (PFA) (Date et al. 1999; de Lecea et al. 1998; Nambu et al. 1999; Peyron et al. 1998; Sakurai et al. 1998), *OX1R* and *OX2R* mRNA show wide distributions within the brain with partly overlapping but distinct and complementary distributions (Marcus et al. 2001; Mieda et al. 2011). 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. Histaminergic neurons in the tubelomammillary nucleus (TMN) exclusively express *OX2R*, while noradrenergic neurons in the locus coeruleus (LC) exclusively express *OX1R*. In the dorsal raphe (DR) and median raphe (MnR), serotonergic neurons express *OX1R* and/or

*OX2R*. In the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT), cholinergic neurons express *OX1R* but not *OX2R* mRNA (Marcus et al. 2001; Mieda et al. 2011). Intriguingly, there are many GABAergic neurons expressing orexin receptors which are intermingled with these monoaminergic and cholinergic neurons.

### **Disruption of the Orexin System Causes Narcolepsy**

Degenerative loss of orexin neurons in humans is associated with narcolepsy (type I narcolepsy: narcolepsy with cataplexy), 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*<sup>-/-</sup>) 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 rapid eye movement (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*<sup>-/-</sup> mice display a phenotype strikingly similar to human narcolepsy, with 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).

### **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*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice) have provided a deeper insight into the differential roles of OX1R and OX2R (Fig. 1)(Sakurai 2007). First, *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice demonstrate a narcoleptic phenotype nearly similar to that in *orexin*<sup>-/-</sup> mice, confirming that orexinergic regulation of sleep/wakefulness is mediated by these two receptors. *OX1R*<sup>-/-</sup> mice show little abnormality in the states of sleep and wakefulness in the baseline condition (Hondo et al. 2010; Sakurai 2007). In contrast, *OX2R*<sup>-/-</sup> mice have clear narcoleptic symptoms, although their phenotype is milder as

compared to that found in *orexin*<sup>-/-</sup> mice (Sakurai 2007; Willie et al. 2003). During the dark phase, *OX2R*<sup>-/-</sup> 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 (Fig. 1). However, the frequency of such arrests was far less as compared to *orexin*<sup>-/-</sup> mice (31-fold lower frequency in *OX2R*<sup>-/-</sup> mice than in *orexin*<sup>-/-</sup> mice). Importantly, close observation of the behavior of *OX2R*<sup>-/-</sup> 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*<sup>-/-</sup> mice with a frequency similar to *OX2R*<sup>-/-</sup> 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*<sup>-/-</sup> mice are fragmented to an extent similar to those of *orexin*<sup>-/-</sup> mice, indicating that these two strains of mouse suffer from comparable sleepiness (Fig. 1). In contrast, *OX2R*<sup>-/-</sup> mice show normal REM sleep latency and amount of REM sleep, which are profoundly shortened and increased, respectively, in both *orexin*<sup>-/-</sup> mice and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice (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 loss of signaling through both OX1R- and OX2R-dependent pathways.

This conclusion has been further confirmed by a complementary experiment, i.e., comparing the arousal effects of ICV orexin-A administration between wild-type,

*OX1R*<sup>-/-</sup>, and *OX2R*<sup>-/-</sup> mice (Fig. 2)(Mieda et al. 2011). The effects of orexin-A on wakefulness and NREM sleep were significantly attenuated in both *OX1R*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice as compared to wild-type mice, with substantially larger attenuation in *OX2R*<sup>-/-</sup> than in *OX1R*<sup>-/-</sup> 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*<sup>-/-</sup> and *OX2R*<sup>-/-</sup> mice, suggesting a comparable contribution of the two receptors to REM sleep suppression (Mieda et al. 2011). The supplementary role of OX1R in the suppression of NREM sleep is consistent with the fact that *OX2R*<sup>-/-</sup> mice with a C57BL/6J, but not C57BL/6J-129/SvEv-mixed, genetic background show less fragmented wakefulness than *orexin*<sup>-/-</sup> mice and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> 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.

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).

## **Sites of expression of orexin receptors relevant to the physiological control of sleep/wakefulness**

The application of exogenous orexins has been shown to excite many types of neurons. 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 (Bayer et al. 2001; Brown et al. 2001; Burlet et al. 2002; Eggermann et al. 2001; Eriksson et al. 2001; Horvath et al. 1999; Liu et al. 2002; van den Pol et al. 2002; Yamanaka et al. 2002; Mieda et al. 2013). Furthermore, the administration of orexin-A directly into the LC (Bourgin et al. 2000), TMN (Huang et al. 2001), BF cholinergic area (España et al. 2001; Thakkar et al. 2001), and LDT (Xi et al. 2001) has also been reported to increase wakefulness. 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 the arousal effect of orexin. 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*<sup>-/-</sup> 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 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).

Recently, we 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*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice (Fig. 3A)(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 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 (designer receptor

exclusively activated by designer drug) technology ameliorated narcolepsy in mice that lacked orexin neurons (Fig. 3B). These results suggest that DR serotonergic and LC noradrenergic neurons 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 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, our observations 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 DR 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 (Sakurai 2007)(Fig. 4A). DR 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) (Pace-Schott and Hobson 2002; Luppi et al. 2011). Indeed, we observed dense projections of DR serotonergic neurons to these brain areas, as well as to the amygdala (Hasegawa et al. 2014), which suggests that DR 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*<sup>-/-</sup> mice than in *orexin*<sup>-/-</sup> mice and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> 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*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice stabilized wakefulness episodes to an extent comparable to those in *OX2R*<sup>-/-</sup> 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. 4B). Recent optogenetic studies have provided supports 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).

### **Pharmacological dissection of sleep/wakefulness regulation by orexin receptors**

A series of non-selective (dual) antagonists for orexin receptors (DORA), as well as subtype-selective antagonists (SORA), have been developed. On the one hand, these drugs are drawing people's attention as novel medications for insomnia and other diseases (Scammell and Winrow 2011; Sakurai 2014). 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. Selective blockade of OX2R efficiently increases NREM sleep and shortens NREM sleep latency (Dugovic et al. 2009; Betschart et al. 2013; Dugovic et al. 2014; Etori et al. 2014; Gozzi et al. 2011). Blockade of both OX1R and OX2R does increase NREM sleep, but also causes disproportionately large increase in REM sleep (Bonaventure et al. 2015; Dugovic et al. 2009; Dugovic et al. 2014; Etori et al. 2014; Hoyer et al. 2013). Except one study showing increases in REM and NREM sleep with SB-334867 (Morairty et al. 2012), selective blockade of OX1R alone does not cause any effects with statistical significance on baseline sleep/wakefulness (Bonaventure et al. 2015; Dugovic et al. 2009; Dugovic et al. 2014; Gozzi et al. 2011; Steiner et al. 2013). Thus, OX2R is the principal regulator of wakefulness/NREM sleep transition, while both OX1R- and OX2R-mediated pathways are critical for gating REM sleep.

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 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).

### **Conclusions and future directions**

Genetic and pharmacological studies have dissected the differential roles of orexin receptors in sleep/wakefulness regulation. These two approaches have both merits and demerits. Knockout mice provide clear animal models with specific and complete removal of one of two subtypes. However, chronic compensatory changes should always be taken into account. In contrast, SORAs can acutely block one of two, eliminating any compensatory effects. On the other hand, specificity, potency, efficacy, occupancy, and stability of the administered drugs should be considered.

Broad expression of orexin receptors throughout the brain complicates identifying neurons and orexin receptor subtypes directly regulated by endogenous orexins, mediating their wake-stabilizing effects. We and others have tackled this problem with a strategy of brain region/cell type-specific rescues of orexin receptors. In addition to such an approach, future studies utilizing brain region/cell type-specific genetic deletions of orexin receptors and pharmacological studies of focal administration of SORAs, as well as optogenetic and pharmacogenetic strategies, would

further uncover the differential roles of orexin receptors and neuronal pathways downstream to orexin neurons in the regulation of sleep/wakefulness.

## Figure legends

Fig. 1. 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 with 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 (Sakurai 2007).

Fig. 2. Differential effects on sleep/wakefulness states following activation of OX1R and OX2R. Dose responses of the effect of ICV orexin-A in wild-type (WT), *OX1R*<sup>-/-</sup>, *OX2R*<sup>-/-</sup>, and *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice on time spent in wakefulness, NREM sleep, and REM sleep following administration. Modified from (Mieda et al. 2011).

Fig. 3. Search for the downstream targets of orexin neurons to prevent narcolepsy. (A) Restoration of orexin receptor expression in the DR and LC inhibits cataplexy-like episodes and consolidates wakefulness, respectively, in narcoleptic *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice. Orexin receptor expression was restored in the nuclei indicated in *OX1R*<sup>-/-</sup>;*OX2R*<sup>-/-</sup> mice by recombinant AAV vectors. (B) Pharmacogenetic activation of DR serotonergic and LC noradrenergic neurons suppresses cataplexy-like episodes and

consolidates wakefulness, respectively, in narcoleptic mice lacking orexin neurons (*orexin/ataxin-3* mice). *Orexin/ataxin-3* mice with DR serotonergic neuron-selective or LC noradrenergic neuron-selective expression of excitatory DREADD hM3Dq were injected with saline or CNO, the artificial ligand for DREADDs. Hourly plots of number of cataplexy-like episodes and mean duration of wakefulness episodes within 12 hours after saline or CNO administration at ZT 12 (arrows) are shown. Modified from (Hasegawa et al. 2014).

Fig. 4. Proposed model for OX1R- and OX2R-mediated pathways in suppressing narcoleptic symptoms. **(A)** Prevention of cataplexy-like episodes. **(B)** Consolidation of wakefulness episodes. Modified from (Hasegawa et al. 2014).

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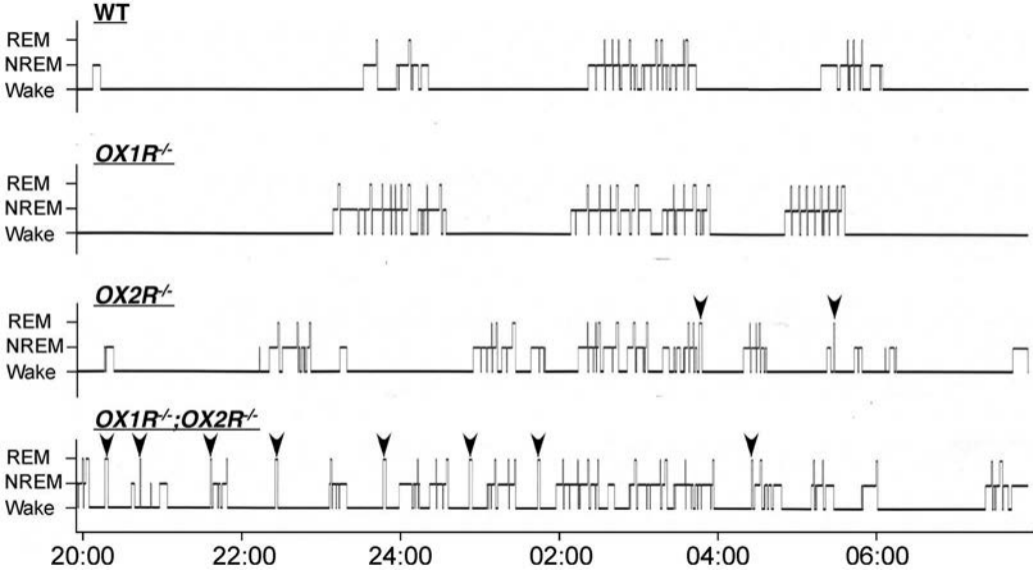
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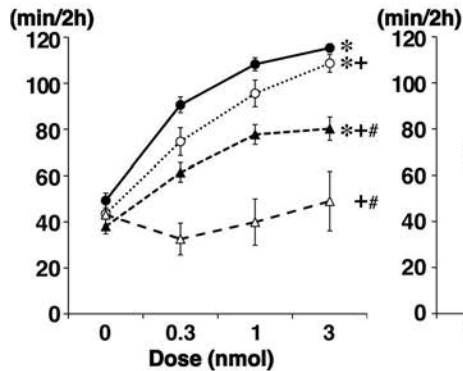
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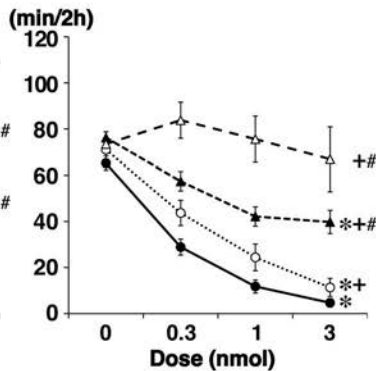
G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins. *Journal of pharmacological sciences* 92 (3):259-266



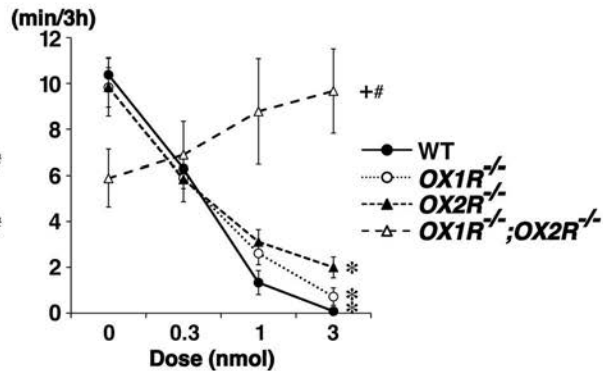
### Wakefulness

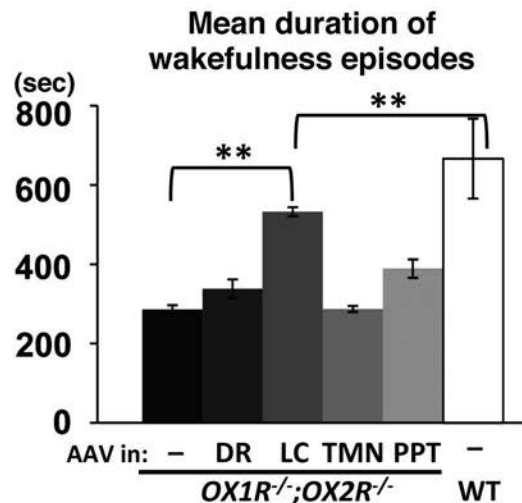
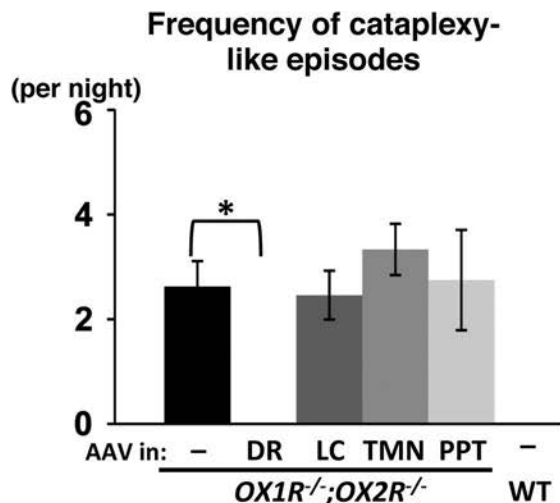
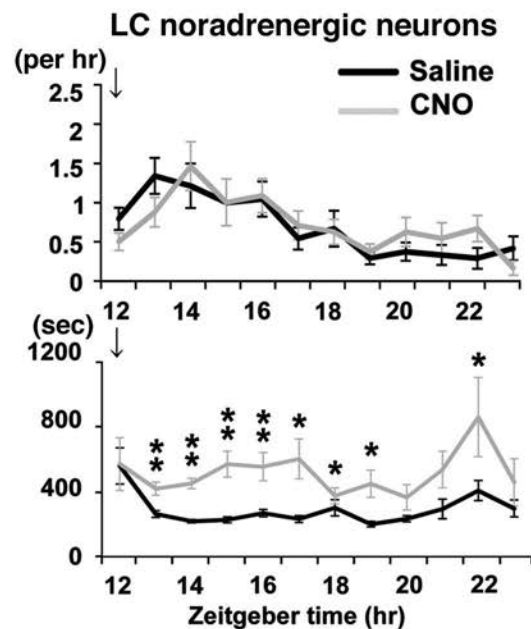
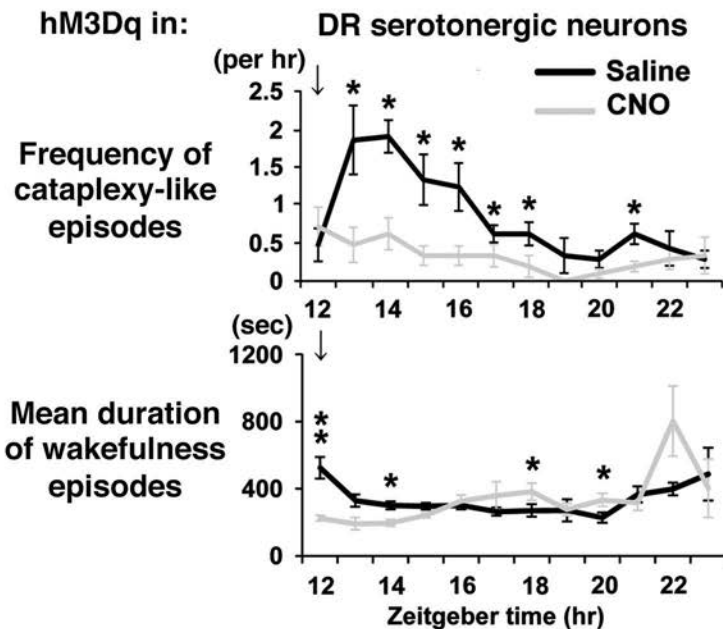


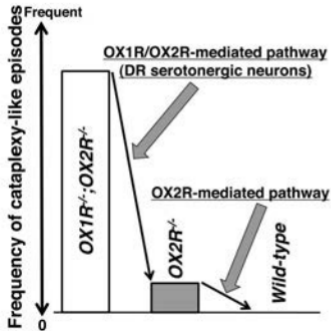
### NREM sleep



### REM sleep



**A****B**

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