Histamine-1 receptor is not required as a downstream effector of orexin-2 receptor in maintenance of basal sleep/wake states

Title

Histamine-1 receptor is not required to maintain basal sleep/wake states as a downstream effecter of orexin-2 receptor.

Short Title

Histamin-1 Receptor in Orexin Signaling

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Keywords

Electroencephalographic/electromyographic (EEG/EMG), histamine, histamine H_1 receptor, orexin, orexin receptor-1, orexin receptor-2, sleep/wake states, tuberomammillary nucleus (TMN)

Abstract

Aim: The effect of orexin on wakefulness has been shown to be largely mediated by activation of histaminergic neurons via orexin receptor-2 (OX_2R). Consistently, *OX_2R* mRNA is abundantly expressed in histaminergic neurons in the tuberomammillary nucleus (TMN). However, orexin receptors in the other regions of the brain might also play important roles in maintenance of wakefulness. To dissect the role of histaminergic system as downstream mediator of orexin system in regulation of sleep/wake states without compensation by the OX_1R-mediated pathway, we analyzed phenotype of *$H_1R^{-/-};OX_1R^{-/-}$* mice. This mice lack OX_1R-mediated regulation of noradrenergic neurons in the locus coeruleus and either H_1R system belonging to downstream of OX_2R in the TMN. By analyzing *$H_1R^{-/-};OX_1R^{-/-}$* mice, we aimed to find out the function of OX_2R–mediated pathways other than histaminergic system in the TMN in sleep/wake regulation.

Methods: We used *$H_1R^{-/-};OX_1R^{-/-}$* mice, *$H_1R^{-/-}$* mice, and their littermate wild type mice. REM sleep, NREM sleep, and awake states were determined by polygraphic electroencephalographic/electromyographic (EEG/EMG) recording.

Results: We did not observe detectable abnormality in sleep/wake states of *$H_1R^{-/-}$* mice, consistent with previous studies. *$H_1R^{-/-};OX_1R^{-/-}$* mice also showed comparable sleep/wake phenotype as wild type mice. This result indicates that regulation of sleep/wake system is

completely achieved by OX_2R-expressing neurons without involving H_1R-mediated pathways.

Conclusion: Our observation showed that the maintenance of basal physiological sleep/wake states is fully achieved without both H_1 and OX_1 receptors. Downstream pathways of OX_2R other than histaminergic system might play an important role in maintenance of sleep/wake states.

Introduction

The neuropeptides orexin A and orexin B (hypocretin 1 and hypocretin 2) are found as endogenous ligands for two orphan G-protein coupled receptors (Sakurai et al., 1998). There are two receptor subtypes, named orexin receptor-1 (OX_1R) and orexin receptor-2 (OX_2R). Orexin-producing neurons, localized in the lateral hypothalamic area (LHA), send projections to all over the central nervous system except the cellebellum. Especially dense orexin-immunoreactive fibers were found in monoaminergic and cholinergic nuclei in the brain stem regions (Marcus et al., 2001). Moreover, these nuclei abundantly express orexin receptors. Recent studies on efferent and afferent systems of orexin neurons, and phenotypic characterization of genetically modified mice in the orexin system have suggested roles of orexin system in regulation of sleep and wakefulness through interactions with systems that regulate emotion, reward system and energy homeostasis (Boutrel et al., 2005), (Sakurai et al., 2005), (Yamanaka et al., 2003), (Yoshida et al., 2006), (Mieda et al., 2004), (Narita et al., 2006), (Harris et al., 2005), (Akiyama et al., 2004).

Mice with targeted deletion of the *prepro-orexin* gene display a phenotype strikingly similar to human narcolepsy (Chemelli et al., 1999). Besides, functionally-null mutations in the *OX_2R* gene were found in familial narcoleptic dogs (Lin et al., 1999). Consistently, *$OX_2R^{-/-}$* mice show fragmented sleep/wake behavior and direct transitions from wakefulness

to REM sleep, although phenotype is significantly milder than that of *orexin*$^{-/-}$ mice, whereas *OX$_1$R*$^{-/-}$ mice do not have any overt behavioral abnormalities (Willie et al., 2001). Narcoleptic human brains have been shown to be associated with markedly reduced numbers of orexin neurons (Nishino et al., 2000), (Peyron et al., 2000), (Thannickal et al., 2000). These observations suggest that the OX_2R-mediated pathway has pivotal roles, although OX_1R has an additional role in the regulation of sleep/wake states.

Orexin receptors are distributed in a pattern consistent with orexin projections. mRNAs for *OX$_1$R* and *OX$_2$R* were differentially expressed throughout the brain (Marcus et al., 2001). *OX$_1$R* is most abundantly expressed in the locus cerules (LC), whereas *OX$_2$R* is most abundantly expressed in the tubaromamillary nucleus (TMN). The raphe nuclei contain both receptors mRNA. In vitro electrophysiological studies have also showed that all these monoaminergic neurons are activated by orexins. These monoaminergic neurons are implicated in regulation of sleep/wake states (Hara et al., 2001), (Lin et al., 1999), (Peyron et al., 2000), (Thannickal et al., 2000), (Chemelli et al., 1999).

Several studies showed that the arousal effect of orexin A largely depends on the activation of the histaminergic neurotransmission mediated by the histamine receptor-1 (H_1R) (Huang et al., 2001). Furthermore, histamine concentrations in the brain were decreased in *OX$_2$R* mutated narcoleptic dogs (Lin et al., 1999). These observations have

suggested the importance of interaction between orexin system and the TMN histaminergic system in regulation of sleep/wake states.

Histaminergic neurons are exclusively localized in the TMN (Watanabe T, 1984), and practically project to all brain regions with especially dense innervations in the hypothalamus, basal forebrain and amygdala (Panula P, 1984), (Takeda N, 1984). Those neurons are large and have a multi-dendritic shape and contain histidine decarbosylase (HDC), which converts L-histidine to histamine. The central histaminergic system is involved in many important function of the central nervous system, such as regulation of food intake (Morimoto T, 2001), thermoregulation and sleep/wake regulation (Lin JS, 1988), (Vanni-Mercier G, 1984) . It has been reported that the firing rates of histamine neurons vary across the sleep/wake cycle (Sakai, 1990), and intracerebroventricular (i.c.v.) injection of histamine or H_1 agonist induces wakefulness and diminution of non-rapid eye movement (NREM) sleep (Monti JM, 1986), (Tasaka K, 1989), (Monti, 1993). These results suggest that the brain histaminergic system plays a critical role in regulation of sleep/wake states.

Orexin neurons densely innervate TMN neurons, where OX_2R is abundantly expressed (Marcus et al., 2001), further suggesting the possibility that orexin neurons regulate vigilance and arousal through modulating the activity of histaminergic neurons in the TMN. However, *H_1R*-deficient mice show normal sleep/wake phenotype. This

contradicts the hypothesis that histaminergic system the most important as the downstream systems of the OX_2R. A possible explanation of this discrepancy is the compensation by other systems such as noradrenergic neurons in the LC, serotonergic neurons in the raphe, and cholinergic neurons in the LDT/PPT. *$OX_2R^{-/-}$* mice are affected with cataplexy like attacks of REM sleep and sleep/wake fragmentation(Willie et al., 2003), suggesting that solo deficiency of OX_2R-signaling pathway can affect stability of sleep/wake states. On the otherhand, single OX_1R-deficiency might be compensated by OX_2R-mediated pathways. These observations suggest that solo deficiency of OX_2R might be also compensated by OX_1R-mediated pathway, because the phenotype of *OX_2R*-deficient mice is milder than *prepro-orexin* knockout mice.

In the present study, we analyzed the role of histaminergic system as a downstream signaling of OX_2R-mediated pathway using *H_1R* and *OX_1R* double deficient mice (*$H_1R^{-/-};OX_1R^{-/-}$*). In these mice, OX_1R-mediated pathway was totally abolished, while OX_2R-mediated pathway remained intact, but H_1R-mediated pathway is deficient system. Therefore, these mice enable us to analyze the roles of the H_1R-mediated pathway without compensation by OX_1R-mediated pathways.

Materials and Methods

Animal preparation

All experimental procedures involving animals were approved and conducted in accordance with the University of Tsukuba and Kanazawa University Animal Care Committees. All efforts were made to minimize animal suffering from discomfort and to reduce the number of animals used. $H_1R^{-/-}$ mice were previously reported in detail (Inoue I, 1996). $OX_1R^{-/-}$ and $OX_2R^{-/-}$ mice were described previously (Hara et al., 2001, Willie et al., 2003). Mice were housed under controlled lighting (12 hr light-dark cycle; light-on at 8:00 a.m., off at 8:00 p.m.) and temperature conditions. Food and water were available *ad libitum*.

All mice were backcrossed with C57BL/6J mice more than 6 times.

Surgery

Male mice (10-12 weeks old, 20-25 g at the time of surgery) were prepared for chronic monitoring of EEG/EMG signals using a lightweight implant and cabling procedure. Full details of this technique were published previously (Chemelli et al., 1999). Briefly, the EEG/EMG implant was based on a six-pin double inline microcomputer connector, modified to form four EEG electrodes, each 1.3 mm x 0.3 mm (h x w) positioned 4.6 mm x 2.9 mm (l x

w) apart with two EMG electrodes soldered to the enter pins. Mice were anesthetized with sodium pentobarbital (Nembutal, 50-60 mg/kg i.p.), and standard sterile surgical and stereotaxic procedures were employed for implant placement. Four burr holes were drilled on the cranium, anterior and posterior to bregma, bilaterally (AP 1.1, ML 1.4 and AP-3.6, ML1.4) according to the atlas of Franklin and Paxinos (1997). The implant was then inserted into these holes, fixed to the skull with adhesive dental cement, and the EMG electrodes were placed into the nuchal musculature, and wounds were closed by sutures.

Electrophysiological recording

Immediately after surgery, mice were housed singly for a recovery period of a week. The head-mounted connector was coupled via a light weight cable to a slip ring commutator, which was suspended from a counter-balanced arm mounted to a standard shoebox cage (19 cm x 30 cm, Allentown Caging, Allentown, NJ). This allowed mice full freedom of movement. The cage was modified to provide side delivery of food and water that were available *ad libitum*. All mice were habituated to these conditions for at least 7 days before recording was begun. Then EEG/EMG recording for two consecutive 24 hr period, beginning at lights-on at 8:00 pm was performed. Infrared video recording was simultaneously performed. EEG/EMG signals were amplified using a multichannel amplifier

(Nihon Koden, Tokyo, Japan) and filtered (EEG: 0.5-100 Hz, EMG: 0.5-100 Hz) before being digitized at a sampling rate of 250 Hz, displayed on a paperless polygraph system, and archived for off-line sleep staging and analysis.

Sleep scoring and data analysis

EEG/EMG records were visually scored into 16 s epochs of Awake, REM sleep, and NREM sleep. Data were analyzed by two-way ANOVA followed by post hoc analysis of significance with the Bonferroni's or Student's t test using the Stat View 5.0 software package (Abacus Concepts, Berkeley, CA). In all cases, $P<0.001$ was taken as the level of significance.

Results

Sleep/Wake States Characteristics

Sleep state patterns of $H_1R^{-/-}$;$OX_1R^{-/-}$ mice, $H_1R^{-/-}$ mice, and wild type mice were revealed by simultaneous EEG/EMG recording as previously reported(Chemelli et al., 1999). Typical representative 12 hour dark period (20:00-8:00) hypnograms for $H_1R^{-/-}$ mice and $H_1R^{-/-}$;$OX_1R^{-/-}$ mice are shown in Figure 1. Under baseline conditions, the patterns of sleep/wake states were statistically not different among wild type, $H_1R^{-/-}$ mice, $OX_1R^{-/-}$ mice, $H_1R^{-/-}$ mice, and $H_1R^{-/-}$;$OX_1R^{-/-}$ mice. There were no significant differences between $H_1R^{-/-}$;$OX_1R^{-/-}$ mice, $H_1R^{-/-}$ mice, and their littermate wild type mice, in either the amounts and duration of NREM sleep, REM sleep and wakefulness during dark period(Figure 2). These parameters were not distinguishable among all genotypes during light period (table 1). Hourly analysis of quantities of NREM sleep, REM sleep and wakefulness also revealed that there are no detectable differences between control, $H_1R^{-/-}$ and $H_1R^{-/-}$;$OX_1R^{-/-}$ mice (Figure 3). In contrast, $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice show significantly shorter duration of wakefulness and direct transitions from wakefulness to REM sleep as previously described (Hara et al., 2001).

Discussion

The actions of orexins are mediated via two G-protein coupled receptors, OX_1R and OX_2R. Although these orexin receptors are expressed in a pattern consistent with orexin projections, mRNAs for *OX_1R* and *OX_2R* were differentially distributed in the brain. Several studies have indicated that the effect of orexin is largely mediated by activation of the histaminergic system through the OX_2R in the TMN (Huang et al., 2001), (Shigemoto Y, 2004), (Yamanaka A, 2002) . Consistently, *$OX_1R^{-/-}$* mice showed normal sleep/wake phenotype, while *$OX_2R^{-/-}$* mice are affected with cataplexy like attacks of REM sleep and sleep/wake fragmentation (Willie et al., 2001), (Sakurai, 2007). However, phenotype of *$OX_2R^{-/-}$* mice was significantly milder than *$OX_1R^{-/-}$*;*$OX_2R^{-/-}$* mice (Sakurai, 2007). From these observations, loss of OX_1R has additional effects on sleep/wake states, although chronic loss of OX_1R in mice might be fully compensated by OX_2R function.

From these observations, the histaminergic pathway is currently thought to be the most important pathway in the OX_2R-pathway in arousal regulation.

Three distinct subtypes of histamine receptors, H_1 receptor (H_1R), H_2 receptor (H_2R), and H_3 receptor (H_3R) are distributed in the brain and exhibit well defined distribution patterns (Bouthenet ML, 1988), (Martinez-Mir MI, 1990) . H_1R and H_2R are postsynaptic receptors coupled to $G_{q/11}$ and Gs protein, and H_3R is a presynaptic autoreceptor coupled to

$G_{i/o}$ protein. Previous pharmacological studies demonstrated that clinical use of H_1R antagonists promotes sleepiness (Nicholson AN, 1985). The depletion of endogenous histamine by injection of α-fluoromethylhistidine, an HDC inhibitor, decreased the amount of nocturnal wakefulness in rats (Kiyono S, 1985) . Also, slow-wave sleep is induced in cats by microinjections of the selective H_1R antagonist pyrilamine into the preoptic area (Lin JS, 1994) or the dorsal pontine tegmentum (Lin JS, 1996) . These results suggest that H_1R mediates the waking effect of histamine.

However, Huang et al. reported that $H_1R^{-/-}$ mice showed sleep/wake states essentially identical to those of wild type mice but with fewer incidents of brief awakening (<16-sec epoch), and prolonged durations of non-rapid eye movement (NREM) sleep episodes, although the physiological significance of brief awakening has not been well understood yet (Huang ZL, 2006) . This suggests that the physiological function of H_1R does not contribute in regulating the basal amount of sleep/wake states. Alternatively, chronic deficiency of H_1R-mediated pathways is compensated by other arousal-related pathways such as noradrenergic system in the LC, in which OX_1R is abundantly expressed.

In this study, we analyzed $H_1R^{-/-};OX_1R^{-/-}$ mice to define importance of the role of histaminargic system as a downstream effector of the orexinergic system in sleep/wake regulation. This mice lack both OX_1R-mediated regulation of noradrenergic neuron in the

locus coeruleus (LC), and H_1R system belonging to downstream of OX_2R in the TMN. Signaling pathways in the downstream of OX_2R, such as serotonergic neuron in the raphe nuclei, cholinergic neuron in the LDT/PPT, dopaminergic neuron in the VTA remain intact. The raphe nuclei, LDT/PPT, and VTA also abundantly express OX_1R in wild type mice, but in $H_1R^{-/-};OX_1R^{-/-}$ mice, only OX_2R is expressed in these regions. By analyzing these mice, we analyzed the role of H_1R system belonging to downstream of OX_2R. We found that $H_1R^{-/-};OX_1R^{-/-}$ mice showed normal sleep/wake phenotype as compared with wild type mice. These results are not consistent with the hypothesis that “activation of the histaminergic system via OX_2R is the most important pathway in sleep/wake regulation”. In consistent with these results, Blanco-Centurion et al. reported that saporin-induced lesions in the TMN of the histamine neurons, along with the cholinergic neurons in the basal forebrain (BF), and the noradrenergic neurons in the locus ceruleus (LC) did not change waking amount, although the lesions effected the transition time of light-dark phase (Blanco-Centurion C, 2007). Our observations suggest that the regulations of basal physiological sleep/wake states are fully achieved without H_1R even when OX_1R is absent. Loss of H_1R-mediated pathway might be fully compensated by OX_2R-mediated pathways without contribution of OX_1R. Considering that fact that $OX_2R^{-/-}$ show mild but definite narcoleptic phenotype, OX_2R-mediated pathways other than histaminergic pathways might highly important for

basal regulation of sleep/wake states.

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Conflict of interest

The authors declare no conflicts of interest in the present study.

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Legends to figures

Figure 1. Representative 12hr dark period (20:00-8:00) hypnograms for wild type (WT), $H_1R^{-/-}$, $H_1R^{-/-}$;$OX_1R^{-/-}$ and $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice. The height of the horizontal line above baseline indicates the vigilance state of the mouse at the time. W, wakefulness; NR, non-REM sleep; R, REM sleep. There were no significant sleep/wake abnormalities between them. Hypnograms were obtained by simultaneous EEG/EMG recording.

Figure 2. (A) Total time (minutes, mean±SEM) spent in each state (awake, non-REM sleep, and REM sleep) in $H_1R^{-/-}$, $H_1R^{-/-}$;$OX_1R^{-/-}$ and wild type mice during dark period. (B) Episode duration (seconds±SEM) spent in each state (awake, non-REM sleep, and REM sleep) during dark period in $H_1R^{-/-}$, $H_1R^{-/-}$;$OX_1R^{-/-}$, wild type mice, and $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice. $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice show characteristic of narcoleptic disorder induced by deficiency of both orexin receptors. However mice with other genotypes did not show abnormality in sleep/wake states. The bar graphs summarize the data during the 12h dark period.

*$p<0.001$ (statistical analyses)

Figure 3. Hourly analysis of sleep/wake amounts in control, $H_1R^{-/-}$, $H_1R^{-/-}$;$OX_1R^{-/-}$, and $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice. The shaded area represent the 12h dark cycle. There are no

significant differences between $H_1R^{-/-}$;$OX_1R^{-/-}$ mice, $H_1R^{-/-}$ mice, and their littermate wild type mice, while $OX_1R^{-/-}$;$OX_2R^{-/-}$ mice show abnormal circadian distribution of REM sleep.

Table 1. Total time spent in each state (minutes, mean ± SEM), episode duration (seconds ± SEM), REM latency (minutes, mean ± SEM) over 24 hr and itemized separately for light and dark periods. There are no significant differences between $H_1R^{-/-}$;$OX_1R^{-/-}$ mice, $H_1R^{-/-}$ mice, and their littermate wild type mice. *$p<0.05$ (statistical analyses)

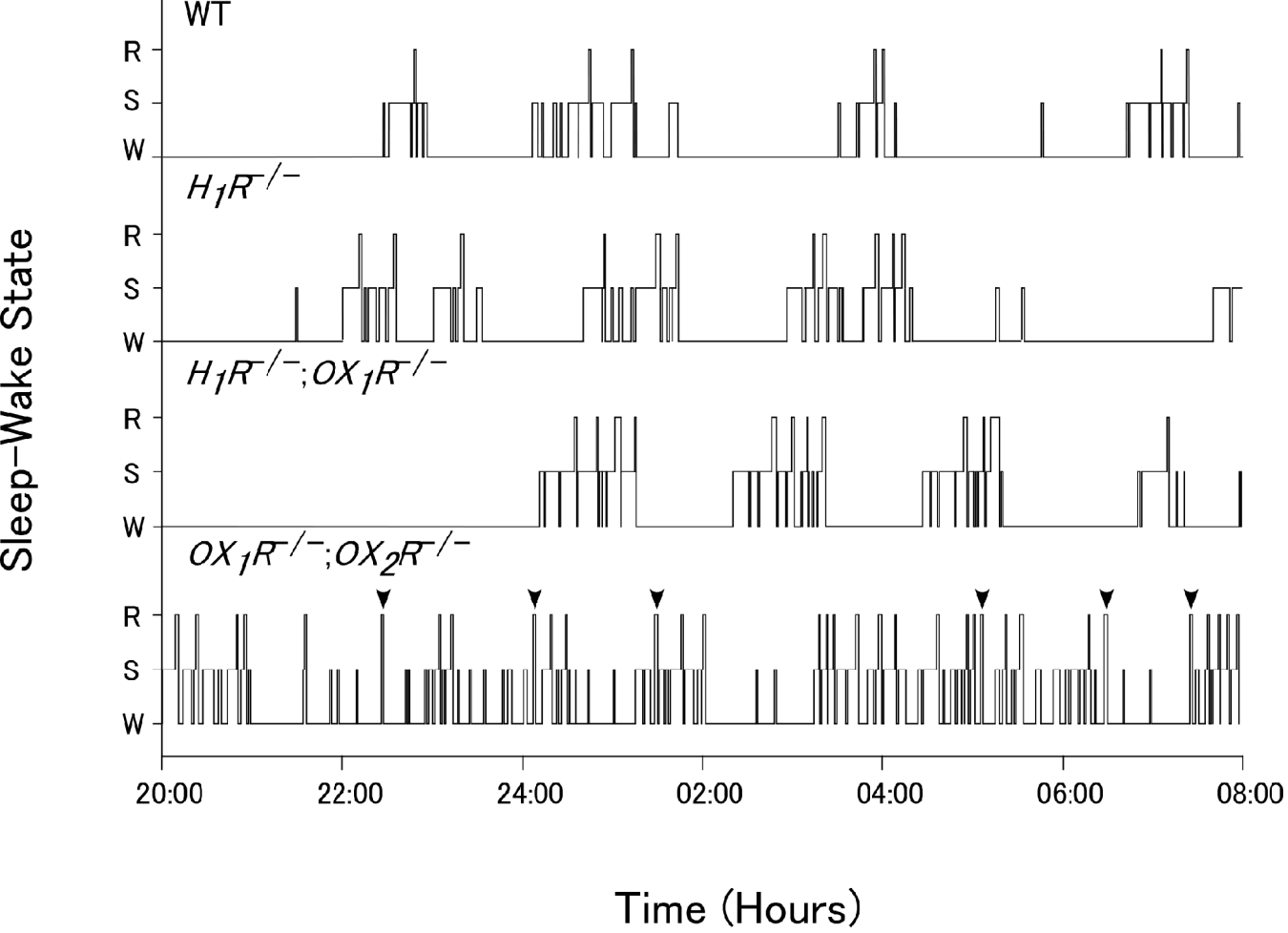

WT
R
S
W
H1R-/-
R
S
W
H1R-/-;OX1R-/-
R
S
W
OX1R-/-;OX2R-/-
R
S
W
20:00
22:00
24:00
02:00
04:00
06:00
08:00
Sleep–Wake State
Time (Hours)

Figure 1

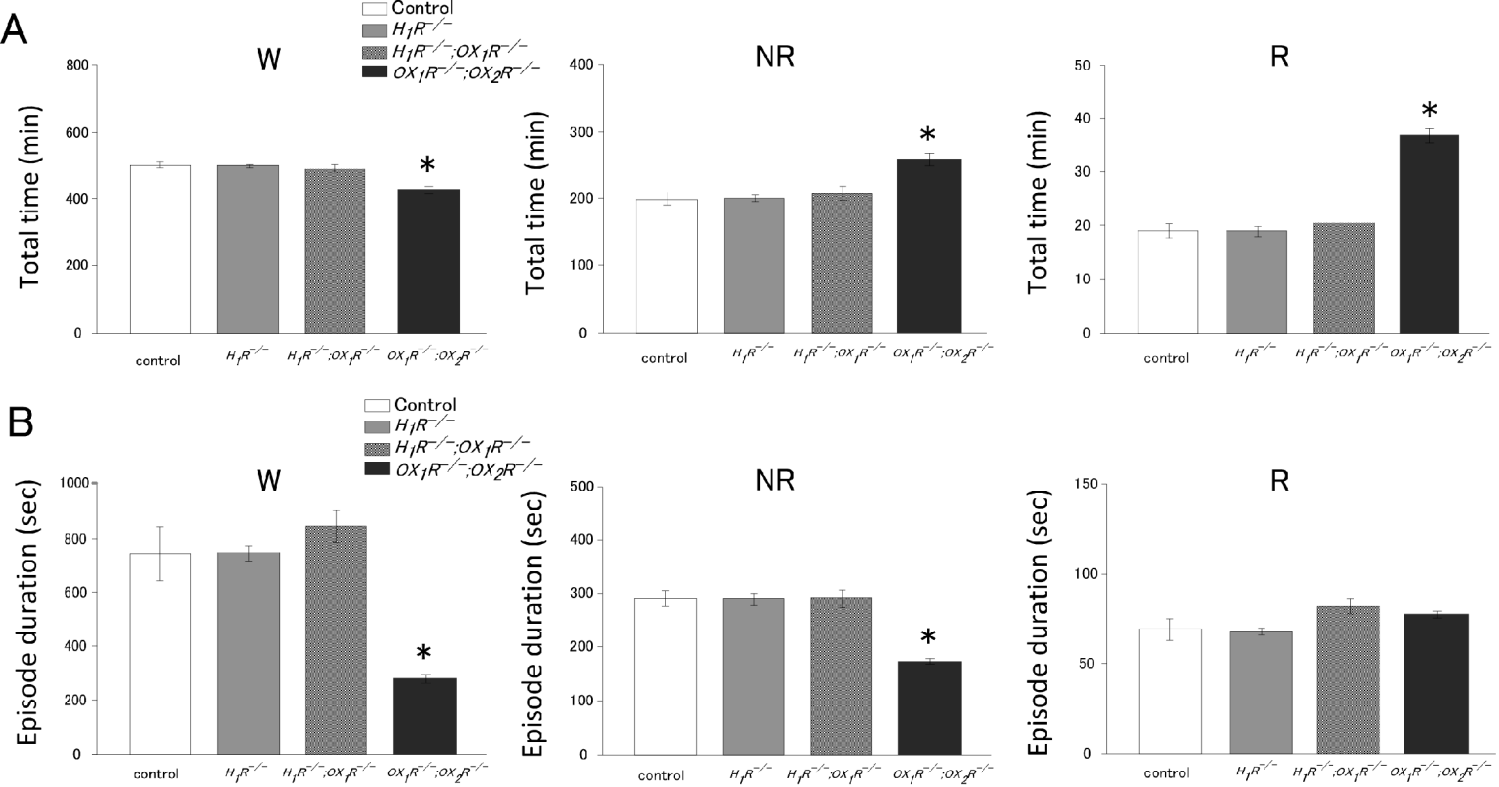
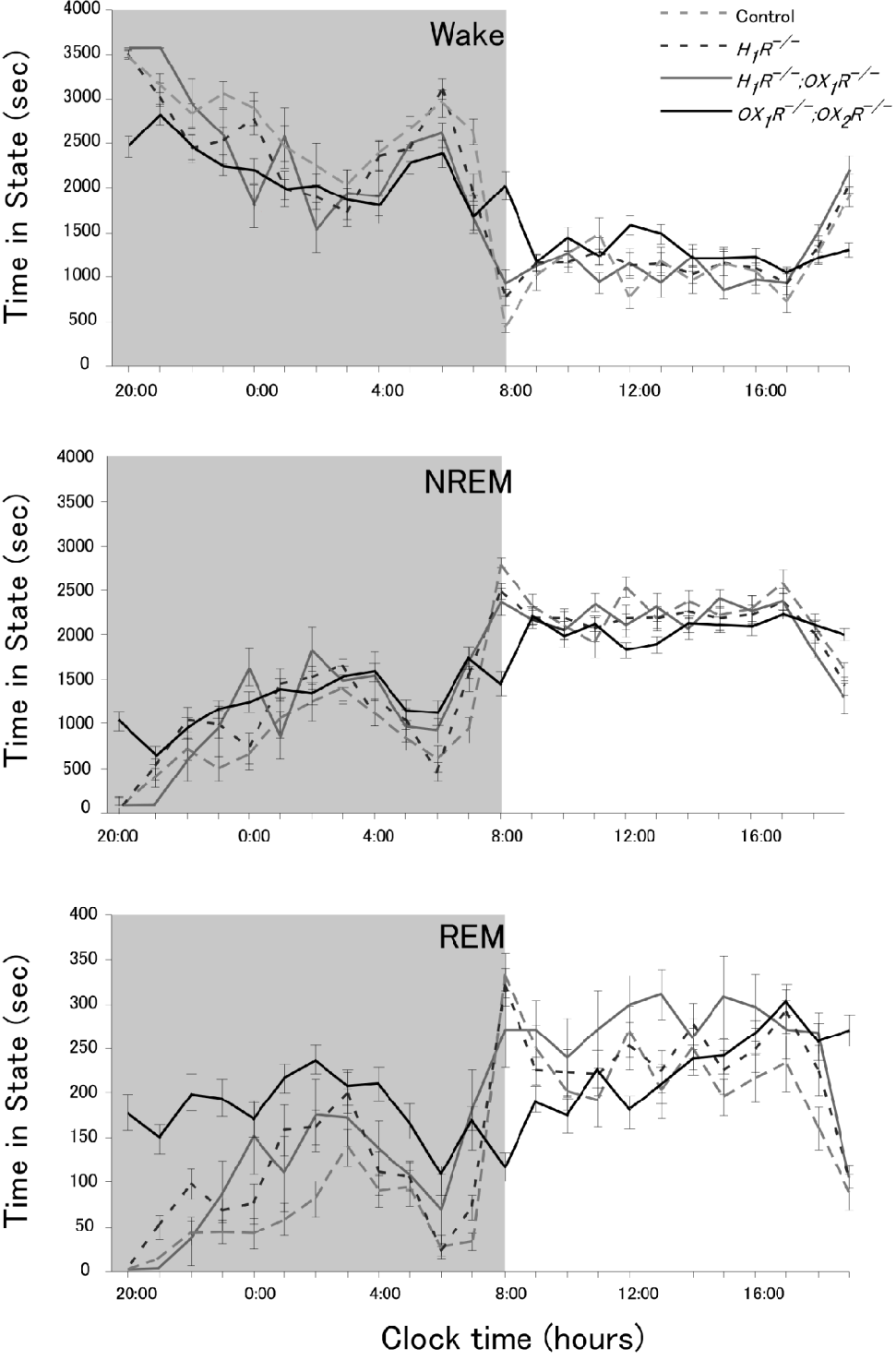

A
Control
H1R−/−
H1R−/−;OX1R−/−
OX1R−/−;OX2R−/−
W
NR
R
Total time (min)
control
H1R−/−
H1R−/−;OX1R−/−
OX1R−/−;OX2R−/−
B
Episode duration (sec)

Figure 2


Wake
NREM
REM
Control
$H_1R^{-/-}$
$H_1R^{-/-}$;$OX_1R^{-/-}$
$OX_1R^{-/-}$;$OX_2R^{-/-}$
Time in State (sec)
Clock time (hours)
20:00
0:00
4:00
8:00
12:00
16:00

	REM Sleep			Non-REM Sleep			Awake		
	WT	$H_1R^{-/-}$	$H_1R^{-/-}/OX_1R^{-/-}$	WT	$H_1R^{-/-}$	$H_1R^{-/-}/OX_1R^{-/-}$	WT	$H_1R^{-/-}$	$H_1R^{-/-}/OX_1R^{-/-}$
24 hr									
Total time (min)	65.8±1.6	66.9±2.4	70.0±4.2	636.1±13.4	635.2±6.6	639.5±13.7	735.1±11.5	737.9±7.0	726.2±16.8
Episode duration (s)	71.5±3.2	70.0±1.5	82.8±2.4	288.9±16.2	287.3±7.0	289.9±9.5	456.1±107.9	452.7±49.4	531.7±69.7
REM latency (min)	368.3±19.1	353.0±9.9	364.1±15.8						
REM counts	25.4±2.1	25.8±2.0	28.2±3.3						
Light Period									
Total time (min)	46.8±1.3	47.9±1.6	49.4±3.0	435.7±5.7	433.4±4.9	430.5±6.7	233.4±5.0	238.6±5.5	235.8±7.4
Episode duration (s)	73.6±3.0	71.8±2.2	83.5±2.7	286.4±31.0	284.0±9.0	287.8±9.6	165.6±18.3	155.8±4.5	214.6±13.0
REM latency (s)	328.3±18.7	323.1±10.1	328.5±12.9						
REM counts	35.13±2.3	37.1±2.1	36.8±4.7						
Dark Period									
Total time (min)	19.0±1.2	19.0±1.0	20.6±2.1	200.4±9.7	201.8±5.4	209.0±10.4	501.7±8.1	499.2±5.6	490.4±12.0
Episode duration (s)	69.4±6.0	68.2±2.0	82.1±4.2	291.4±14.6	290.4±10.8	292.1±16.8	746.6±99.5	749.5±27.1	848.8±57.5
REM latency (s)	403.2±29.6	382.2±13.7	399.7±25.6						
REM counts	15.7±1.2	16.7±0.9	19.6±3.2						

Table 1