# Selective loss of GABAB receptors in orexin/hypocretin-producing neurons results in disrupted sleep/wakefulness architecture

Taizo Matsuki[1,2], Mika Nomiyama[1], Hitomi Takahira[2], Noriko Hirashima[2], Thomas S. Kilduff[3], Satoshi Kunita[4], Satoru Takahashi[4,5], Ken-ichi Yagami[4], Bernhard Bettler[6], Masashi Yanagisawa[1,7], Takeshi Sakurai[1,2,*]

[1]Yanagisawa Orphan Receptor Project, Exploratory Research for Advanced Technology (ERATO), Japan Science and Technology Agency, Tokyo 135-0064, Japan; [2]Department of Pharmacology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305-8575, Japan; [3]Neurobiology Laboratory, Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA; [4]Laboratory Animal Resource Center, and [5]Department of Anatomy and Embryology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305-8575, Japan; [6]Department of Biomedicine, Institute of Physiology, Pharmazentrum, University of Basel, CH-4056 Basel, Switzerland; [7]Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390

**Address for correspondence:** Dr. Takeshi Sakurai, Department of Pharmacology, Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8575, Japan; Phone & Fax: +81-29-853-3276, E-mail: stakeshi@md.tsukuba.ac.jp

**Number of Figures and Tables:** 6 figures and 1 table

**Number of Pages:** 40 pages

**Acknowledgements**

**Author Contributions**

T.M. and T.S. conducted the experiments and performed the analyses, and also participated in the

design of the experiments. All authors contributed to discussion of the data and writing of the paper.

**Competing Interests Statement**

The authors declare that they have no competing financial interests.

**Abstract**

We generated mice with a selective loss of $GABA_B$ receptors in orexin neurons. Orexin neurons in these $GABA_{B1}^{-/-(orexin)}$ mice showed reduced responsiveness to $GABA_A$ receptor agonists due to a compensatory increase in $GABA_A$ receptor-mediated inhibition. This increased $GABA_A$ receptor-mediated inhibition of orexin neurons is due to orexin-1 receptor-mediated activation of local GABAergic interneurons. Surprisingly, orexin neurons were also less responsive to glutamate, apparently because the augmented $GABA_A$ receptor-mediated inhibition increases the membrane conductance and shunts excitatory currents. These observations indicate that absence of $GABA_B$ receptors decreases the sensitivity of orexin neurons to both excitatory and inhibitory inputs. $GABA_{B1}^{-/-(orexin)}$ mice exhibited severe fragmentation of sleep/wake states during both the light and dark periods without affecting total sleep time or inducing cataplexy, indicating that $GABA_B$ receptors are crucial regulators of orexin neurons and that "fine tuning" of orexin neurons by inhibitory and excitatory inputs is important for the stability of sleep/waking states.

Orexin A and orexin B, also known as hypocretin-1 and hypocretin-2, are critical regulators of sleep/wakefulness states[1]. Orexin neurons are localized exclusively in the lateral hypothalamic area (LHA) and send excitatory projections to the waking-active monoaminergic neurons in the hypothalamus and brainstem regions[1]. The importance of orexins in the maintenance of consolidated sleep/wakefulness states is demonstrated by the fact that the sleep disorder narcolepsy is caused by orexin deficiency in human and animals[2-5]. Investigations of the efferent and afferent systems of orexin neurons and phenotypic characterization of genetically-modified mice have suggested additional functions for the orexins in the coordination of emotions, energy homeostasis, reward systems, drug addiction, and arousal[1].

The regulatory mechanisms of orexin neurons are important for understanding the physiological basis of sleep/wake control. *In vitro* studies showed that the activity of orexin neurons is influenced by several neuropeptides and neurotransmitters[1]. Recent studies showed that orexin neurons receive innervation from several brain regions, including the limbic system (amygdala, bed nucleus of the stria terminalis, and septal regions), preoptic area, and monoaminergic neurons[1,6].

Orexin neurons also receive glutamatergic and GABAergic innervation from local interneurons[1,7]. *In vitro* electrophysiological studies showed that both $GABA_A$ and $GABA_B$ agonists inhibit the activity of orexin neurons[7-9], but the physiological relevance of such regulation is unknown.

$GABA_B$ receptors ($GABA_B$Rs) are heterodimers composed of $GABA_{B1}$ and $GABA_{B2}$ subunits, both of which are required for normal receptor function[10,11]. $GABA_B$ receptors are coupled to G-proteins and modulate synaptic transmission by activating postsynaptic inwardly rectifying Kir3 $K^+$ channels (GIRK channels) and by controlling neurotransmitter release through coupling to $Ca^{2+}$ channels[12,13]. Dysfunction of $GABA_B$-mediated synaptic transmission in the central nervous system occurs in several neurological disorders[13-15]. Among sleep disorders, the partial $GABA_B$R agonist gamma-hydroxybutyrate (GHB)[16] is used to treat both the excessive sleepiness and cataplexy symptoms of human narcolepsy. $GABA_B$R activation inhibits presynaptic release of both glutamate and GABA onto orexin neurons[9] and both pre- and postsynaptic $GABA_B$Rs modulate the activity of orexin neurons[9]. In the present study, we engineered mice with a selective deletion of the *$GABA_{B1}$* gene in orexin neurons (orexin neuron-restricted knockout mice; *$GABA_{B1}^{-/-(orexin)}$* mice) to study the consequences of the absence of $GABA_B$Rs on orexin neuron function at the cellular and behavioral levels.

**Results**

**Mice Selectively Lacking Functional GABA$_B$ Receptors in Orexin Neurons**

The GABA$_{B1}$ subunit is essential for the assembly of functional GABA$_B$ receptors and mice lacking

the *GABA$_{B1}$* subunit show a complete absence of GABA$_B$ responses[17]. To obtain mice with a deletion

of the *GABA$_{B1}$* subunit gene restricted to orexin-producing neurons (*GABA$_{B1}$$^{-/-(orexin)}$* mice), *orexin/Cre*

transgenic mice (see supplementary information) were mated with homozygous floxed GABA$_{B1}$ mice

possessing exon 7 and 8 of the *GABA$_{B1}$* allele flanked by lox-P sites[18]. Double immunofluorescence

studies in wild-type control mice showed that many neurons in the LHA had GABA$_{B1}$-ir in their soma

and dendrites (Figure 1). Among these, many neurons in the LHA were positive for both orexin-ir

and GABA$_{B1}$-ir (Figure 1). In contrast, neurons positive for both GABA$_{B1}$-ir and orexin-ir were rarely

detectable in the brain of *GABA$_{B1}$$^{-/-(orexin)}$* mice, although there were many GABA$_{B1}$-ir-positive neurons

in the LHA that were negative for orexin (Figure 1). We found that less than 10% of orexin neurons in

*GABA$_{B1}$$^{-/-(orexin)}$* mice were double-labelled for orexin and GABA$_{B1}$-ir. This may be due to incomplete

penetrance of Cre expression in *orexin/Cre* transgenic mice and/or incomplete deletion of loxP sites

in *GABA$_{B1}$$^{-/-(orexin)}$* mice. These observations indicate that in *GABA$_{B1}$$^{-/-(orexin)}$* mice, more than 90% of

orexin neurons lack expression of $GABA_B$ receptors. Gross anatomical and histological studies failed to detect any structural abnormalities in the brain of $GABA_{B1}{}^{-/-(orexin)}$ mice. Specifically, the number of orexin neurons in the LHA remained normal; the number of immunoreactive cells (located from 0.76 mm to 2.52 mm posterior to bregma) were 3420±124 and 3560±108 for control littermates and $GABA_{B1}{}^{-/-(orexin)}$ mice, respectively (n=5).

$GABA_{B1}{}^{-/-(orexin)}$ mice showed normal development of body weight; body weights at 22 weeks of age were 35.9±2.9, 34.9±2.5, and 34.8±1.8 g for $GABA_{B1}{}^{flox/+}$, *orexin/Cre* Tg mice (n=9), $GABA_{B1}{}^{flox/flox}$ (*orexin/Cre* negative) mice (n=6), and $GABA_{B1}{}^{-/-(orexin)}$ mice (n=6), respectively (p=0.35, ANOVA with Bonferroni correction). Expression levels of hypothalamic neuropeptides, including orexin, neuropeptide Y, proopiomelanocortin, and melanin concentrating hormone, did not differ between $GABA_{B1}{}^{-/-(orexin)}$ and control mice, as determined by quantitative RT-PCR using Taqman probes or immunohistochemical analysis (data not shown). In contrast to conventional $GABA_{B1}$ KO mice that exhibit contextual hyperactivity,[17] locomotor activity of $GABA_{B1}{}^{-/-(orexin)}$ mice across the 24 hr period was not altered (data not shown).

**Electrophysiological Characteristics of Orexin Neurons Lacking GABA$_B$ Receptors**

The electrophysiological properties of orexin neurons were examined in $GABA_{B1}^{-/-(orexin)}$ mice by

patch-clamp recordings using brain slice preparations (Figure 2). Bath application of GABA (0.6 mM)

or muscimol (30 μM) produced hyperpolarization of orexin neurons in both control ($GABA_{B1}^{flox/+}$,

*orexin/Cre* Tg or $GABA_{B1}^{flox/flox}$, *orexin/Cre* negative) and $GABA_{B1}^{-/-(orexin)}$ mice (Figure 2a, b). These

GABA- and muscimol-induced effects rapidly reversed upon removal of these reagents from the

external solution (Figure 2a, b). To specifically examine GABA$_B$ receptor-mediated effects on

neuronal activity, we applied the GABA$_B$ agonist baclofen. Bath application of baclofen (100 μM)

hyperpolarized all orexin neurons tested in control slices (n=6) (Figure 2a). As expected from the

histological data in Figure 1, baclofen failed to induce hyperpolarization in 10 out of 12 orexin

neurons tested in slices from six $GABA_{B1}^{-/-(orexin)}$ mice (Figure 2b). Application of 5-hydroxytryptamine

(5-HT) induced strong hyperpolarization of orexin neurons regardless of genotype, suggesting that

downstream signaling systems of G$_{i/o}$-coupled receptors, including the activation of Kir3 channels,

remain intact in $GABA_{B1}^{-/-(orexin)}$ mice (Figure 2a, b).

In current-clamp mode, orexin neurons lacking $GABA_B$ receptors showed a resting

membrane potential and firing frequency similar to that of orexin neurons in control littermates

($GABA_{B1}^{-/-(orexin)}$: -59.2±5.8 mV, 5.1±2.6 Hz, n=12; control: -55.0±2.9 mV, 4.0±0.7 Hz, n=12),

indicating that $GABA_B$ receptor-deficient orexin neurons have a basal activity comparable to that of

control neurons, despite the absence of a major inhibitory receptor. However, the frequency of fast

spontaneous inhibitory postsynaptic currents (sIPSCs) of orexin neurons in $GABA_{B1}^{-/-(orexin)}$ slices

was significantly increased compared to that in slices from control littermates ($GABA_{B1}^{-/-(orexin)}$:

2.65±0.32 Hz, n=9; control: 1.59±0.12 Hz, n=13; p=0.011) (Figure 3a, b). Bath application of

baclofen (100 µM) decreased the frequency of sIPSCs to 3.4% and 23% of control values (in the

absence of baclofen) in control littermates and $GABA_{B1}^{-/-(orexin)}$ mice, respectively (Figure 3a, b). This

suggests that sIPSCs on orexin neurons are elicited by GABA release from GABAergic terminals

having presynaptic $GABA_B$ receptors (Figure 3a, b). Next, we examined the effects of the $GABA_A$

and $GABA_B$ receptor antagonists bicuculline (BIC) and CGP54626, respectively, on the activity of

orexin neurons. Consistent with our previous study[9], orexin neurons in wild-type control slices did

not respond to applications of the antagonists BIC (25 μM) or CGP54626 (12 μM) (Figure 3c and

data not shown). In contrast, orexin neurons in slices from $GABA_{B1}^{-/-(orexin)}$ mice responded with

depolarization to BIC application (n=4) (Figure 3c). These findings indicate that $GABA_A$ receptors

are tonically activated in orexin neurons of $GABA_{B1}^{-/-(orexin)}$ mice but not in wild-type mice. The

spontaneous excitatory postsynaptic currents (sEPSCs) of orexin neurons from $GABA_{B1}^{-/-(orexin)}$ and

control littermate mice were comparable ($GABA_{B1}^{-/-(orexin)}$: 5.1±0.73 Hz, n=4; control: 5.8±1.2 Hz, n=3;

p= 0.74). These results indicate that the inhibitory $GABA_A$ input to orexin neurons is increased, while

excitatory glutamatergic input to orexin neurons remains unchanged in $GABA_{B1}^{-/-(orexin)}$ mice. This

mechanism might prevent offset of the resting membrane potential and firing frequency of orexin

neurons in $GABA_{B1}^{-/-(orexin)}$ mice.

To further analyze the mechanisms that increase sIPSC frequency in orexin neurons lacking

$GABA_B$ receptors, we recorded miniature IPSCs (mIPSCs). mIPSCs were isolated by adding

tetrodotoxin (TTX) (1 μM) to the sIPSC recording solution. We found that the mean frequency and

peak currents of mIPSCs in orexin neurons of $GABA_{B1}^{-/-(orexin)}$ mice were comparable to that of control

mice ($GABA_{B1}^{-/-(orexin)}$: 1.1±0.29 Hz, 94.5±21.0 pA, n=5; control: 0.99±0.26 Hz, 104.5±11.2 pA, n=6;

p>0.05 by Student's t test).  These results suggest that the increase of sIPSCs in $GABA_{B1}$-deleted

orexin neurons is due to an increase in the local GABAergic tone, rather than to an increase in

$GABA_A$ receptors or the number of GABAergic synapses. Due to increase in $GABA_A$ input, the

membrane resistance of orexin neurons in $GABA_{B1}^{-/-(orexin)}$ mice was significantly decreased (control:

466 ± 29 MΩ, n=25; $GABA_{B1}^{-/-(orexin)}$: 364 ± 27 MΩ, n=12; p= 0.017, Student's *t* test) (Figure 3e).

Since the genetic deletion of the $GABA_{B1}$ gene is restricted to orexin neurons (Figure 1), the

increase in sIPSP frequency in orexin neurons must be attributable to a dysregulation of these

neurons. Interestingly, we found that application of orexin A (1 μM) increased the frequency of

sIPSCs in wild-type orexin neurons (baseline: 1.6 ± 0.25 Hz; orexin A: 2.7 ± 0.33 Hz; washout: 1.5 ±

0.32 Hz; n= 5; p <0.05 by one-way ANOVA), suggesting that orexin-induced activation of local

GABAergic neurons is involved in the increase of inhibitory input to orexin neurons in $GABA_{B1}^{-/-(orexin)}$

mice.  We hypothesized that the activity of local GABAergic neurons expressing the orexin receptor

is increased in $GABA_{B1}^{-/-(orexin)}$ mice due to increased orexin release onto these interneurons. To

evaluate this possibility, the orexin receptor antagonist SB334867 was used to block the orexin

receptor-1 ($OX_1R$).  Preincubation with SB334867 (5 μM) reduced the frequency of sIPSCs in orexin

neurons of $GABA_{B1}^{-/-(orexin)}$ mice (basal: 2.7 ± 0.43 Hz, n = 6; SB334867: 1.1 ± 0.30 Hz, n = 7; p=

0.012 by Student *t* test), but had no effect in control littermate mice  (1.1 ± 0.36 Hz, n = 4  vs 1.0 ±

0.13 Hz, n = 9, p= 0.769 by Student *t* test) (Fig, 3d). These results indicate that orexin activates local

GABAergic neurons via the $OX_1R$ resulting in an increase of the GABAergic synaptic input onto

orexin neurons, and that activity of these local circuits is increased in $GABA_{B1}^{-/-(orexin)}$ mice.

**Decreased Responsiveness of Orexin Neurons Lacking GABA$_B$ Receptors to Excitatory and**

**Inhibitory Inputs**

Although the basal membrane potential and firing frequency of orexin neurons in $GABA_{B1}^{-/-(orexin)}$ mice

were comparable to that of wild-type mice due to the compensatory increase in GABAergic input, the

absence of GABA$_B$ receptors caused abnormalities in these cells.  We found that the effect of the

GABA$_A$ agonist muscimol on orexin neuron activity was markedly attenuated in $GABA_{B1}^{-/-(orexin)}$ mice

(Figure 4a, b). In current clamp mode, the threshold for hyperpolarization of orexin neurons by

muscimol was 1.2 μM and 6 μM in littermate control slices and $GABA_{B1}^{-/-(orexin)}$ slices, respectively.

These findings indicate that the responses of orexin neurons to the GABA$_A$ agonist muscimol are

decreased in slices from $GABA_{B1}^{-/-(orexin)}$ mice. Tonic activation of GABA$_A$ receptors might explain

why the exogenous application of the GABA$_A$ agonist muscimol had a less potent effect on orexin

neurons in $GABA_{B1}^{-/-(orexin)}$ mice since, under basal conditions, GABA$_A$ receptors in these neurons are

already tonically activated by GABA (Figure 3c).

Next, we examined the effect of an excitatory neurotransmitter, glutamate, on the activity of

orexin neurons. The effects of glutamate were markedly attenuated in $GABA_{B1}^{-/-(orexin)}$ mice (Figure

4c, d). These findings suggest a decrease in the membrane resistance of orexin neurons in

$GABA_{B1}^{-/-(orexin)}$ mice (Figure 3e) due to excess GABA$_A$R activation by increased local GABAergic

neuron activity which results in decreased sensitivity to both excitatory and inhibitory inputs. This

decreased sensitivity might be due to the increase in membrane conductance (Figure 3e), which

caused shunting of excitatory input, although the resting membrane potential was comparable to

control.

## $GABA_{B1}$ $^{-/-(orexin)}$ Mice Exhibit Severe Behavioral State Disruption

To examine the *in vivo* consequences of dysregulation of orexin neurons resulting from the absence

of $GABA_B$ receptors, behavioral state patterns of $GABA_{B1}$ $^{-/-(orexin)}$ mice and control littermate mice

were studied by simultaneous EEG/EMG recordings[5,19,20]. Typical hypnograms (graphic

representations of sleep-waking states over time) of $GABA_{B1}$ $^{-/-(orexin)}$ mice and control littermates

during the 24 hr dark and light periods are shown in Figure 5a and 5b. Although the overall amounts

of wakefulness, non-REM, and REM sleep were similar between the two strains (Figure 5c),

$GABA_{B1}$ $^{-/-(orexin)}$ mice displayed severe fragmentation of sleep/wakefulness states indicated by

decreased awake, non-REM, and REM sleep episode durations and reduced REM latency (Figure

5d and Table 1). The fragmentation of sleep/wakefulness states in $GABA_{B1}$ $^{-/-(orexin)}$ mice was more

severe than that previously described in *orexin*-knockout mice or even in *orexin/ataxin-3* transgenic

mice in which orexin neurons are specifically ablated[5,20]. However, neither the direct transitions from

an awake state to REM sleep nor the cataplexy-like behavioral arrests that are occasionally

observed in both orexin-deficient mice and orexin neuron-ablated mice were observed in

$GABA_{B1}^{-/-(orexin)}$ mice[5,20]. Interestingly, in contrast to the findings in orexin-deficient mice in which

fragmentation is only observed during the dark period,[5,20] fragmentation of behavioral states in

$GABA_{B1}^{-/-(orexin)}$ mice occurred during both the light and the dark periods (Figure 5 and Table 1).

Although the total sleep time of $GABA_{B1}^{-/-(orexin)}$ mice tended to increase when compared to control

mice, this difference was not statistically significant (Figure 5 and Table 1). These changes along

with a decrease in REM sleep latency indicate increased pressure for entry into REM sleep in

$GABA_{B1}^{-/-(orexin}$ mice.

**Discussion**

Orexin neurons are indispensable components of the regulatory mechanism that controls

sleep/wake states. We have previously studied the regulatory mechanisms of orexin neurons in

vitro[7,8,21-24]. However, the physiological relevance of the regulatory mechanisms or inputs affecting

the activity of orexin neurons remains unknown. In this study, we addressed the importance of

GABA, the major inhibitory neurotransmitter in the mammalian central nervous system, because the

proper functioning of neurons is dependent upon the regulation of neural activity by GABA-releasing

neurons. Moreover, GABA has been implicated in the regulation of sleep. For instance, $GABA_A$

receptor activation is well-known to induce sleep-promoting responses[25]. Administration of the

$GABA_B$ agonist baclofen (25 mg) before sleep in a clinical study also significantly prolonged total

sleep time and reduced the time spent awake after sleep onset[26]. The $GABA_B$ antagonist CGP

35348 increased the duration of both non-REM and REM sleep in aged rats compared to those in

saline-injected controls when injected during the night[27] and decreased non-REM sleep while

increasing the duration of waking and REM sleep when injected during the day[28]. These results

suggest an important role for $GABA_B$ receptors in the regulation of sleep/wakefulness states.

The present experiments showed that typical $GABA_B$ responses are almost completely

eliminated in orexin neurons from $GABA_{B1}^{-/-(orexin)}$ mice (Figure 2). $GABA_B$ agonist-induced

postsynaptic effects were also eliminated and the sensitivity of orexin neurons to both $GABA_A$

receptor agonists and glutamate was decreased in $GABA_{B1}^{-/-(orexin)}$ mice (Figure 4). As shown in

Figure 3, we also found that inhibitory synaptic inputs to orexin neurons are increased in

$GABA_{B1}^{-/-(orexin)}$ mice. However, in the presence of TTX, mIPSC frequencies are comparable between

$GABA_{B1}^{-/-(orexin)}$ and control orexin neurons (data not shown). These observations suggest that

spontaneous GABA release onto orexin neurons was not increased, but that activity-dependent

inhibitory signals from local GABAergic neurons onto orexin neurons were increased in slice

preparations from $GABA_{B1}^{-/-(orexin)}$ mice.

Because deletion of the $GABA_BR$ was confined to orexin neurons (Figure 1), the increase

in GABAergic influence must be attributable to the abnormality of the orexin neurons. We suggest

that orexin neurons innervate local GABAergic neurons that, in turn, send inhibitory projections onto

the orexin neurons (Figure 6). In the parental $GABA_{B1}$ floxed line, exons 7 and 8 of $GABA_{B1}$ gene are

flanked by lox-P sites[18]. Therefore, both the $GABA_{B1a}$ and $GABA_{B1b}$ subtypes are deficient in the

orexin neurons of the $GABA_{B1}^{-/-(orexin)}$ mice, which therefore lack functional $GABA_B$ receptors

altogether. Presumably, in the $GABA_{B1}^{-/-(orexin)}$ mice, orexin release is increased due to the lack of

functional GABA$_B$ receptors in the nerve terminal or the somatodendritic compartment of orexin

neurons, leading to excessive activation of local GABAergic neurons. Consistent with this hypothesis,

preincubation of $GABA_{B1}^{-/-(orexin)}$ slices with the OX$_1$R antagonist reduced the sIPSC frequency to a

level similar to that found in control slices (Figure 3d).

These alterations in the synaptic input of orexin neurons due to a lack of functional GABA$_B$

receptors result in severe sleep/waking fragmentation in $GABA_{B1}^{-/-(orexin)}$ mice during both the light

and dark periods (Figure 5 and Table 1), indicating that the GABA$_B$ receptor in orexin neurons is

indispensable for proper regulation of sleep/wake states.  As to why $GABA_{B1}^{-/-(orexin)}$ mice showed

robust fragmentation of sleep/wake behavior in both the dark and light periods, we have previously

hypothesized that orexin neurons are appropriately regulated to maintain proper vigilance states

according to the animals' environment[1].  In $GABA_{B1}^{-/-(orexin)}$ mice, the effects of both GABA and

glutamate in orexin neurons were severely impaired. Therefore, orexin neurons in these mice are

unlikely to be appropriately and tightly regulated (Figure 4).

Orexin knockout mice and orexin neuron-deficient mice show behavioral instability and

sleep/wake fragmentation only during the dark (active) period[5,20]. In contrast, transgenic mice with

constitutive overexpression of orexin show a fragmented sleep/wake phenotype only during the light

(rest) period. The activity of orexin neurons is high during the dark period[29]; therefore, abnormality of

*orexin*$^{-/-}$ mice during this period might be expected. On the other hand, since the activity of orexin

neurons is low or quiescent during the light period, the sleep/wake abnormality of

orexin-overexpressing mice becomes apparent during this period[30]. However, *GABA$_{B1}$*$^{-/-(orexin)}$ mice

showed robust fragmentation of sleep/wake behavior during both the dark and light periods. In the

light (rest) period, orexin neurons might be inhibited by GABAergic influences from GABAergic

neurons in the preoptic area[1], which are likely important for stably silencing orexin neurons[31,32]. In

*GABA$_{B1}$*$^{-/-(orexin)}$ mice, both GABA$_A$- and GABA$_B$-mediated inhibition of orexin neurons was severely

impaired (Figures 2, 3, and 4). These defects may explain why these mice showed fragmented sleep

in the light period, because orexin neurons in *GABA$_{B1}$*$^{-/-(orexin)}$ mice cannot be effectively inhibited by

GABA. On the other hand, during the dark (active) period, orexin neurons would be activated by

various excitatory inputs including glutamatergic influences[1]. In *GABA$_{B1}$*$^{-/-(orexin)}$ mice, the effect of

glutamate on orexin neurons was also severely impaired (Figure 4); consequently, the activity of

orexin neurons is not appropriately regulated to maintain wakefulness according to various inputs

(Figure 6).

In $GABA_{B1}{}^{-/-(orexin)}$ mice, we did not observe the cataplexy-like behavioral arrests or the direct

transitions from wakefulness to REM sleep that are occasionally seen in orexin-deficient animals.

This result is consistent with the observation that mice overexpressing orexin do not show cataplexy,

although these mice show fragmentation of sleep/wakefulness states during the light period[30].

Together, these results suggest that fine tuning of orexinergic tone may not be necessary for

inhibition of cataplexy and direct transition from wakefulness to REM sleep.  On the other hand, the

present study showed that fine tuning of orexin neurons is crucial for stability of sleep/wakefulness

states and maintenance of normal sleep/wake architecture (Fig. 5d).

This study demonstrated the importance of $GABA_B$ activity in a defined neuronal circuit and the

importance of orexin neuron $GABA_BRs$ for maintenance of wakefulness and sleep state

consolidation.  The phenotype of $GABA_{B1}{}^{-/-(orexin)}$ mice is characterized by impaired ability to maintain

any behavioral state, which is one of the primary symptoms of human narcolepsy. Therefore,

$GABA_{B1}^{-/-(orexin)}$ mice may be a useful model in which to study narcolepsy without cataplexy. Since the

partial $GABA_B$ agonist GHB is used clinically to extend both sleep bouts and subsequent

wakefulness, thereby allowing narcoleptic humans to engage in longer periods of purposeful activity

during their waking hours, $GABA_{B1}^{-/-(orexin)}$ mice may also be a useful model in which to study the

therapeutic action of GHB. Impaired ability to maintain wakefulness and consolidate sleep is also

one of the hallmarks of sleep in the elderly. Given the widespread use of hypnotic medications that

target GABAergic signalling, it will be interesting to determine whether age-related defects in

GABAergic control of orexin neurons occur. Lastly, the orexin neurons have been proposed to be a

crucial component of a "flip-flop" switch controlling sleep and wakefulness[25]. The orexin system is

hypothesized to be the stabilizer of the flip-flop switch[1]. The phenotype of the $GABA_{B1}^{-/-(orexin)}$ mice

suggests that fine regulation of orexin neuronal activity is necessary for proper function of the flip-flop

switch.

**Methods**

**Animal Usage**

All experimental procedures involving animals were approved by the Animal Experiment and Use

Committee of University of Tsukuba and were in accordance with NIH guidelines.

**Generation of *Orexin/Cre* Transgenic Mice**

The transgenic construct was made by substituting the *nlacZ* gene (SalI-BamHI fragment) of the

*orexin/nlacZ* transgenic construct[33] with the Cre-recombinase cDNA and an internal ribosome entry

site with the eGFP (*IRES-eGFP*) gene (see supplementary information).  The transgene was

linearized and microinjected into pronuclei of fertilized mouse eggs from the F1 of C57BL/6 x DBA1;

BDF1.  Founder animals were bred with C57BL/6 mice to produce a stable *orexin/Cre* line.

Genotyping of *orexin/Cre* transgenic mice was performed by PCR of tail DNA.  Human *prepro-orexin*

promoter sequences, *Cre recombinase*, and *eGFP* coding sequences were amplified with PCR

primer sets: human *prepro-orexin* genomic fragment, sense: GCAGCGGCCATTCCTTGG,

anti-sense: AAGTCGACGGTGTCTGGCGCTCAGGGTG; Cre recombinase, sense:

GGTTCGTTCACTCATGGAAAATAG, anti-sense: GGTATCTCTGACCAGAGTCATCCT; eGFP,

sense: GAAGGGCATCGACTTCAAGG, anti-sense: ACGAACTCCAGCAGGACCAT.

**Breeding and Maintenance of Mouse Lines**

*Orexin/Cre* mice were mated with $GABA_{B1}^{flox/flox}$ (BALB/cA) mice[18] and a breeding colony for

producing *orexin/Cre*, $GABA_{B1}^{flox/flox}$ ($GABA_{B1}^{-/-(orexin)}$; BALB/cA: C57BL/6: DBA1 mixed background)

was maintained by mating $GABA_{B1}^{flox/flox}$ with *orexin/Cre*, $GABA_{B1}^{flox/+}$ mice. To detect Cre

recombinase expression, we mated *orexin/Cre* Tg mice with 129-$Gt(ROSA)26Sor^{(EGFP)}$/J reporter

mice (Jackson Laboratories) (Supplementary fig. 1).

We produced mice with selective deletion of the $GABA_{B1}$ gene in orexin neurons by crossing

$GABA_{B1}^{flox/flox}$ and *orexin/Cre* Tg mice. The genetic background of the mice used in this study was a

mixture of BALB/c, C57BL/6, and DBA1 (75% : 21.875% : 3.125%). These mice with either a

C57BL/6 background or a C57BL/6 and DBA1 mixed background showed similar sleep/wakefulness

behavior, consistent with previous studies[34,35]. The total daily duration of non-REM sleep did not

significantly differ between strains, even though the BALB/c strain has been reported to exhibit a

shorter period of time in non-REM sleep during the day than the night[36]. These similarities suggest

that differences in genetic background have minimal effects on the results of our study. To further

minimize the effect of genetic background, all experiments were performed with littermates as

controls. We found that wild type mice, *orexin/Cre* Tg mice*, GABA$_{B1}$$^{flox/+}$*, *orexin/Cre* Tg mice and

*GABA$_{B1}$$^{flox/flox}$* (*orexin/Cre*-negative) mice all show the same phenotype regarding sleep/wakefulness

states (data not shown). In this study, *GABA$_{B1}$$^{flox/flox}$*, *orexin/Cre* Tg mice were used as *GABA$_{B1}$*

spatially-restricted KO mice, while *GABA$_{B1}$$^{-/-(orexin)}$* mice and their *GABA$_{B1}$$^{flox/flox}$*, *orexin/Cre*-negative

littermates were used as controls unless otherwise stated.

For electrophysiological analysis, *GABA$_{B1}$$^{-/-(orexin)}$* mice were mated with *orexin/eGFP*

transgenic mice (C57BL/6 background) to prepare *GABA$_{B1}$$^{-/-(orexin)}$* mice with expression of eGFP as a

marker of orexin neurons[19].

**Histological Analysis**

Mouse brains were fixed and prepared as previously described[37].  Mice were anesthetized by

intraperitoneal injection of sodium pentobarbital and perfused via the heart with phosphate-buffered

saline (PBS), followed by 0.1 M phosphate buffer containing 4% paraformaldehyde.  The whole brain

was postfixed for 2 hr in 4% paraformaldehyde in PBS.  Sections were washed with 0.3% Triton

X-100 in PBS and incubated with 1% bovine serum albumin in PBS for 1 hr, and then incubated with

primary antibodies in the same solution for 1 hr at room temperature.  Staining was performed with

standard procedures using rabbit or guinea pig anti-orexin antisera[37], mouse anti-Cre antibody

(Nacalai), or guinea pig anti-GABA$_{B1}$ antibody (Chemicon) and Alexa 488 or 594-conjugated

secondary antibodies (Invitrogen).

**EEG/EMG Recording**

Male $GABA_{B1}^{-/-(orexin)}$ mice (n=10) and their weight-matched male control littermates ($GABA_{B1}^{flox/+}$,

$orexin/Cre$ Tg mice, n=5; or $GABA_{B1}^{flox/flox}$ ($orexin/Cre$-negative) mice, n=6) were implanted with

electrodes at 12 weeks of age to record simultaneous EEG/EMG, as described previously[5]. Animals

were allowed to recover and habituate for 2 weeks under a constant 12 hr light/dark cycle at 25°C

with free access to food and water. Continuous EEG/EMG traces were recorded with ad libitum

feeding for the first 48 hr (baseline). Traces were captured and digitized in 20 s epochs. Each of two

investigators, blinded to genotype, scored and categorized all epochs as wakefulness, REM sleep,

or non-REM sleep according to standard criteria of rodent sleep. REM latency was determined as

the time between the onset of sleep and the first REM sleep period. Inter-REM interval was

calculated as the duration between successive REM sleep episodes. Data were analyzed by

ANOVA for repeated measurements to detect interactions between genotype and vigilance state

parameters.

**Electrophysiological Studies**

Male and female $GABA_{B1}^{-/-(orexin)}$ ($GABA_{B1}^{flox/flox}$, $orexin/Cre$ Tg), control ($GABA_{B1}^{flox/+}$; $orexin/Cre$ Tg or

$GABA_{B1}^{flox/flox}$, $orexin/Cre$ negative), and $orexin/eGFP$ mice, 2-5 weeks old, were used for the

experiments. The brain was cut coronally into 300-μm slices with a microtome (Leica) in

Ringer-sucrose buffer bubbled with 95% $O_2$/5% $CO_2$ gas. Slices containing the LHA were

transferred to an incubation chamber filled with a physiological solution containing the following (in

mM): 140 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, at room temperature

(24-26ºC) for at least for 1 hr. For electrophysiological recording, the slices were kept at a controlled

temperature of 34ºC and were superfused with a physiological solution that was warmed by an in-line

heater (Warner Instruments) to 34ºC. A fluorescence microscope equipped with an infrared camera

(Hamamatsu Photonics) for infrared differential interference contrast imaging and a charge-coupled

device camera (Olympus Optical) was used for fluorescent imaging. For whole cell patch-clamp

recording, pipettes were filled with an internal solution, HEPES-buffered potassium chloride (KCl) or

potassium methanesulfonate ($KMeSO_4$) solution, as previously described[9]. Pipette resistance was

5-10 MΩ. The series resistance during recording was 10-40 MΩ and was not compensated. The

liquid junction potential of the patch pipette solution and perfused HEPES solution was estimated to

be 3.9 mV and was applied to the data. The membrane patch was then ruptured by suction, and

membrane current and potential were monitored using an Axopatch 200B patch-clamp amplifier

(Axon Instruments). Data were recorded on a computer through a Digidata 1322A analog-to-digital

converter using pClamp 10.0 software (Axon Instruments). sEPSCs were recorded using KCl

internal solution supplemented with the sodium channel blocker QX-314 (1 mM) to inhibit action

potentials in the neuron, and bicuculline (BIC; 20 μM) and picrotoxin (PTX; 100 μM) were added to

the physiological HEPES solution to block $GABA_A$ receptor-mediated neurotransmission. sIPSCs

were recorded using the same pipette solution but with addition of

6-cyano-7nitroquinoxaline-2,3-dione (CNQX, 20 μM) and DL-2-amino-5-phosphono-pentanoic acid

(AP-5, 50 μM) to the bath solution to block AMPA and NMDA receptor-mediated neurotransmission.

mIPSCs were recorded in the presence of 1 μM TTX, 20 μM CNQX and 50 μM AP-5. Frequencies

and amplitudes of spontaneous and miniature synaptic events were measured using the Mini

Analysis Program version 6.0 (Synaptosoft) from recordings before, during and after washout of the

drug. Only those events with amplitudes > 10 pA were used. Electrophysiological traces were

processed for presentation using Origin 7.5 (Origin Lab Corporation).

**Drugs**

The drugs used were glutamate, GABA, muscimol, baclofen, BIC, PTX, CNQX, 5-HT, and AP5

(Sigma), TTX (Wako), CGP54626 and SB334867 (Tocris), and CCK, orexin A and orexin B (Peptide

Institute).  Drugs were dissolved in DMSO or the buffers suggested by the suppliers and were diluted

with bath HEPES buffer.

**Statistical Analysis**

Data are presented as mean±SEM and were analyzed by one-way or repeated-measurements

ANOVA followed by *post hoc* analysis of significance by Scheffé's F test or Fisher's Protected Least

Significant Difference test using the GraphPad Prism version 4 software package (GraphPad

Software).

## References

1.     Sakurai, T. The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness. *Nat Rev Neurosci* **8**, 171-81. (2007).

2.     Lin, L. et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* **98**, 365-376 (1999).

3.     Peyron, C. et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat. Med.* **9**, 991-997 (2000).

4.     Thannickal, T.C. et al. Reduced number of hypocretin neurons in human narcolepsy. *Neuron* **27**, 469-474 (2000).

5.     Chemelli, R.M. et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* **98**, 437-451 (1999).

6.     Yoshida, K., McCormack, S., Espana, R.A., Crocker, A. & Scammell, T.E. Afferents to the orexin neurons of the rat brain. *J Comp Neurol.* **494**, 845-861 (2006).

7.     Li, Y., Gao, X.B., Sakurai, T. & van den Pol, A.N. Hypocretin/Orexin excites hypocretin neurons via a local glutamate neuron-A potential mechanism for orchestrating the hypothalamic arousal system. *Neuron* **36**, 1169-1181 (2002).

8.     Yamanaka, A., Muraki, Y., Tsujino, N., Goto, K. & Sakurai, T. Regulation of orexin neurons by the monoaminergic and cholinergic systems. *Biochem Biophys Res Commun.* **303**, 120-129 (2003).

9.     Xie, X. et al. GABA(B) receptor-mediated modulation of hypocretin/orexin neurones in mouse hypothalamus. *J Physiol.* **574**, 399-414 (2006).

10.    Marshall, F.H., Jones, K.A., Kaupmann, K. & Bettler, B. GABAB receptors - the first 7TM heterodimers. *Trends Pharmacol Sci* **20**, 396-9 (1999).

11.    Mohler, H. & Fritschy, J.M. GABAB receptors make it to the top--as dimers. *Trends Pharmacol Sci* **20**, 87-9 (1999).

12.    Bowery, N.G. et al. International Union of Pharmacology. XXXIII. Mammalian gamma-aminobutyric acid(B) receptors: structure and function. *Pharmacol Rev.* **54**, 247-264

(2002).

13.     Bettler, B., Kaupmann, K., Mosbacher, J. & Gassmann, M. Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev.* **84**, 835-867 (2004).

14.     Vigot, R. et al. Differential compartmentalization and distinct functions of GABAB receptor variants. *Neuron* **50**, 589-601 (2006).

15.     Shaban, H. et al. Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition. *Nat Neurosci* **9**, 1028-35 (2006).

16.     Lingenhoehl, K. et al. Gamma-hydroxybutyrate is a weak agonist at recombinant GABA(B) receptors. *Neuropharmacology* **38**, 1667-1673.

17.     Schuler, V. et al. Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). *Neuron* **31**, 47-58. (2001).

18.     Haller, C. et al. Floxed allele for conditional inactivation of the GABAB(1) gene. *Genesis* **40**, 125-30 (2004).

19.     Yamanaka, A. et al. Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* **38**, 701-713 (2003).

20.     Hara, J. et al. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30**, 345-354 (2001).

21.     Muraki, Y. et al. Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A receptor. *J. Neurosci.* **24**, 7159-7166 (2004).

22.     Sakurai, T. Reverse pharmacology of orexin: from an orphan GPCR to integrative physiology. *Regul Pept* **126**, 3-10 (2005).

23.     Sakurai, T. et al. Input of Orexin/Hypocretin Neurons Revealed by a Genetically Encoded Tracer in Mice. *Neuron* **46**, 297-308 (2005).

24.     Yamanaka, A. et al. Orexin neurons are directly and indirectly regulated by catecholamines in a complex manner *J. Neurophysiol.* **96**, 284-298 (2006).

25.     Saper, C.B., Scammell, T.E. & Lu, J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* **437**, 1257-63 (2005).

26. Finnimore, A.J., Roebuck, M., Sajkov, D. & McEvoy, R.D. The effects of the GABA agonist, baclofen, on sleep and breathing. *Eur Respir J* **8**, 230-4. (1995).

27. Puigcerver, A., van Luijtelaar, E.L., Drinkenburg, W.H. & Coenen, A.L. Effects of the GABAB antagonist CGP 35348 on sleep-wake states, behaviour, and spike-wave discharges in old rats. *Brain Res Bull* **40**, 157-62. (1996).

28. Gauthier, P., Arnaud, C., Gandolfo, G. & Gottesmann, C. Influence of a GABA(B) receptor antagonist on the sleep-waking cycle in the rat. *Brain Res* **773**, 8-14. (1997).

29. Estabrooke, I.V. et al. Fos expression in orexin neurons varies with behavioral state. *J Neurosci.* **21**, 1656-1662 (2001).

30. Mieda, M. et al. Orexin peptides prevent cataplexy and improve wakefulness in an orexin neuron-ablated model of narcolepsy in mice. *Proc Natl Acad Sci U S A.* **101**, 4649-4654 (2004).

31. Alam, M.N. et al. GABA-mediated control of hypocretin- but not melanin-concentrating hormone-immunoreactive neurones during sleep in rats. *J Physiol.* **563**, 569-582 (2005).

32. Suntsova, N. et al. The median preoptic nucleus reciprocally modulates activity of arousal-related and sleep-related neurons in th perifornical lateral hypothalamus. *J Neurosci.* **27**, 1616-1630 (2007).

33. Sakurai, T. et al. Structure and function of human prepro-orexin gene. *J Biol Chem* **274**, 17771-17776 (1999).

34. Maret, S. et al. Retinoic acid signaling affects cortical synchrony during sleep. *Science* **310**, 111-3. (2005).

35. Tafti, M. & Franken, P. Genetic dissection of sleep. *J Appl Physiol* **92**, 1339-47. (2002).

36. Toth, L.A. Strain differences in the somnogenic effects of interferon inducers in mice. *J Interferon Cytokine Res* **16**, 1065-72. (1996).

37. Nambu, T. et al. Distribution of orexin neurons in the adult rat brain. *Brain Res.* **827**, 243-260 (1999).

38. Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H. & Orkin, S.H. Activation of EGFP

expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* **97**,

324-6 (2001).

**Figure 3. Alteration of Spontaneous Synaptic Input to Orexin Neurons of $GABA_{B1}^{-/-(orexin)}$ Mice.**

(a) sIPSC recordings from brain slices of littermate (control) and $GABA_{B1}^{-/-(orexin)}$ mice. Basal (upper),

baclofen (100 μM) application (middle), and after drug washout (lower). IPSCs were recorded in the

presence of AP-5 (50 μM) and CNQX (20 μM). (b) Effects of baclofen on sIPSC frequencies in

control (n=13) and $GABA_{B1}^{-/-(orexin)}$ (n=9) mice recorded for at least 3 minutes. (c) In current-clamp

recording mode, the GABA$_A$ receptor antagonist BIC (25 μM) was applied to orexin neurons of

control (upper, n=4) and $GABA_{B1}^{-/-(orexin)}$ (lower, n=4) mice. (d) Basal (control, n = 4, cKO, n = 6) and

mean effect of pre-incubation with OX$_1$R antagonist, SB334867 (5 μM; control n = 9, cKO, n = 7), on

sIPSC frequency in $GABA_{B1}^{-/-(orexin)}$ (cKO) mice. Data shown are mean ± SE. *, P < 0.05. Data were

analyzed by one way ANOVA or Student's t test. (e) Membrane potential of orexin neurons in control

(n=25) vs. $GABA_{B1}^{-/-(orexin)}$ mice (n=12) in response to a series of 100 msec current steps (in 20 pA

increments, -200 to 80 pA) from resting potential (-60mV). Data shown are mean±SE. *, P < 0.05.

Data were analyzed by one way ANOVA or Student's t test.

**Figure 4. Action of GABA$_A$ Agonist and Glutamate on Orexin Neurons of $GABA_{B1}^{-/-(orexin)}$ Mice.**

(a) In voltage clamp mode with $V_m$ held at -60 mV, muscimol (10 μM) induced an outward current in orexin neurons of control (upper) and $GABA_{B1}^{-/-(orexin)}$ (lower) mice. The effect of muscimol was smaller in $GABA_{B1}^{-/-(orexin)}$ mice. (b) Dose effects of muscimol on orexin neurons of control (n=6-10) vs $GABA_{B1}^{-/-(orexin)}$ mice (n=9-12). (c) In voltage clamp mode with with $V_m$ held at -60 mV, glutamate (100 μM) induced an inward current in orexin neurons of control (upper) and $GABA_{B1}^{-/-(orexin)}$ (lower) mice. The effect of glutamate was smaller in $GABA_{B1}^{-/-(orexin)}$ mice. (d) Dose effects of glutamate on orexin neurons of control (n=5-13) vs $GABA_{B1}^{-/-(orexin)}$ mice (n=3-8). Representative recordings (a and c) and summary data are shown as mean±SE (b and d).

**Figure 5. Severe Fragmentation of Sleep Stages in $GABA_{B1}^{-/-(orexin)}$ Mice.** Representative 24 hr hypnograms for a $GABA_{B1}^{-/-(orexin)}$ mouse (a) and a control littermate (b). W, wakefulness; NR, non-REM sleep; R, REM sleep. Note the severe sleep/waking fragmentation, reduced wakefulness duration, and reduced REM latency in the $GABA_{B1}^{-/-(orexin)}$ mouse relative to control. Hourly amounts

(c) and average episode duration (d) of awake, non-REM, and REM sleep states (mean±SE) plotted over 24 hr for control littermate (n=11) and $GABA_{B1}^{-/-(orexin)}$ mice (n=10).  Data for the dark and light phases are displayed on light gray and white backgrounds, respectively. *P < 0.05 by Student's t test.

**Figure 6. Proposed Model in $GABA_{B1}^{-/-(orexin)}$ Mice Displaying Sleep/Wakefulness Fragmentation.** $GABA_A$ input is increased by compensatory mechanisms, resulting in increased membrane conductance of orexin neurons in $GABA_{B1}^{-/-(orexin)}$ mice.  Therefore, orexin neurons in $GABA_{B1}^{-/-(orexin)}$ mice show decreased responses to both excitatory and inhibitory input.  In vivo, responses of orexin neurons in $GABA_{B1}^{-/-(orexin)}$ mice to wake-active excitatory input or sleep-active inhibitory input are inappropriate, resulting in severe instability of sleep/wakefulness states.

**Supplementary information**

**Generation of Transgenic Mice in which Orexin Neurons Specifically Express Cre**

**Recombinase**

To achieve orexin neuron-specific *Cre* expression, a transgene was constructed with the 3.2 kb

fragment of the 5′-upstream region of the human *prepro-orexin* gene as a promoter [33], which was

ligated to the Cre recombinase cDNA fused to an *IRES-eGFP* construct (Supplementary figure 1a).

Using this transgene, several transgenic mouse lines, *orexin/Cre* transgenic mice, were established.

To confirm *Cre* expression, immunohistochemical staining was carried out using an anti-Cre

antibody (Nacalai). Cre-immunoreactivity (ir) was observed in most (~80%) of the orexin-containing

neurons (Supplementary figure 1b). Expression of eGFP could not be detected by

immunohistochemical or direct GFP fluorescence analysis, suggesting that the *IRES-eGFP* system

was non-functional in orexin neurons. To examine if functional Cre protein is expressed in orexin

neurons, *orexin-Cre* mice were crossed with 129-*Gt(ROSA)26Sor*$^{(EGFP)}$/J reporter mice[38]. This

confirmed that Cre activity expressed as eGFP-fluorescence was restricted to orexin neurons in the

LHA (Supplementary figure 1c). Similar results were obtained using ROSA-LacZ reporter mice and

LacZ staining (data not shown). Extensive examination throughout the brain failed to identify ectopic

GFP expression other than that in orexin neurons (data not shown). These observations suggest

that functional Cre recombinase is exclusively expressed in orexin neurons in *orexin/Cre* transgenic

mice.

**Legend for Supplementary figure 1 Transgenic Mice Specifically Expressing Cre**

**Recombinase Only in Orexin Neurons.** (a) Structure of the *orexin/Cre* (*IRES-eGFP*) transgene,

consisting of human prepro-orexin promoter, *Cre recombinase* (*Cre*) cDNA, *IRES-eGFP* cDNA, and

murine *protamine-1* gene fragment (mPrm1 polyA). (b) Specific expression of Cre recombinase by

orexin-containing neurons in the lateral hypothalamic area (LHA) of *orexin/Cre* transgenic mice is

shown by immunofluorescence. Left, Cre recombinase-like immunoreactivity (Alexa 594, red);

middle, orexin-like immunoreactivity (Alexa 488, green); right, merged images. (c) Expression of Cre

recombinase was monitored by crossing ROSA-eGFP reporter mice. Left, orexin-like

immunoreactivity (red); middle, eGFP fluorescence (green); right, merged images.

**Table 1. Vigilance State Parameters Recorded from $GABA_{B1}^{-/-(orexin)}$ and Control Mice**

| Light period (8:00~20:00) | REM | | Non-REM | | Awake | |
|---|---|---|---|---|---|---|
| | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) |
| Total time (min) | 61.8±3.7 | 54.6±5.1 | 402.2±11.6 | 384.0±16.2 | 254.1±11.7 | 280.1±18.5 |
| Duration (sec) | 67.3±3.5 | 78.8±3.2 | 240.6±24.0** | 369.4±27.0 | 208.0±22.5** | 498.2±71.5 |
| Episode counts | 57.0±5.1* | 42.1±3.9 | 108.5±11.8** | 65.5±3.6 | 79.9±7.9** | 40.0±4.9 |
| REM latency (sec) | 295.0±30.5* | 410.3±24.8 | | | | |
| Inter-REM interval (sec) | 759.6±79.9 | 1060.1±103.8 | | | | |

| Dark period (20:00~8:00) | REM | | Non-REM | | Awake | |
|---|---|---|---|---|---|---|
| | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) | $GABA_{B1}^{-/-(orexin)}$ (n=10) | Control (n=11) |
| Total time (min) | 36.7±2.7 | 32.0±3.0 | 282.4±12.7 | 286.9±15.3 | 400.2±15.0 | 405.4±20.2 |
| Duration (sec) | 69.1±3.2** | 84.2±3.2 | 231.6±22.2** | 392.4±40.4 | 387.1±65.7* | 883.7±127.4 |
| Episode counts | 32.9±3.3* | 23.5±2.5 | 82.8±11.8** | 46.7±3.6 | 68.5±12.0** | 32.4±3.9 |
| REM latency (sec) | 285.6±28.3* | 503.2±53.6 | | | | |
| Inter-REM interval (sec) | 1356.9±204.2 | 2091±417.5 | | | | |

Total time spent in each state (min, mean±SEM), episode duration (sec±SEM), REM latency, and interval between successive REM sleep episodes (min, mean±SEM) over 24 hr are itemized separately for the light and dark periods. Significant differences (*P<0.05,**P<0.01; one-way ANOVA and *post hoc* Scheffé's F test) between $GABA_{B1}^{-/-(orexin)}$ and control ($GABA_{B1}^{flox/flox}$, *orexin/Cre* negative) mice are indicated with asterisks.
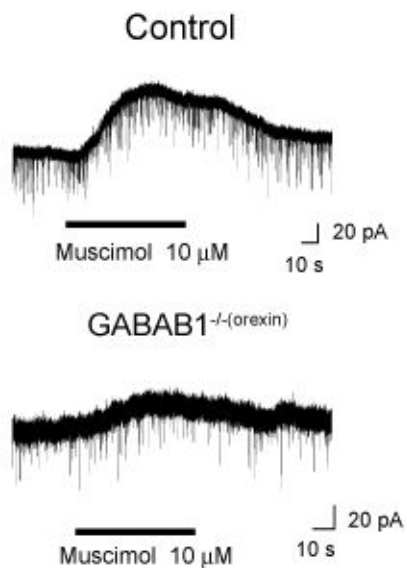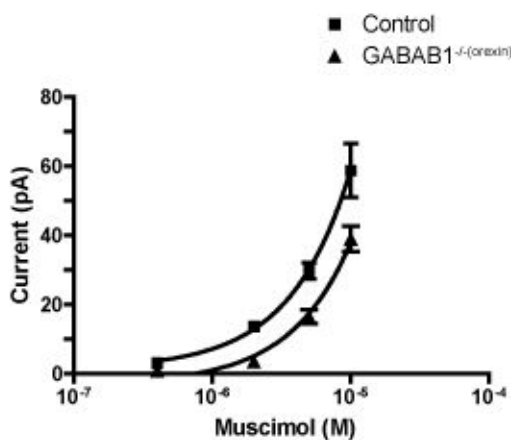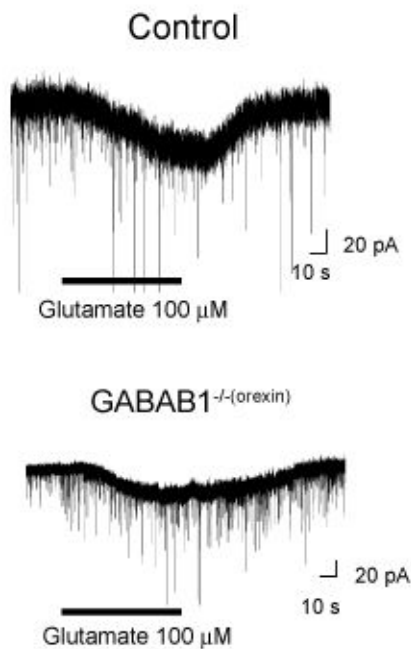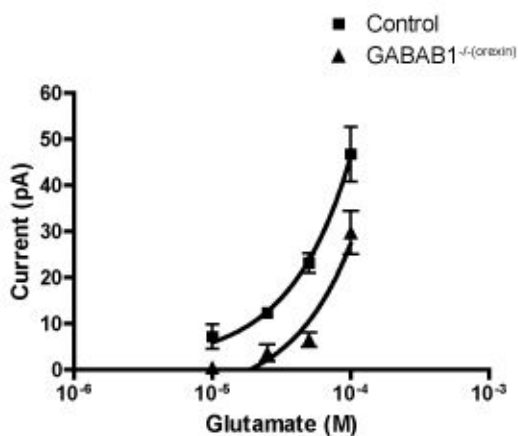
Figure 1. Matsuki et al.,

Control

GABAB1-/-(orexin/ataxin-3)

Orexin-ir      GABAB1-ir      Merge

Figure 2. Matsuki et al.,

Figure 3. Matsuki et al.,

Figure 4. Matsuki et al.,

Figure 5. Matsuki et al.,

Figure 6. Matsuki et al.

**a**



IRES-eGFP

Cre

mPrm1 polyA

Human prepro-orexin promoter

**b**

Cre-ir          Orexin-ir          Merge

50 μm

**c**



Orexin-ir          EGFP          Merge

50 μm