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

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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.

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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.

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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 ($GABA_BRs$) 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 $GABA_BR$ agonist gamma-hydroxybutyrate (GHB)[16] is used to treat both the excessive sleepiness and cataplexy symptoms of human narcolepsy. $GABA_BR$ activation inhibits presynaptic release of both glutamate and GABA onto orexin neurons[9] and both pre- and postsynaptic $GABA_BRs$ 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 $GABA_BRs$ on orexin neuron function at the cellular and behavioral levels.

Results

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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

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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).

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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,

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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

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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

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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

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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.

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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 $GABA_AR$ 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

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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

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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

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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.

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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

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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_1R 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

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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

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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 $GABA_B$Rs for maintenance of wakefulness and sleep state consolidation. The phenotype of $GABA_{B1}^{-/-(orexin)}$ mice is characterized by impaired ability to maintain

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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.

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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.

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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

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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].

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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).

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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.

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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

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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).

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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.

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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).

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Figure legends

Figure 1. Selective Loss of $GABA_B$ Receptors in Orexin Neurons in $GABA_{B1}^{-/-(orexin)}$ Mice.

$GABA_{B1}^{-/-(orexin)}$ ($GABA_{B1}^{flox/flox}$, *orexin/Cre* Tg) mice showed loss of $GABA_{B1}$ receptor gene expression in orexin neurons. Matched brain sections from $GABA_{B1}^{-/-(orexin)}$ and wild-type littermate mice were double-stained with anti-orexin and anti-$GABA_{B1}$ receptor serum. Left: orexin-like immunoreactive neurons in LHA; middle: $GABA_{B1}$ receptor-like immunoreactive neurons; right: merged image. Lower panels are high-power views of the areas surrounded by the white rectangles in the corresponding upper panels.

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Figure 2. Lack of Functional $GABA_B$ Receptors in Orexin Neurons of $GABA_{B1}^{-/-(orexin)}$ Mice. (a) Effects of GABA (0.6 mM), muscimol (30 μM), baclofen (100 μM), and 5-HT (10 μM) in orexin neurons in control mice during current-clamp recording (n=4-8). Drugs were applied during the periods indicated by bars. (b) Effects of GABA (0.6 mM), muscimol (30 μM), baclofen (100 μM), and 5-HT (10 μM) in orexin neurons from $GABA_{B1}^{-/-(orexin)}$ mice (n=4-12).

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_1R 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.

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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).

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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.

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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

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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.

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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

	REM		Non-REM		Awake	
Light period (8:00~20:00)	$GABA_{B1}^{-/-(orexin)}$ (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				

	REM		Non-REM		Awake	
Dark period (20:00~8:00)	$GABA_{B1}^{-/-(orexin)}$ (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.

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Figure 1. Matsuki et al.,

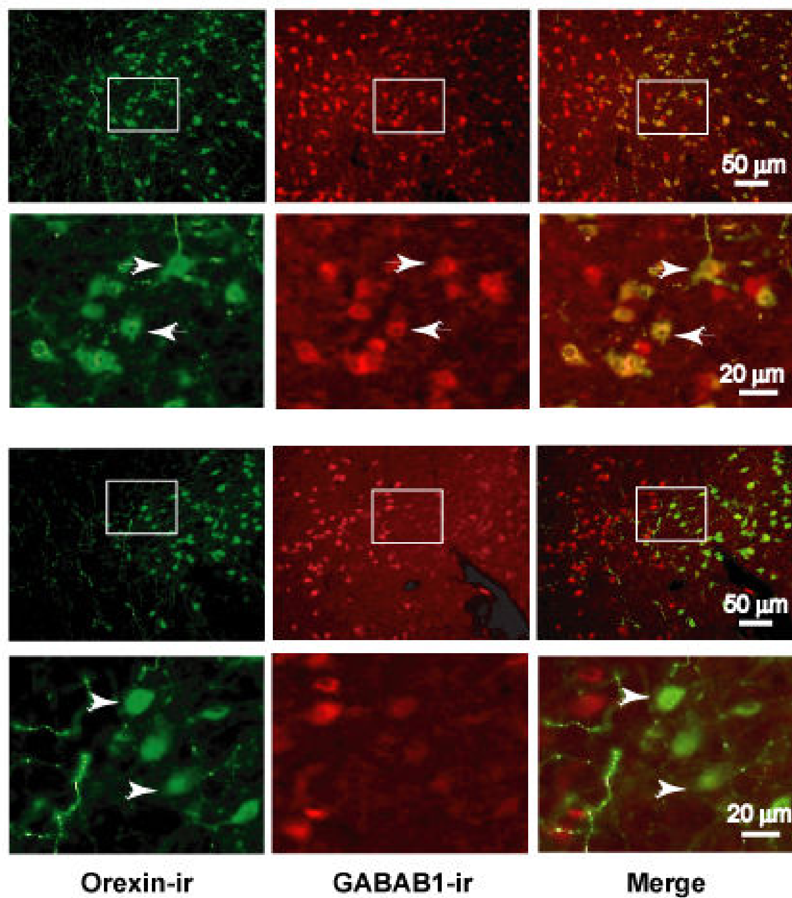

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Figure 2. Matsuki et al.,

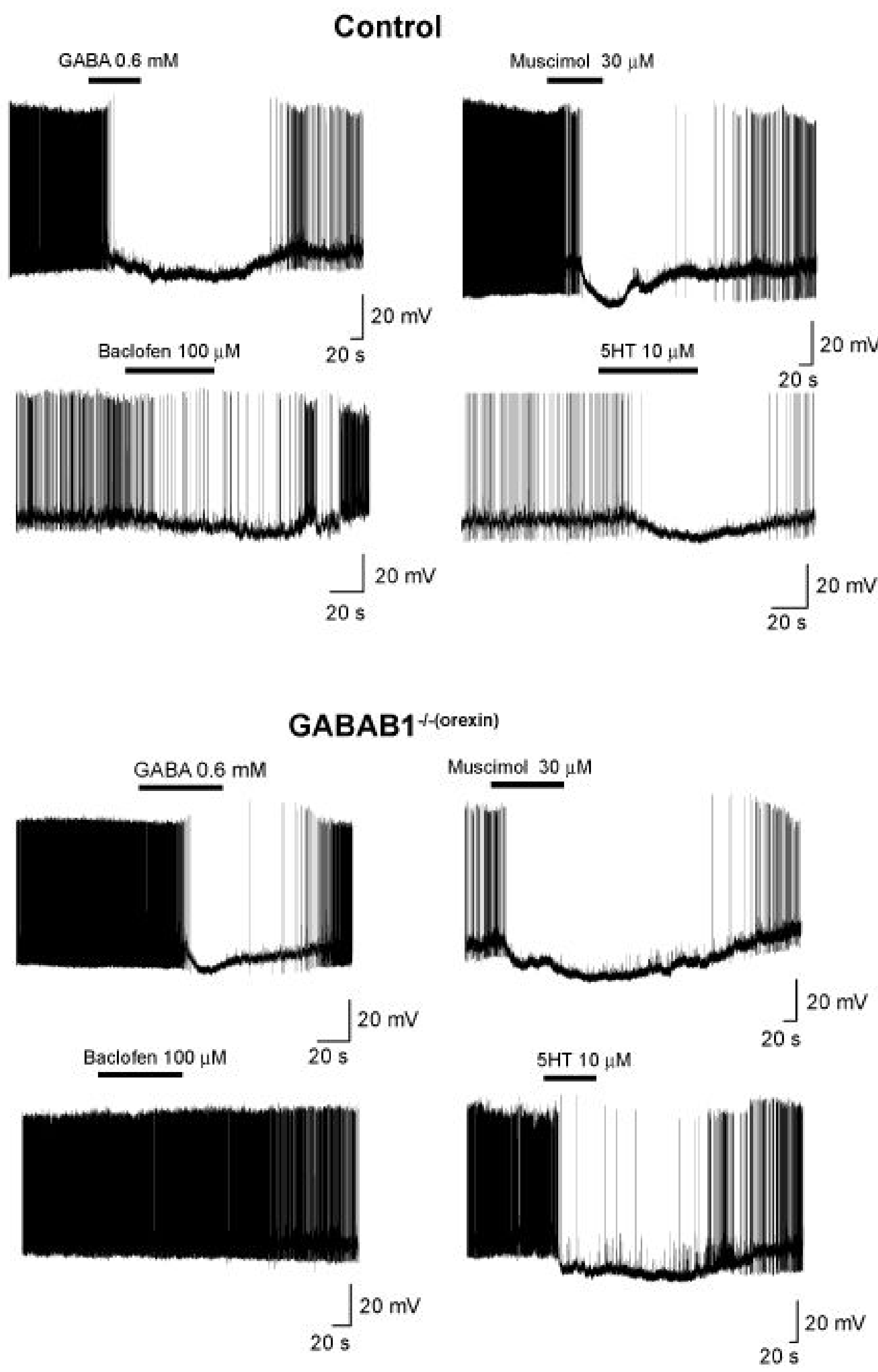

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Figure 3. Matsuki et al.,

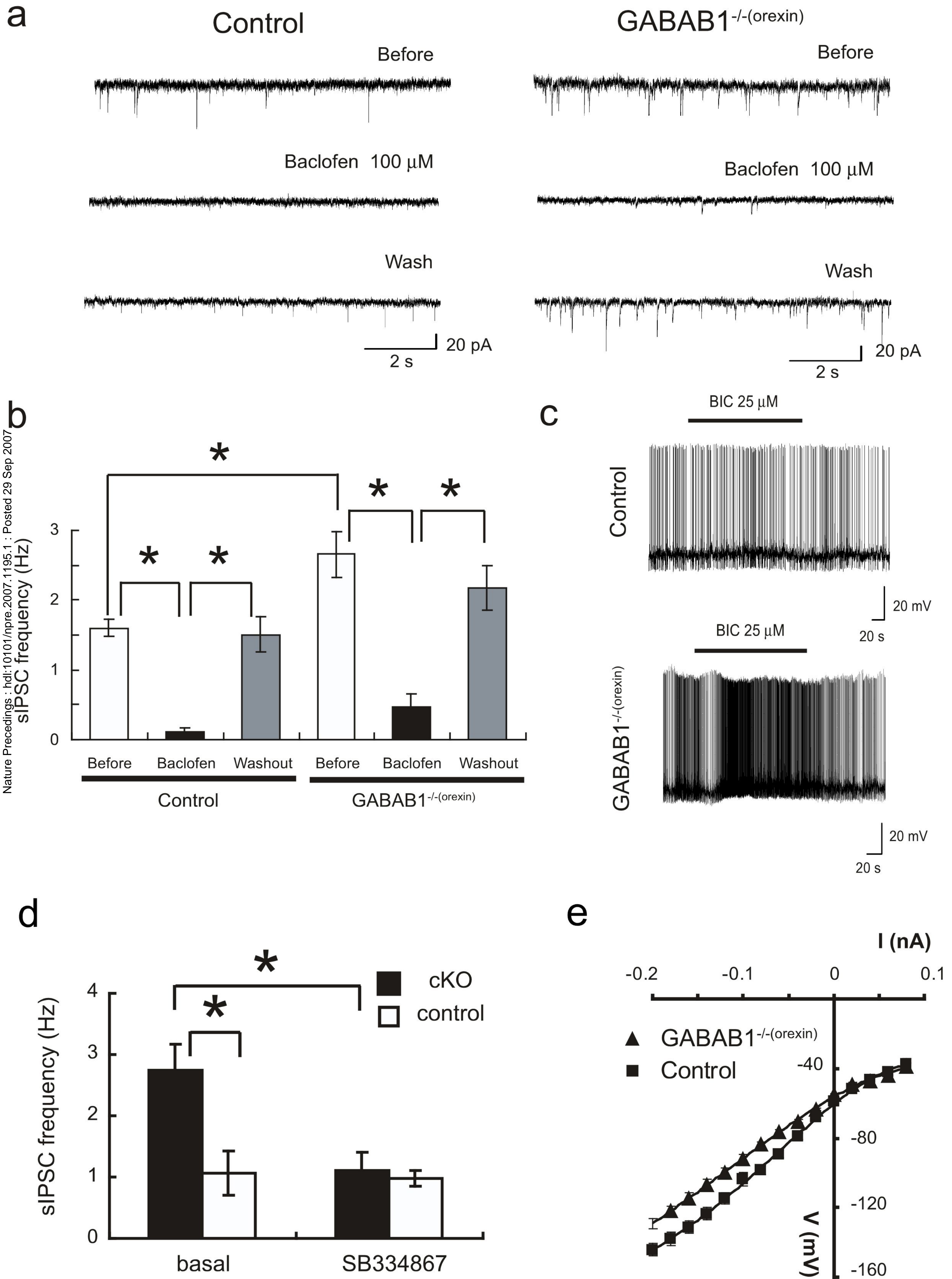

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Figure 4. Matsuki et al.,

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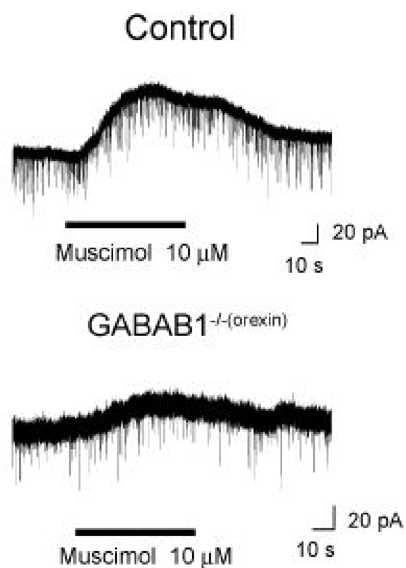
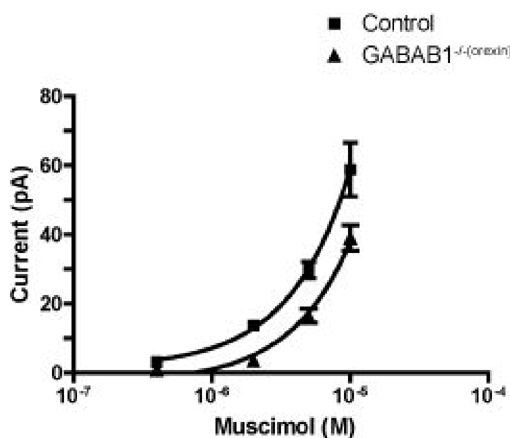
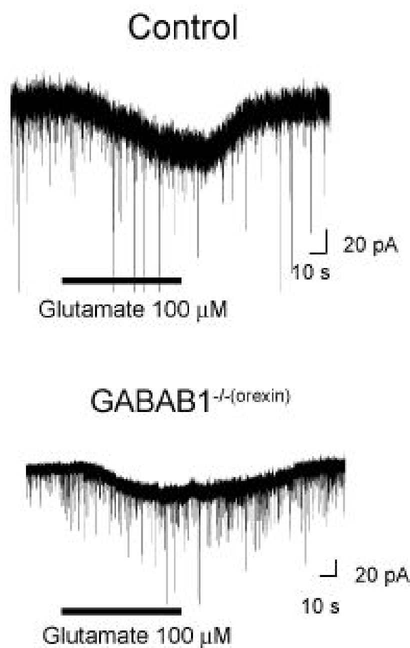
a



b



c



d

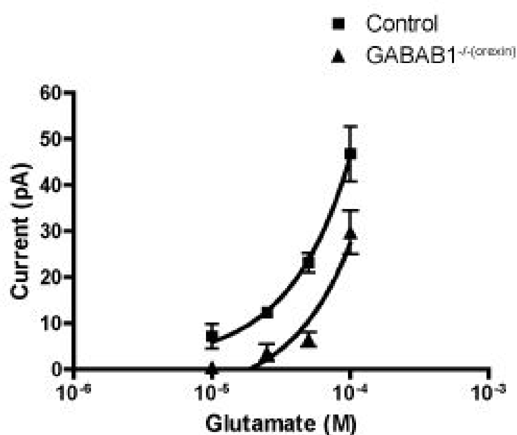


Figure 5. Matsuki et al.,

a

GABAB1$^{-/-(orexin)}$

R NR W

20:00 23:00

2:00 5:00

8:00 11:00

14:00 17:00

b

Control

R NR W

20:00 23:00

2:00 5:00

8:00 11:00

14:00 17:00

c

Awake Non-REM REM

■ GABAB1$^{-/-(orexin)}$

□ Control

Time in state (min)

Clock time (hour)

d

Awake Non-REM REM

■ GABAB1$^{-/-(orexin)}$

□ Control

Episode duration (min)

Clock time (hour)

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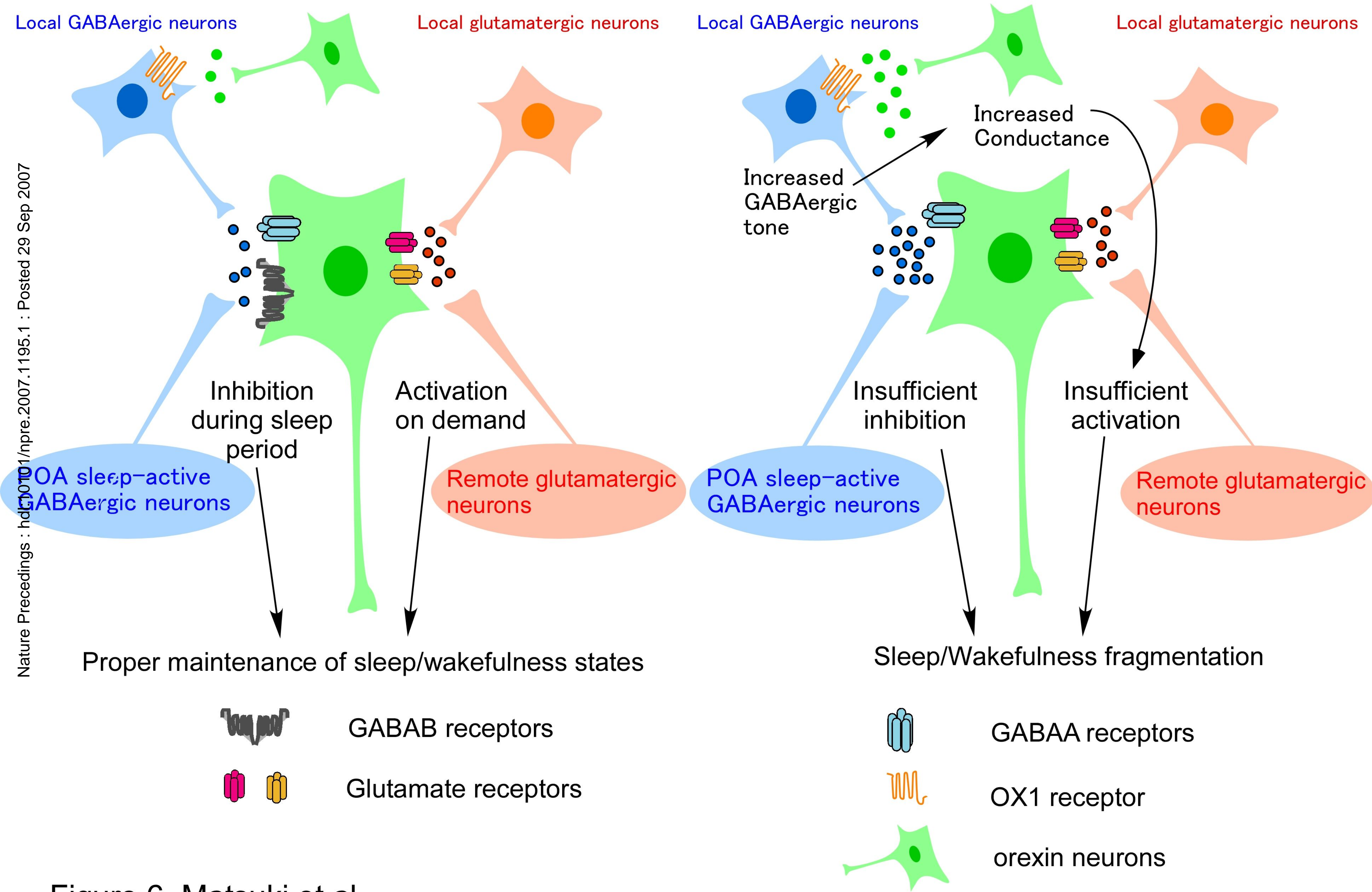

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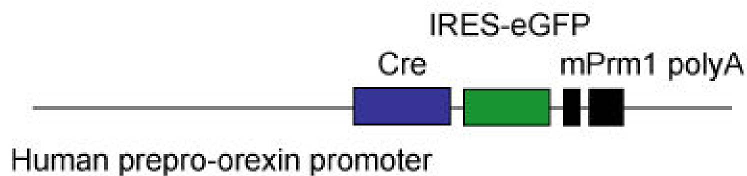
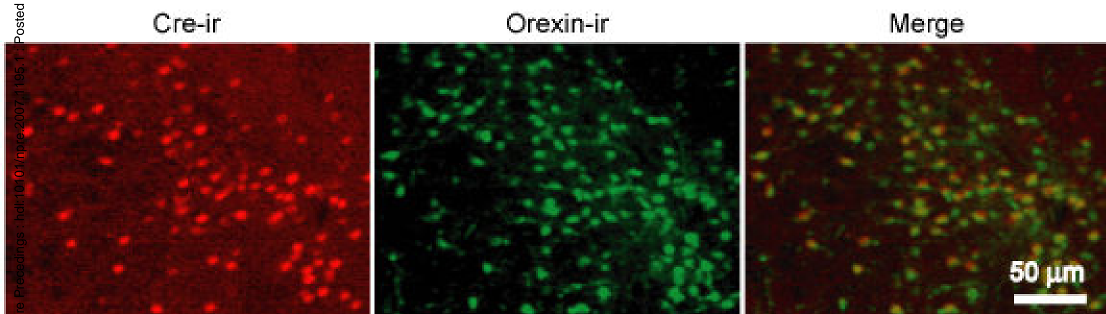
Figure 6. Matsuki et al.

Supplementary figure 1. Matsuki et al.,

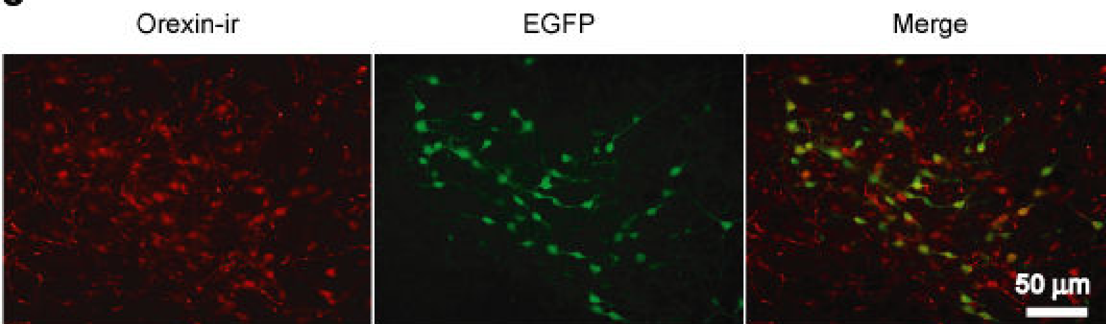
a



b



c



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