

1 **Title:**

2 **Dissociating orexin-dependent and -independent functions of orexin neurons**
3 **using novel *orexin-Flp* knock-in mice**

4
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1 **Abstract**

2 Uninterrupted arousal is important for survival during threatening situations. Activation of
3 orexin/hypocretin neurons is implicated in sustained arousal. However, orexin neurons
4 produce and release orexin as well as several co-transmitters including dynorphin and
5 glutamate. Thus, it is important to disambiguate orexin peptide-dependent and
6 -independent physiological functions of orexin neurons. To attain this, we generated a
7 novel *orexin-flippase* (Flp) knock-in (KI) mouse line. Crossing with Flp-reporter or
8 Cre-expressing mice showed gene expression exclusively in orexin neurons.
9 Histological studies confirmed that orexin was completely knock-out (KO) in KI/KI
10 homozygous mice. Orexin neurons without orexin showed altered electrophysiological
11 properties, as well as received decreased glutamatergic inputs. Selective chemogenetic
12 activation revealed that both orexin and co-transmitters functioned to increase
13 wakefulness, however, orexin was indispensable to promote sustained arousal.
14 Surprisingly, activation of orexin neurons without orexin caused a significant increase in
15 the total time spent in cataplexy. Taken together, orexin is essential to maintain basic
16 membrane properties and input-output computation of orexin neurons, as well as to exert
17 awake-sustaining aptitude of orexin neurons.

18

19 **Keywords:** Orexin/hypocretin, flippase, electrophysiology, chemogenetics,
20 sleep/wakefulness, cataplexy

1 **Introduction**

2 Orexin A (hypocretin-1) and orexin B (hypocretin-2) (de Lecea et al., 1998;
3 Sakurai et al., 1998), generated from the same precursor protein called prepro-orexin,
4 are endogenous ligands for two closely related G-protein-coupled receptors termed
5 orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R) (Sakurai et al., 1998). Although
6 orexin was named for its effect on inducing feeding behavior, it gained immense interest
7 in sleep research as the knockout (KO) of prepro-orexin or dysfunction of OX2R in
8 canines or mice reportedly mimics the human sleep disorder narcolepsy (Chemelli et al.,
9 1999; Lin et al., 1999). Narcolepsy is a neurological disorder characterized by
10 fragmented sleep/wakefulness, persistent daytime sleepiness and brief episodes of
11 muscle weakness called cataplexy, which is often triggered by positive emotions.
12 Narcolepsy patients showed a loss of overall prepro-orexin mRNA, confirming the
13 association between orexin neuronal loss and the pathogenesis of narcolepsy (Peyron et
14 al., 2000; Thannickal et al., 2000).

15 A small number of orexin-producing neurons (orexin neurons) are exclusively
16 distributed in the lateral hypothalamic area (LHA) and perifornical area, but send
17 projections widely throughout the major brain areas (Nambu et al., 1999; Peyron et al.,
18 1998). Thus, it is no surprise that orexin neurons have diverse physiological roles,
19 including the regulation of sleep/wakefulness (Chemelli et al., 1999; Lin et al., 1999),
20 energy homeostasis (Yamanaka et al., 2003), thermoregulation (Tupone et al., 2011), as
21 well as regulation of heart rate and blood pressure (Zhang et al., 2006). Using
22 optogenetic and/or chemogenetic techniques, we and others showed that orexin
23 neurons modulate sleep/wake cycles in rodents (Adamantidis et al., 2007; Sasaki et al.,
24 2011; Tsunematsu et al., 2011). In addition to these, employing transgenic mice, we
25 reported that chemogenetic activation of orexin neurons increased locomotion, feeding
26 behavior and metabolism (Inutsuka et al., 2014). Thus, orexin neurons are thought to

1 interact with the neuroregulatory, autonomic and neuroendocrine systems, and perform
2 critical roles in the regulation of sleep/wakefulness and energy homeostasis.

3 The physiological activity of orexin neurons is modulated by multiple neural
4 inputs and humoral factors. These inputs include GABAergic neurons in the preoptic
5 area, serotonergic neurons in the dorsal and median raphe nuclei, central amygdala,
6 basal forebrain cholinergic neurons, the bed nucleus of the stria terminalis,
7 supraventricular zone, and the dorsomedial, lateral and posterior hypothalamus (Sakurai
8 et al., 2005; Yoshida et al., 2006). Recently, we reported that serotonergic neurons in the
9 raphe nucleus inhibit orexin neurons both directly and indirectly (Chowdhury and
10 Yamanaka, 2016). Orexin neurons are also found to respond to multiple humoral factors
11 and neuropeptides (Inutsuka and Yamanaka, 2013; Sakurai, 2014). Most interestingly,
12 orexin neurons form a positive-feedback circuitry among themselves in the LHA by
13 activating other orexin neurons through OX2R to maintain the wake-active network at
14 optimum level and/or for a sustained period (Yamanaka et al., 2010a).

15 However, orexin neurons contain other neurotransmitters including glutamate
16 (Rosin et al., 2003), dynorphin (Chou et al., 2001) and galanin (Hakansson et al., 1999).
17 Therefore, to better understand the physiology and pathophysiology of orexin/hypocretin
18 system, it is essential to disambiguate the roles of orexin from other co-transmitters. To
19 address this, we generated novel *orexin-Flppase (Flp)* knock-in mice, which express Flp
20 recombinase under control of prepro-orexin gene in mice. Employing this novel mouse
21 line, we found that orexin performs critical roles in maintaining basic electrophysiological
22 properties of orexin neurons as well as to initiate feed-forward activation of orexin
23 neurons by facilitating excitatory glutamatergic inputs. Focusing on sleep/wakefulness,
24 we also manipulated orexin neuronal activity and evaluated their physiological effects in
25 freely-moving mice. To achieve this, we employed a chemogenetic technique, Designer
26 Receptors Exclusively Activated by Designer Drugs (DREADD) (Armbruster et al.,

1 2007). Our results showed orexin plays the major role in the awake-sustaining aptitude
2 of orexin neurons. Although the phenotypic consequences of orexin neurons activation
3 in increased wakefulness was reinforced for by co-transmitters, these co-transmitters
4 rather deteriorated cataplexy. Together, these data clearly identified the importance of
5 orexin neuropeptides for the physiological function of orexin neurons and their
6 postsynaptic partners.

1 **Results**

2 **Generation of novel *orexin-Flp (OF)* mice**

3 We previously generated *orexin-Cre* (Inutsuka et al., 2014) or
4 *orexin-tetracycline-controlled trans-activator (orexin-tTA)* (Tabuchi et al., 2013)
5 transgenic mice in which Cre recombinase or tTA was expressed in orexin neurons to
6 allow regulation of transgene expression in the orexin neurons. However, expression of
7 Cre and tTA in those transgenic mice was driven by a 3.2 kb short gene fragment
8 upstream of the human prepro-orexin gene. Thus, we generated a new mouse by
9 targeting the prepro-orexin gene of the mouse genome. Employing a homologous
10 recombination system, here we generated *orexin-Flippase (OF)* mice by knocking in the
11 EGFP-2A-Flp transgene just downstream of the translation initiation site of the
12 prepro-orexin gene in-frame (Figure 1A). To visualize the function of Flp recombinase,
13 we bred these mice with Flp-reporter (*FSF-mTFP1*) mice (Imayoshi et al., 2012) in which
14 fluorescent protein mTFP1 is expressed in a Flp-dependent manner (Figure 1B).
15 Immunohistochemical analysis showed mTFP expression in $80.9 \pm 4.4\%$ of
16 orexin-immunoreactive neurons. We observed no ectopic expression of EGFP and/or
17 mTFP1 protein in melanin-concentrating hormone (MCH)-immunoreactive ($n = 4$ mice;
18 Figure 1C and 1D) or in any other non-orexin-immunoreactive neurons in these bigenic
19 mice. Together, these data confirmed that functional Flp was expressed exclusively in
20 the orexin neurons of newly generated *OF* mice.

21

22 ***OF* mice enable the dual targeting of adjacent cell types**

23 By taking advantage of the vast repertoire of cell type-specific Cre- or tTA-expressing
24 mice, the new *OF* mice may enable the expression of various genes in different subsets
25 of neurons (Figure 2A). To confirm this, we next generated two different bigenic mice by
26 crossing *OF* mice with *MCH-Cre* or *Gad67-Cre* mice (Figure 2B and 2E). *MCH-Cre* or

1 *Gad67-Cre* mice exclusively express Cre recombinase in MCH or GABA neurons,
2 respectively (Higo et al., 2009; Kong et al., 2010). To test whether we could target dual
3 cell types simultaneously inside the LHA, we performed injection of an AAV cocktail
4 composed of an equal volume of AAV(DJ)-CMV-FLEX-tdTomato and
5 AAV(DJ)-CMV-dFRT-hrGFP in both *orexin-Flp; MCH-Cre* and *orexin-Flp; Gad67-Cre*
6 bigenic mice (Figure 2C and 2F). Given that MCH and GABA neurons are distinct
7 neuronal subsets from orexin neurons in the LHA (Broberger et al., 1998; Rosin et al.,
8 2003), we expected a discrete expression of tdTomato and hrGFP in both bigenic mice.
9 Indeed, we observed that Flp and Cre recombinase-driven fluorescent protein
10 expression did occur in distinct populations (Figure 2D and 2G) in coronal brain sections
11 (n = 3 mice for each). These data demonstrated that a broader range of opportunities for
12 cell type-specific manipulation and/or activity recording is possible with *OF* mice.

13

14 ***OF* (KI/KI) homozygote knockout orexin peptides**

15 Since the EGFP-2A-Flp sequence was inserted in-frame at the start codon of the
16 prepro-orexin gene using the KI method, we reasoned that KI/KI homozygous mice are
17 essentially orexin-KO mice. To confirm, immunohistochemical studies were conducted to
18 compare the expression of EGFP, orexin and dynorphin in the LHA of heterozygous *OF*
19 (KI/-) and homozygous *OF* (KI/KI) mice (Figure 3A and 3B). As expected, we found that
20 EGFP expressing neurons were distributed in the LHA, however,
21 orexin-immunoreactivity was absent in the LHA in *OF* (KI/KI) homozygous mice (Figure
22 3B). We stained every 4th slice of mouse brain containing LHA and counted 567 ± 25
23 orexin-positive and 673 ± 55 dynorphin-positive cells/animal in heterozygous mice (n = 3
24 mice). Among the counted neurons, $84.9 \pm 4.4\%$ of dynorphin-positive neurons
25 co-expressed orexin. However, while we counted 661 ± 15 dynorphin-positive
26 cells/animal, we did not find any orexin-positive neurons in the homozygous mice (n = 3

1 mice, Hetero vs Homo, $p = 0.847$ (dynorphin), $p = 2.4e-5$ (orexin), unpaired t -test),
2 showing that the number of dynorphin-positive cells were comparable to that in
3 heterozygous animals. These immunohistochemical studies confirmed that orexin
4 neurons in homozygous mice did not express orexin, however, expression of dynorphin
5 was unaffected.

6

7 **Electrophysiological properties of orexin neurons lacking orexin**

8 The expression of EGFP in the *OF* mice allowed us to visualize orexin neurons in the
9 acute brain slice preparations. To this end, we sought to evaluate the importance of
10 orexin neuropeptides in conserving the electrophysiological properties of orexin
11 neurons. Therefore, we recorded and compared resting membrane potentials (V_{rest}),
12 firing frequency, input resistance and capacitance in *OF* (KI/-), *OF* (KI/KI), and
13 *orexin-EGFP* (Tg/Tg) (*OE*) mice (Yamanaka et al., 2003) (Figure 4A-4E). Orexin neurons
14 in the *OF* (KI/KI) mice were found to have significantly hyperpolarized membrane
15 potential (-58.8 ± 1.2 mV; $n = 21$) compared to *OF* (KI/-) mice (-51.6 ± 0.9 mV; $n = 21$, $p =$
16 $1.0e-5$) and *OE* mice (-50.4 ± 0.9 mV; $n = 22$, $p = 3.5e-7$; Figure 4A-i to 4A-iv).
17 Spontaneous firing frequency measured by cell-attached recordings were also found to
18 be significantly lower in orexin neurons in *OF* (KI/KI) mice (1.5 ± 0.2 Hz; $n = 25$)
19 compared to *OF* (KI/-) mice (2.5 ± 0.3 Hz; $n = 21$, $p = 0.015$) and *OE* mice (2.6 ± 0.2 Hz;
20 $n = 25$, $p = 0.004$; Figure 4B-i to 4B-iv). This lower discharge rate could be attributed to
21 the hyperpolarized membrane potential of orexin neurons in homozygous mice. We next
22 measured the input resistance of identified neurons by measuring the voltage deviation
23 generated by current injection from -100 to +100 pA in the current clamp protocol. In mice
24 lacking orexin peptide (*OF* (KI/KI) mice), orexin neurons were found to have significantly
25 lower input resistance (485.9 ± 35.8 M Ω ; $n = 23$) compared to *OF* (KI/-) mice ($639.9 \pm$
26 48.2 M Ω ; $n = 21$, $p = 0.037$) and *OE* mice (638.2 ± 43.4 M Ω ; $n = 24$, $p = 0.032$; Figure

1 4C-i to 4C-iv). The lower input resistance in neurons lacking orexin peptides suggests
2 that greater synaptic inputs (current injection) are necessary to generate changes in
3 membrane potential. We also compared the membrane capacitance measured during
4 whole-cell recordings. Interestingly, we observed that orexin neurons in *OF* (KI/KI) mice
5 had significantly higher membrane capacitance (35.4 ± 1.3 pF; $n = 25$) than *OF* (KI/-)
6 mice (29.9 ± 1.5 pF; $n = 25$, $p = 0.012$) and *OE* mice (30.0 ± 1.3 pF; $n = 25$, $p = 0.014$,
7 one-way ANOVA post-hoc Tukey; Figure 4E). This higher capacitance in orexin neurons
8 that lack orexin peptides reflects the increased surface area of the plasma membrane.
9 Taken together, these data clearly suggest that orexin neuropeptide is essential for
10 maintaining the active and passive electrophysiological properties of orexin neurons.

11

12 **Orexin mediates feed-forward activation of orexin neurons via facilitation of** 13 **glutamatergic inputs**

14 To characterize whether the excitatory and inhibitory synaptic inputs onto orexin neurons
15 are regulated by the presence or absence of orexin peptides, we recorded glutamatergic
16 and GABAergic inputs using the voltage clamp method. We recorded spontaneous
17 excitatory post-synaptic currents (sEPSCs) from EGFP-expressing orexin neurons in the
18 presence of picrotoxin ($400 \mu\text{M}$), a GABA-A receptor antagonist. We found that orexin
19 neurons lacking orexin protein had significantly lower sEPSC frequency while the
20 amplitudes were unaffected (Figure 5A-5E). The average inter-event interval for all
21 recorded sEPSCs of orexin neurons in *OF* (KI/KI) mice was 247.2 ± 48.6 ms ($n = 29$)
22 while that of *OF* (KI/-) mice was 116.7 ± 7.6 ms ($n = 25$, $p = 0.01$ vs *OF* (KI/KI)) and *OE*
23 mice was 77.9 ± 7.4 ms ($n = 26$, $p = 5.8\text{e-}4$ vs *OF* (KI/KI), one-way ANOVA pot-hoc
24 Tukey; Figure 5D). The sEPSC amplitude of orexin neurons in *OF* (KI/KI) mice was 22.6
25 ± 1.3 pA ($n = 29$) while that of *OF* (KI/-) mice was 22.6 ± 1.3 pA ($n = 25$, $p = 1.0$ *OF* (KI/KI))
26 and in *OE* mice was 20.5 ± 1.1 pA ($n = 26$, $p = 0.46$ vs *OF* (KI/KI), one-way ANOVA

1 post-hoc Tukey; Figure 5E).

2 Next, we recorded spontaneous inhibitory post-synaptic currents (sIPSCs)
3 from orexin neurons in the presence of CNQX (20 μ M) and AP-5 (50 μ M) to block
4 glutamatergic inputs. Although sIPSCs in neurons lacking orexin peptides showed a
5 tendency toward decreasing frequency and amplitude, these changes were not
6 statistically significant. The inter-event interval for sIPSCs in orexin neurons in *OF* (KI/KI)
7 mice was 835.0 ± 124.6 ms ($n = 17$) while that of *OF* (KI/-) mice was 642.4 ± 70.9 ms (n
8 $= 22$, $p = 0.35$ vs *OF* (KI/KI)) and in *OE* mice was 595.0 ± 96.7 ms ($n = 21$, $p = 0.21$ vs *OF*
9 (KI/KI), one-way ANOVA post-hoc Tukey; Figure 5H). The sIPSC amplitude in orexin
10 neurons in *OF* (KI/KI) mice was 48.4 ± 6.1 pA ($n = 17$) while that of *OF* (KI/-) mice was
11 52.5 ± 3.9 pA ($n = 22$, $p = 0.86$ vs *OF* (KI/KI)) and in *OE* mice was 57.7 ± 6.5 pA ($n = 21$,
12 $p = 0.48$ vs *OF* (KI/KI), one-way ANOVA post-hoc Tukey; Figure 5I). These, along with
13 previous, results indicate that orexin plays a role in maintaining the physiological
14 input-output functions in orexin neurons.

15

16 ***OF* (KI/KI) mice showed symptoms in narcolepsy**

17 We reasoned that if orexin is successfully knocked out from *OF* (KI/KI) mice, it must
18 show the sign of behavioral arrest, cataplexy, which is defined as the sudden and
19 reversible episodes of the drop of voluntary muscle tone while remains fully conscious
20 during the episodes (Tabuchi et al., 2014a). Thus, we recorded and analyzed the
21 baseline sleep/wakefulness cycle in *OF* (KI/KI) mice. Behavioral states shown by *OF*
22 (KI/KI) mice were classified in 4 states which includes either wakefulness, REM, NREM
23 or cataplexy (see methods). All recorded *OF* (KI/KI) mice showed cataplexy attack,
24 especially during the start of the dark period. Sleep state parameters for orexin knockout
25 mice are presented in Table 1. These values were comparable with our previously
26 generated orexin neuron-ablated mice (Tabuchi et al., 2014a), as well as with the

1 previously generated orexin-knockout mice (Chemelli et al., 1999).

2

3 **Chemogenetic activation of orexin neurons lacking orexin neuropeptides**

4 Next, we sought to isolate the physiological effects of orexin from those of all other
5 neurotransmitters co-released by orexin neurons. To achieve this, we employed
6 chemogenetics, DREADD. Using Flp recombinase-dependent gene expression, hM3Dq
7 was exclusively expressed in orexin neurons. Here, hM3Dq was fused with mCherry to
8 detect expression and localization. AAV(9)-CMV-dFRT-hM3Dq-mCherry was injected
9 bilaterally into the LHA of both homozygous *OF* (KI/KI) and heterozygous *OF* (KI/-) mice
10 (Figure 6A). We quantified the number of orexin-positive cells in every 4th brain slices.
11 The number of orexin-immunoreactive cells was 439 ± 23 cells/mouse in the brain of
12 heterozygous mice and, among these, $92.1 \pm 0.6\%$ expressed mCherry ($n = 3$ mice;
13 Figure 6B). Moreover, a very similar number of LHA neurons expressed mCherry in both
14 heterozygous and homozygous mice: 422 ± 80 cells/mouse ($n = 6$) in *OF* (KI/-) and $455 \pm$
15 74 cells/mouse ($n = 6$) in *OF* (KI/KI) mice expressed mCherry ($p = 0.76$, unpaired *t*-test).
16 To confirm the selective activation of hM3Dq-expressing neurons *in vivo*, we measured
17 the expression of an immediate early gene product, c-Fos, which is a surrogate
18 molecular marker of neuronal activity. Following the behavioral studies, 6 randomly
19 selected mice from both the heterozygous and homozygous groups received i.p.
20 administration of either saline or clozapine N-Oxide (CNO) (1.0 mg/kg). Animals were
21 perfused, and tissues were collected 90 min after the injection. c-Fos staining of brain
22 slices confirmed that CNO selectively activated hM3Dq-expressing neurons in both
23 heterozygous and homozygous mice. In heterozygous mice, the ratio of c-Fos
24 expression in mCherry-positive cells of saline or CNO injected mice was $11.6 \pm 1.9\%$ or
25 $89.2 \pm 1.4\%$, respectively ($n = 3$ mice/group, $p = 9.2e-5$, Figure 6C-6D). In homozygous
26 mice, the ratio of c-Fos expression in mCherry-positive cells of saline or CNO injected

1 mice was $10.6 \pm 1.8\%$ or $90.3 \pm 1.1\%$, respectively ($n = 3$ mice/group, $p = 9.6e-4$, paired
2 t -test, Figure 6C-6D). Thus, we concluded that the DREADD system successfully
3 enabled selective activation of orexin neurons in both heterozygous and homozygous
4 mice.

5

6 **Orexin, and not other co-transmitters, was critical to promote sustained**
7 **wakefulness and preventing cataplexy**

8 To this end, we compared the effect of chemogenetic activation of orexin neurons on
9 sleep/wakefulness. Mice were injected with either saline or CNO during the light (L)
10 period (at 10:00 AM) and the dark (D) period (at 10:00 PM; Figure 6E). Expectedly in *OF*
11 (*KI/-*) mice, activation of orexin neurons increased total time spent in wakefulness ($n = 9$,
12 Figure 7B-i and 8D-i) and decreased time in REM sleep (Figure 7B-ii and 7D-ii) and in
13 NREM sleep (Figure 7B-iii and 7D-iii) after CNO administration during both the light and
14 dark periods. However, while the effects of orexin neuronal activation in *OF* (*KI/KI*) mice
15 were comparable to the *OF* (*KI/-*) control during the dark period, it was dampened during
16 the light period ($n = 8$, Wakefulness: Figure 7B-i and 7D-i); REM sleep: Figure 7B-ii and
17 7D-ii; and NREM sleep: Figure 7B-iii and 7B-iii).

18 This clear difference could be explained by the ability of co-transmitters to
19 partially compensate for the increased wakefulness. Therefore, we hypothesized that the
20 co-transmitters could eventually rescue *OF* (*KI/KI*) mice from cataplexy as well.
21 Surprisingly, however, activation of orexin neurons that lacked orexin peptide rather
22 deteriorate the cataplexy during the light periods while it showed similar propensity
23 during the dark period (saline (L): 0.2 ± 0.1 min/hr; CNO (L): 0.9 ± 0.3 min/hr, $n = 8$, $p =$
24 0.04 ; saline (D): 3.0 ± 1.2 min/hr; CNO (D): 5.2 ± 1.4 min/hr, $n = 8$, $p = 0.14$; Figure 7B-iv
25 and 7D-iv). These results clearly suggested that neurotransmitters other than orexin
26 could partially compensate for abnormalities in the regulation of sleep/wakefulness,

1 excluding cataplexy.

2 We also compared the effect of orexin neuronal activation in the number of
3 episodes (bouts) and average time spent in each vigilance state. Whereas, in *OF* (KI/-)
4 mice, total observed bouts decrease in all 3 vigilance states for 2 hours after CNO
5 injection (Wakefulness: Figure 7C-i and 7E-i; REM: Figure 7C-ii and 7E-ii, NREM: Figure
6 7C-iii and 7E-iii), they remain unaffected in case of *OF* (KI/KI) mice (Wakefulness: Figure
7 7C-i and 7E-i; REM: Figure 7C-ii and 7E-ii, NREM: Figure 7C-iii and 7E-iii; Cataplexy:
8 Figure 7C-iv and 7E-iv). Conversely, orexin neuronal activation increased average wake
9 duration in *OF* (KI/-) mice (Figure 7C-i and 7E-i); and decreased REM sleep (Figure 7C-ii
10 and 7E-ii) and NREM sleep duration (Figure 7C-iii and 7E-iii). However, in case of *OF*
11 (KI/KI) mice, total wake time was increased after chemogenetic activation of orexin
12 neurons (Figure 7B and 7E i-iv), duration of wake was not extended (Wakefulness:
13 Figure 7C-i and 7E-i; REM: Figure 7C-ii and 7E-ii; NREM: Figure 7C-iii and 7E-iii;
14 Cataplexy: Figure 7C-iv and 7E-iv). Together, all these experimental analyses indicated
15 that orexin neuropeptide was indispensable to promote sustained arousal, where the
16 co-transmitters could not compensate.

17

18 **CNO-induced cataplexy was not different from naïve cataplexy**

19 Finally, we sought to answer the question of whether CNO-induced increased in
20 cataplexy attack showed similar properties to naïve cataplexy. We analyzed and
21 compared the relative power spectrum EEG during the cataplexy episode in the daytime
22 after saline or CNO administration as chemogenetic activation of orexin-KO neurons
23 resulted in increased cataplexy in the daytime (Figure 8). Relative power analysis
24 showed no difference in either power of the delta, theta, alpha or beta wave (n = 8 mice,
25 Figure 8C), suggesting that CNO-induced cataplexy showed similar electrocortical
26 activity as the naïve cataplexy attack observed in *OF* (KI/-) mice.

1

2

1 **Discussion**

2 It has been shown that orexin neurons possess several neurotransmitters and
3 release them together with orexin. However, orexin- or OX2R-KO mice closely
4 phenocopy the symptoms observed in human narcolepsy, which is caused by the
5 specific degeneration of orexin neurons. This might suggest that other neurotransmitters
6 have either no or insignificant role in the regulation of sleep/wakefulness. Physiological
7 importance of orexin can be explored in several ways including central administration,
8 KO, functional manipulation or ablation of source neurons, among others. Here, we
9 dissociated the effect of orexin by applying electrophysiological analyses and neural
10 activity manipulation to orexin neurons that lack orexin peptide using novel KI mice.
11 Previously, we generated *orexin-Cre* or *orexin-tTA* transgenic mice in which a short 3.2
12 kb fragment from the 5'-upstream region of the human prepro-orexin gene was used as a
13 promoter (Inutsuka et al., 2014; Sakurai et al., 1999; Tabuchi et al., 2013; Yamanaka et
14 al., 2003). However, the random integration of the *orexin-Cre* or *orexin-tTA* transgene
15 into the genome could result in ectopic gene expression in non-targeted cells as well as
16 unexpected expression during development, as the timing and regulation of gene
17 expression might be affected by genes located near the integrated locus. This could be
18 problematic if these mouse lines were bred with reporter mice. In contrast, KI permits
19 accurate temporo-spatial gene expression control. The Flp function was restricted in
20 orexin neurons in the *OF* mice as confirmed by reporter mice. To our knowledge, *OF*
21 mice represent the first line which enabled exclusive gene expression in orexin neurons
22 by crossing with reporter mice. Moreover, combining Flp and Cre driver mice enable us
23 to express different genes in different neuronal subtypes to apply neural manipulations
24 and/or activity readout simultaneously. By crossing *OF* mice with either *MCH-Cre* or
25 *Gad67-Cre*, we generated *orexin-Flp; MCH-Cre* and *orexin-Flp; Gad67-Cre* bigenic
26 mice, and argued that *OF* mice could be an essential tool for studying the functional

1 connectome between orexin neurons and any other neurons in the hypothalamus.
2 Besides, *OF* mice can also be useful for analyzing long-range neuronal connections. By
3 using the *orexin-Flp; Gad67-Cre* bigenic mice, we recently found that GABAergic
4 neurons in the ventral tegmental area inhibited orexin neuronal activity by making
5 monosynaptic inhibitory projections (Chowdhury et al., *Manuscript submitted*).

6 Previously, we reported that orexin neuropeptide depolarized orexin neurons
7 via OX2R and mediates a positive-feedback loop both directly and indirectly (Yamanaka
8 et al., 2010b). In agreement with this, we also found that orexin facilitated glutamate
9 release onto orexin neurons. However, several basic electrophysiological properties of
10 orexin neurons were also found to be altered in the absence of orexin peptides, including
11 hyperpolarized membrane potential, lower discharge rate and input resistance.
12 Surprisingly, the capacitance of orexin neurons that lack orexin peptide was significantly
13 larger than that of orexin neurons possessing orexin. This indicates that orexin neurons
14 lacking orexin have a larger surface of cell membrane since capacitance reflects the
15 surface area of the cytoplasmic membrane. We did not directly measure the difference in
16 diameter of cell size since an approximately 3 pF increase is estimated to be associated
17 with a 1-2 μm increase in diameter as a spherical body. This might suggest that orexin
18 signaling through either OX2R or glutamatergic excitatory inputs is involved in the
19 regulation of cell size.

20 Orexin neurons are known to play an essential role in maintaining
21 uninterrupted wakefulness as human narcolepsy patients show chronic daytime
22 sleepiness (Crocker et al., 2005; Thannickal et al., 2000). Disruption of orexin signaling
23 in mice, rats and dogs produce a very similar phenotype which includes short bouts of
24 wakefulness and increased transitions of vigilance states (Chemelli et al., 1999;
25 Mochizuki et al., 2004). These evidences suggest that orexin might be the key
26 component released from the orexin neurons in performing the wake-maintaining role of

1 orexin neurons. Our study showed further evidence in support of this hypothesis in
2 mouse model as putative increase in the probability of co-transmitters release from
3 orexin neurons could not rescue from short bout and frequent state-transition
4 phenomenon. Chemogenetic activation of orexin neurons prolonged duration of
5 wakefulness only in the presence of orexin, suggesting that orexin is crucial to achieve
6 long-duration wakefulness. Moreover, orexin administration can also make wakefulness
7 with high cognitive ability in non-human primates (Deadwyler et al., 2007). Such effect of
8 orexin is also expected in human society, and our results indicate the irreplaceability of
9 orexin.

10 On the contrary, we found that other behavioral outcomes of activating orexin
11 neurons, including increased wakefulness and decreased REM and NREM sleep, can
12 be compensated for by co-transmitters. This effect was obvious especially during the
13 dark (active) period when baseline activity of orexin neurons is presumably higher
14 (Estabrooke et al., 2001; Lee et al., 2005; Mileykovskiy et al., 2005). Interestingly, some
15 other behavioral effects, like the prevention of cataplexy, cannot be compensated for by
16 co-transmitters. Rather, it deteriorates the condition. This might suggest that the role of
17 orexin in the prevention of cataplexy is not simply to activate post-synaptic neurons since
18 glutamate (co-transmitter) also activates post-synaptic neurons. Rather, neurons that
19 are not directly innervated by orexin neurons might also be involved in the regulation of
20 cataplexy. It is also possible that the released orexin diffuses in the CSF since
21 intracerebroventricular injection of orexin inhibited cataplexy (Mieda et al., 2004).
22 Moreover, orexin co-transmitter dynorphin, which inhibit orexin neurons both directly and
23 indirectly by depressing glutamatergic afferent inputs to orexin neurons, might also play
24 a key role in such deterioration of cataplexy (Li and van den Pol, 2006).

25 The release of orexin follows a circadian rhythm that is also strongly related to
26 locomotion (Zhang et al., 2004). In rodents, the level of orexin peptide in the CSF is low

1 during the light period and high during the active-wake period (Zeitler et al., 2003).
2 Moreover, orexin neurons supposedly show higher activity during active wakefulness
3 and become less active or inactive during REM or NREM sleep (Lee et al., 2005;
4 Mileykovskiy et al., 2005). Our data also suggest that the activity of orexin neurons is
5 differentially regulated during the light and dark periods. It is possible that the higher
6 endogenous activity of orexin neurons during the dark period may facilitate
7 CNO-induced transmitter release resulting in improved wakefulness in *OF(KI/KI)* mice.

8 In summary, here we dissociated the role of orexin at the cellular and
9 behavioral level. We suggest that the primary function of orexin is to maintain the
10 electrophysiological balance, the input-output functions in orexin neurons and most
11 importantly, to exert the function of orexin neurons in maintaining sustained wakefulness.

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11

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14 analyzed the data; M.K. generated the targeting vector; T.K. and H.B. generated dFRT
15 vector; I.I. generated *FSF-mTFP1* mice; M.A. and K.S. generated *orexin-flippase* mice;
16 S.C. and A.Y. wrote the manuscript.

17

18 **Financial disclosure statements:** None

1 **Methods**

2 **Subjects**

3 All experimental protocols in this study involving the use of mice were approved and
4 were performed in accordance with the approved guidelines of the Institutional Animal
5 Care and Use Committees of the Research Institute of Environmental Medicine, Nagoya
6 University, Japan. Mice were group housed unless stated otherwise, on a 12-hour
7 light-dark cycle (lights were turned on at 8:00 AM), with free access to food and water. All
8 efforts were made to reduce the number of animals used and to minimize the suffering
9 and pain of animals.

10

11 **Generation of *OF* knock-in mice**

12 To generate the *OF* knock-in mice, we designed a targeting vector in which Flp
13 recombinase cDNA was fused to enhanced green fluorescent protein (EGFP) with the 2A
14 peptide gene (EGFP-2A-Flp) and was placed just behind the translational initiation site of
15 the prepro-orexin gene in-frame. The knock-in vector was constructed with the MC1
16 promoter-driven diphtheria toxin gene, a 5.44 kb fragment at the 5' site. Flp recombinase,
17 including a nuclear localization signal cDNA, was fused to EGFP with the T2A peptide
18 sequence, a pgk-1 promoter-driven neomycin phosphotransferase gene (neo) flanked
19 by two Dre recognition target (*rox*) sites, and a 5.16 kb fragment at the 3' site (Figure 1A).
20 The sequence of EGFP-2A-Flp was codon-optimized for expression in mammalian cells.
21 Linearized targeting vector was electroporated into embryonic stem cells from the
22 C57BL/6 mouse line (RENKA), and corrected targeted clones were isolated by southern
23 blotting. Two founders were obtained, and the B line was used in this study. PCR
24 genotyping of mouse tail DNA was performed with the following primers: knock-in
25 forward, 5'-CTCATTAGTACTCGGAACTGCCC-3'; knock-in reverse,
26 5'-AAGCACTATCATGGCCTCAGTAGT-3'.

1

2 **Generation of *orexin-Flp*; *FSF-mTFP1*, *orexin-Flp*; *MCH-Cre* and *orexin-Flp*;**

3 ***Gad67-Cre* bigenic mice**

4 After several generations of breeding, *OF* mice were separately bred with either
5 *R26-CAG-FRT-STOP-FRT-mTFP1* (*FSF-mTFP1*) (Imayoshi et al., 2012), *MCH-Cre*
6 (Kong et al., 2010), or glutamic acid decarboxylase at 67 K-dalton (*Gad67*)-*Cre*
7 (*Gad67-Cre*) (Higo et al., 2009) mice to generate *orexin-Flp*; *FSF-mTFP1*, *orexin-Flp*;
8 *MCH-Cre* or *orexin-Flp*; *Gad67-Cre* bigenic mice, respectively.

9

10 **Generation and microinjection of viral vectors**

11 Adeno-associated viral (AAV) vectors were produced using the AAV Helper-Free System
12 (Agilent Technologies, Inc., Santa Clara, CA, USA). The virus purification method was
13 modified from a previously published protocol (Inutsuka et al., 2016). Briefly, HEK293
14 cells were transfected with a pAAV vector, pHelper and pAAV-RC (serotype 9 or DJ;
15 purchased from Cell Biolabs Inc., San Diego, CA, USA) plasmid using a standard
16 calcium phosphate method. Three days after transfection, cells were collected and
17 suspended in artificial CSF (aCSF) solution (in mM: 124 NaCl, 3 KCl, 26 NaHCO₃, 2
18 CaCl₂, 1 MgSO₄, 1.25 KH₂PO₄ and 10 glucose). Following multiple freeze-thaw cycles,
19 the cell lysates were treated with benzonase nuclease (Merck, Darmstadt, Germany) at
20 37°C for 30 min, and were centrifuged 2 times at 16,000 g for 10 min at 4°C. The
21 supernatant was used as the virus-containing solution. Quantitative PCR was performed
22 to measure the titer of purified virus. Virus aliquots were then stored at -80°C until use.

23 A dFRT cassette was used for Flp-dependent gene expression control. A dFRT cassette
24 is composed of two different FRT sequences (FRT and F3) located in a cis position. In
25 the presence of Flp, sequence between dFRT is reversed and fixed. To express hM3Dq
26 exclusively in Flp-expressing neurons, we stereotactically injected 600 nl of

1 AAV(9)-CMV-dFRT-hM3Dq-mCherry (viral titer: 1.0×10^{12} particles/ml) virus into both
2 brain hemispheres of *OF* mice using the following coordinates: -1.4 mm posterior to the
3 bregma, 0.8 mm lateral to the midline, -5.0 mm ventral to the brain surface. Mice were
4 anesthetized with 1.5-2.0% isoflurane (Wako Pure Chemical Industries, Osaka, Japan)
5 using a Univentor 400 Anaesthesia Unit (Univentor Ltd., Malta) throughout the entire
6 surgery. These mice were used in the behavioral experiments beginning at least 3 weeks
7 post-injection. For Flp-dependent expression of humanized Renilla reniformis green
8 fluorescent protein (hrGFP) and Cre-dependent expression of tdTomato, we
9 stereotactically injected 600 nl of virus cocktail containing AAV(DJ)-CMV-dFRT-hrGFP
10 (viral titer: 3.7×10^{12} particles/ml) and AAV(DJ)-CMV-FLEX-tdTomato (viral titer: 2.0×10^{12}
11 particles/ml) virus into one brain hemisphere of *OF* mice using the same coordinates
12 described above.

13

14 **Immunohistochemistry**

15 Mice were anesthetized with 10% somnopentyl (1.0 mg/kg, Kyoritsu Seiyaku
16 Corporation, Tokyo, Japan) and were perfused transcardially with 20 ml of ice-cold
17 saline. This perfusion was immediately followed by another 20 ml of 10% ice-cold
18 formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Brains were then isolated
19 and postfixed in 10% formalin solution at 4°C overnight. Subsequently, brains were
20 immersed in 30% sucrose in PBS at 4°C for at least 2 days. Coronal brain slices of 40 μ m
21 thickness were generated using a cryostat (Leica CM3050 S; Leica Microsystems,
22 Wetzlar, Germany). For staining, coronal brain sections were immersed in blocking
23 buffer (1% BSA and 0.25% Triton-X in PBS), then incubated with primary antibodies at
24 4°C overnight. The sections were then washed with blocking buffer and incubated with
25 secondary antibodies for 1 hr at room temperature (RT). After washing, brain sections
26 were mounted and examined using a fluorescence microscope (BZ-9000, Keyence,

1 Osaka, Japan or IX71, Olympus, Tokyo, Japan).

2

3 **Antibodies and stains**

4 Primary antibodies were diluted in the blocking buffer as follows: anti-orexin-A goat
5 antibody (Santa Cruz, Dallas, TX) at 1:1000, anti-MCH rabbit antibody (Sigma-Aldrich) at
6 1:2000, anti-prodynorphin guinea pig antibody (Merck Millipore, Billerica, MA) at 1:100,
7 anti-c-Fos rabbit antibody (Santa Cruz) at 1:500 and anti-GFP mouse antibody (Wako,
8 Japan) at 1:1000. Secondary antibodies included: CF 488- or CF 594-conjugated
9 anti-goat antibody (Biotium Inc., Hayward, CA), CF 647-conjugated anti-rabbit antibody
10 (Biotium), CF 680-conjugated anti-guinea pig antibody (Biotium) and CF 488-conjugated
11 anti-mouse antibody (Biotium); all were diluted at 1:1000 in blocking buffer.

12

13 **Acute brain slice preparations and electrophysiological recording**

14 OF and *orexin-EGFP (OE)* (Yamanaka et al., 2003) mice of both sexes, aged 2-5
15 months, were used for electrophysiological recordings. Brain slice preparations and
16 subsequent electrophysiological recording were modified from a previously published
17 protocol (Chowdhury and Yamanaka, 2016). Briefly, mice were deeply anesthetized
18 using isoflurane (Wako) and decapitated at around 11:00 AM. Brains were quickly
19 isolated and chilled in ice-cold oxygenated (95% O₂ and 5% CO₂) cutting solution (in mM:
20 110 K-gluconate, 15 KCl, 0.05 EGTA, 5 HEPES, 26.2 NaHCO₃, 25 Glucose, 3.3 MgCl₂
21 and 0.0015 (±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid). After trimming the
22 brain, coronal brain slices of 300 µm thickness that contained the LHA were generated
23 using a vibratome (VT-1200S; Leica, Wetzlar, Germany) and were temporarily placed in
24 an incubation chamber containing oxygenated bath solution (in mM: 124 NaCl, 3 KCl, 2
25 MgCl₂, 2 CaCl₂, 1.23 NaH₂PO₄, 26 NaHCO₃ and 25 Glucose) in a 35°C water bath for 60
26 min. Slices were then incubated at RT in the same incubation chamber for another 30-60

1 min for recovery.

2 Acute brain slices were transferred from the incubation chamber to a recording
3 chamber (RC-26G; Warner Instruments, Hamden, CT, USA) equipped with an upright
4 fluorescence microscope (BX51WI; Olympus, Tokyo, Japan), and were superfused with
5 oxygenated bath solution at the rate of 1.5 ml/min using a peristaltic pump (Dynamax;
6 Rainin, Oakland, CA, USA). An infrared camera (C3077-78; Hamamatsu Photonics,
7 Hamamatsu, Japan) was installed in the fluorescence microscope along with an electron
8 multiplying charge-coupled device camera (EMCCD, Evolve 512 delta; Photometrics,
9 Tucson, AZ, USA) and both images were separately displayed on monitors.
10 Micropipettes of 4-6 M Ω resistance were prepared from borosilicate glass capillaries
11 (GC150-10; Harvard Apparatus, Cambridge, MA, USA) using a horizontal puller
12 (P-1000; Sutter Instrument, Novato, CA, USA). Patch pipettes were filled with KCl-based
13 internal solution (in mM: 145 KCl, 1 MgCl₂, 10 HEPES, 1.1 EGTA, 2 MgATP, 0.5 Na₂GTP;
14 pH 7.3 with KOH) with osmolality between 280-290 mOsm. Positive pressure was
15 introduced in the patch pipette as it approached the cell. For whole-cell current clamp or
16 voltage clamp recordings, a giga-seal of resistance >1 G Ω was made between the patch
17 pipette and the cell membrane by releasing the positive pressure upon contacting the
18 cell. The patch membrane was then ruptured by gentle suction to form a whole-cell
19 configuration. Electrophysiological properties of the cells were monitored using the
20 Axopatch 200B amplifier (Axon Instrument, Molecular Devices, Sunnyvale, CA). Output
21 signals were low-pass filtered at 5 kHz and digitized at a 10 kHz sampling rate. Patch
22 clamp data were recorded through an analog-to-digital (AD) converter (Digidata 1550A;
23 Molecular Devices) using pClamp 10.2 software (Molecular Devices). Blue light with a
24 wavelength of 475 \pm 17.5 nm was generated by a light source that used a light-emitting
25 diode (Spectra light engine; Lumencor, Beaverton, OR, USA) and was guided to the
26 microscope stage with a 1.0 cm diameter fiber. Brain slices were illuminated through the

1 objective lens of the fluorescence microscope. EGFP-expressing orexin neurons were
2 identified by its fluorescence. For cell-attached recording, a seal of resistance $<1\text{ G}\Omega$
3 was made and spontaneous firing was recorded. The resting membrane potentials
4 (V_{rest}) were measured from offline analysis of current clamp recordings using the
5 predefined fitting function provided by Clampfit. We performed standard exponential
6 fitting with zero shift for the initial 20 s of data to measure the V_{rest} of recorded neurons.
7 Firing frequency was also calculated from offline analysis of the initial 60 s of the
8 cell-attached recording data. sEPSCs were recorded with picrotoxin ($400\text{ }\mu\text{M}$) and
9 sIPSCs were recorded with AP-5 ($50\text{ }\mu\text{M}$) and CNQX ($20\text{ }\mu\text{M}$) in bath solutions. Both
10 sEPSCs and sIPSCs were recorded in the presence of KCl-based pipette solutions that
11 included 1 mM of QX-314.

12

13 **EEG-EMG surgery, data acquisition, and vigilance state determination**

14 Virus injected age-matched male mice were implanted with EEG and EMG electrodes for
15 polysomnographic recording under isoflurane anesthesia following the protocol
16 published elsewhere (Tabuchi et al., 2014a). Immediately after surgery, each mouse
17 received an i.p. injection of 10 ml/kg of analgesic solution containing 0.5 mg/ml of
18 Carprofen (Zoetis Inc., Japan). The same analgesic at the same dose was administered
19 again 1 day after surgery. Mice were singly housed for 7 days during recovery. Mice were
20 then connected to a cable with a slip ring in order to move freely in the cage and were
21 habituated with the cable for another 7 days. The first 3 days were treated as the
22 adaptation period for the animals to acclimate to the new environment and to
23 intraperitoneal (i.p.) administration (10 ml/kg) of saline. On days 4 and 5, the mice were
24 injected with saline (day 4) and CNO (Enzo Life Sciences, Farmingdale, NY, USA) (day
25 5) at 10:00 AM during the light period. On days 7 and 8, they were injected with saline
26 (day 7) and CNO (day 8) at 10:00 PM during the dark period (See Figure 6). CNO was

1 dissolved in water to make a stock solution (10 mg/ml) and was diluted with saline to a
2 final concentration of 100 µg/ml just prior to i.p. administration.
3 EEG and EMG signals were amplified (AB-610J, Nihon Koden, Japan), filtered (EEG
4 1.5-30 Hz and EMG 15-300 Hz), digitized (at a sampling rate of 128 Hz), recorded (Vital
5 Recorder, Kissei Comtec Co., Ltd, Japan) and finally analyzed (SleepSign, Kissei
6 Comtec). Animal behavior was monitored through a CCD video camera (Amaki Electric
7 Co., Ltd., Japan) during both the light and dark periods. The dark period video recording
8 was assisted by infrared photography (Amaki Electric Co., Ltd., Japan) and an infrared
9 sensor (Kissei Comtec). EEG and EMG data were automatically scored in 4 sec epochs
10 and classified as wake, rapid eye movement sleep, and non-rapid eye movement sleep.
11 The EEG analysis yielded power spectra profiles over a 0~20 Hz window with 1 Hz
12 resolution for delta (1-5 Hz), theta (6-10 Hz), alpha (11-15 Hz), and beta (16-20 Hz)
13 bandwidths. All auto-screened data were examined visually and corrected. The criteria
14 for vigilance states were the same as described previously (Tabuchi et al., 2014b). Briefly,
15 (i) wake (low EEG amplitude with high EMG or locomotion score), (ii) non-rapid eye
16 movement (NREM) sleep (low EMG and high EEG delta amplitude), and (iii) rapid eye
17 movement (REM) sleep (low EMG, low EEG amplitude with high theta activity, and
18 should be followed by NREM). Cataplexy was tracked using a combination of multiple
19 criteria: muscle atonia lasting ≥ 10 sec, predominance of theta activity and more than 40
20 seconds of wakefulness before the cataplectic attack.

21

22 **Data analysis and presentation**

23 Immunostaining data were analyzed and processed with ImageJ (US National Institute
24 of Health) and BZ-X Analyzer (Keyence BZ-X710 microscope). Electrophysiological
25 analysis was performed with either Clampfit10 (Molecular Devices, Sunnyvale, CA) or
26 Minianalysis software (Synaptosoft Inc., Decatur, GA). Electrophysiological data were

1 saved as American Standard Code for Information Interchange (ASCII) files and further
2 data calculations were performed in Microsoft Excel. Graphs were generated in Origin
3 2017 (OriginLab, Northampton, MA) using data from Excel. Statistical analysis was also
4 performed with Origin 2017. Graphs were generated using Canvas 15 (ACD Systems,
5 Seattle, WA).

6

7 **Experimental design and statistical analysis**

8 The electrophysiological effects of knocking out a single neuropeptide in its source
9 neurons were analyzed by slice electrophysiology. Individual sample sizes for slice
10 patch-clamp recordings (n number of neurons) are reported separately for each
11 experiment. The physiological effects of activating orexin neurons that lack orexin
12 neuropeptide were also analyzed. In all cases, five or more animals were used for each
13 parameter tested. All statistical tests, including the exact p values, are described when
14 used. No statistical analyses were used to predetermine sample sizes. All data are
15 presented as the mean \pm standard error of the mean (SEM). For all statistical tests *
16 $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered significant and $p > 0.05$ was considered
17 not significant (ns).

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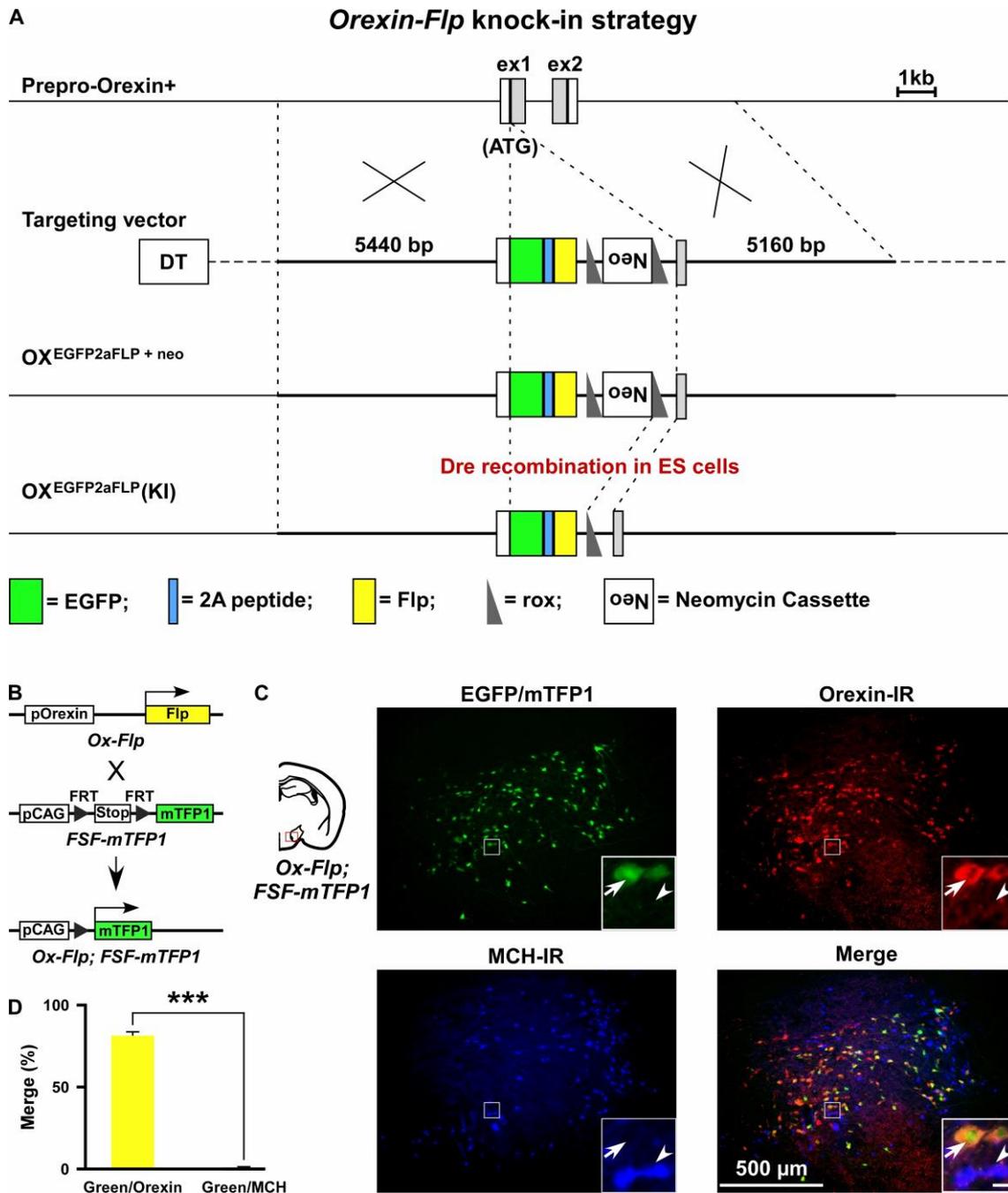
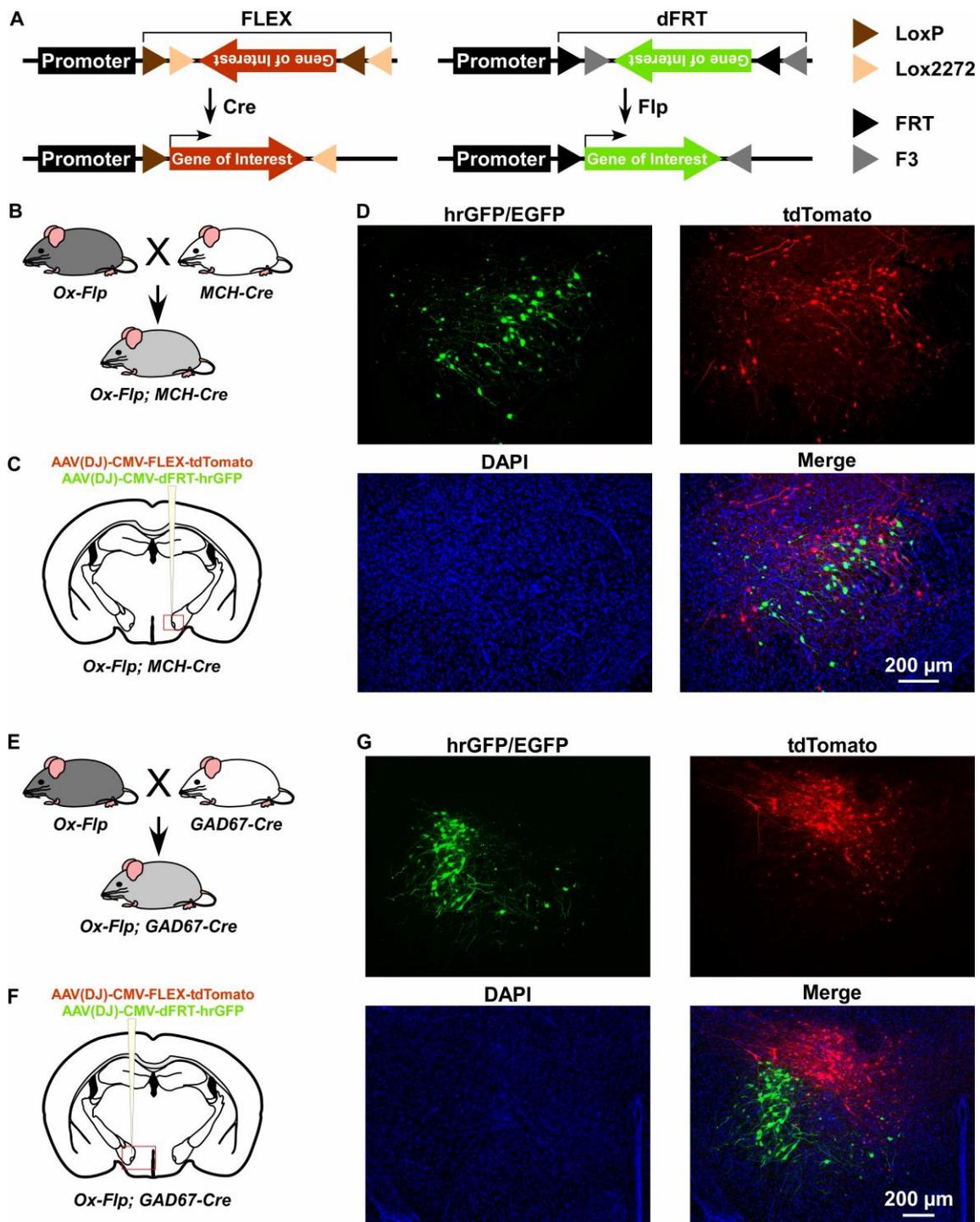


Figure 1: Generation of *OF* mice. A, Schematic representations of the prepro-orexin gene, targeting vector, and targeted gene. To achieve orexin neuron-specific expression of Flp recombinase, we inserted EGFP-2A-Flp just behind the translation initiation site of the prepro-orexin gene in-frame. Viral T2A peptide is cleaved just after translation, and EGFP and Flp recombinase localize independently. DT, diphtheria toxin; Neo, neomycin-resistant gene expression cassette. B, Structure of the reporter gene in the presence of Flp. *Orexin-Flp; FSF-mTFP1* bigenic mice were generated to express

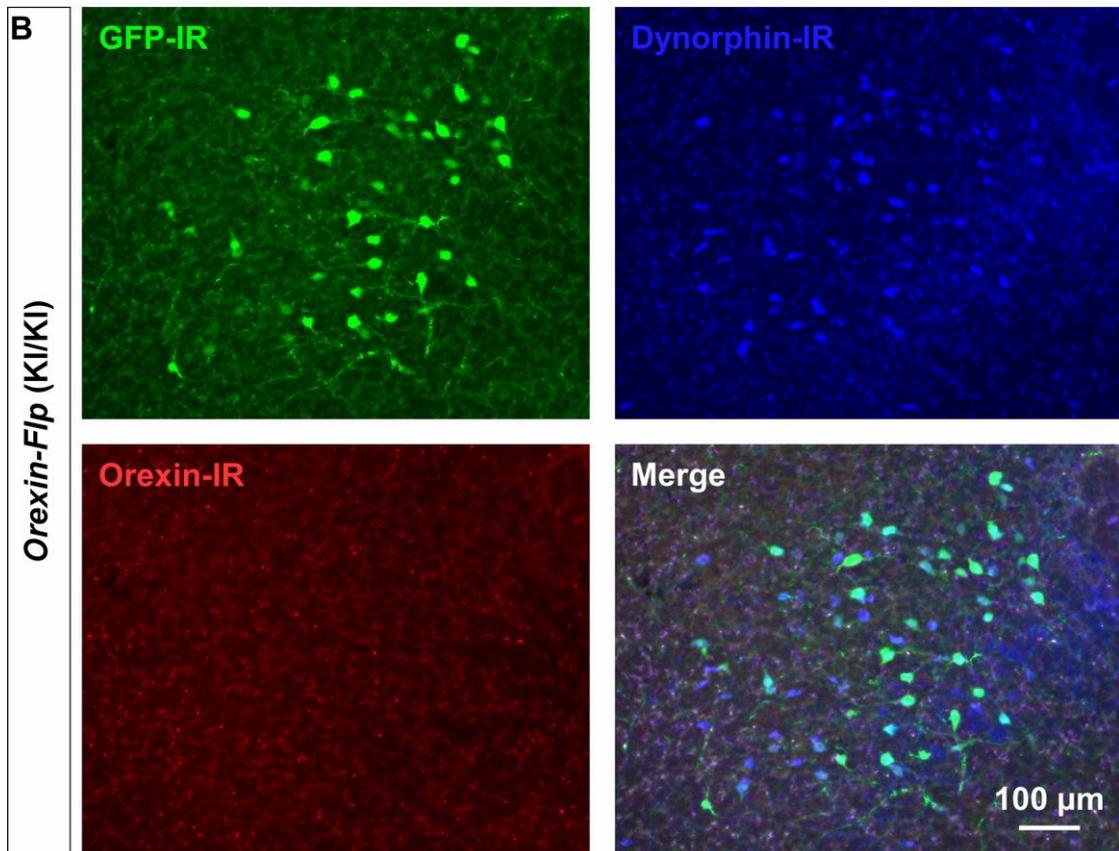
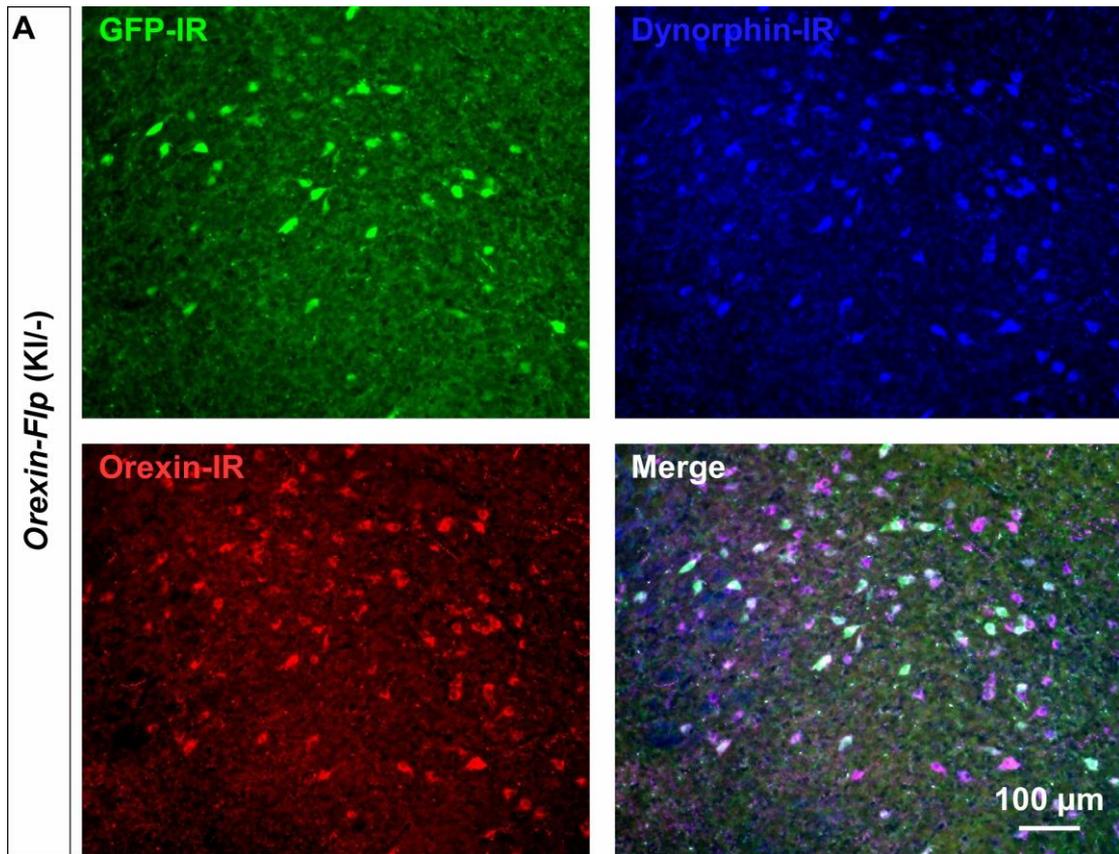
1 mTFP1 in orexin neurons. C) Representative pictures from coronal brain sections of
2 *orexin-Flp; FSF-mTFP1* bigenic mice. Arrow indicates mTFP1 and/or EGFP expressing
3 orexin neurons and the arrowhead indicates the position of the MCH neuron. Inset scale
4 bar: 20 μ m. D) Summary of the co-expression analysis (n = 4 mice). The *p* values were
5 measured by two-tailed paired student's *t*-test. Data represent the mean \pm SEM.



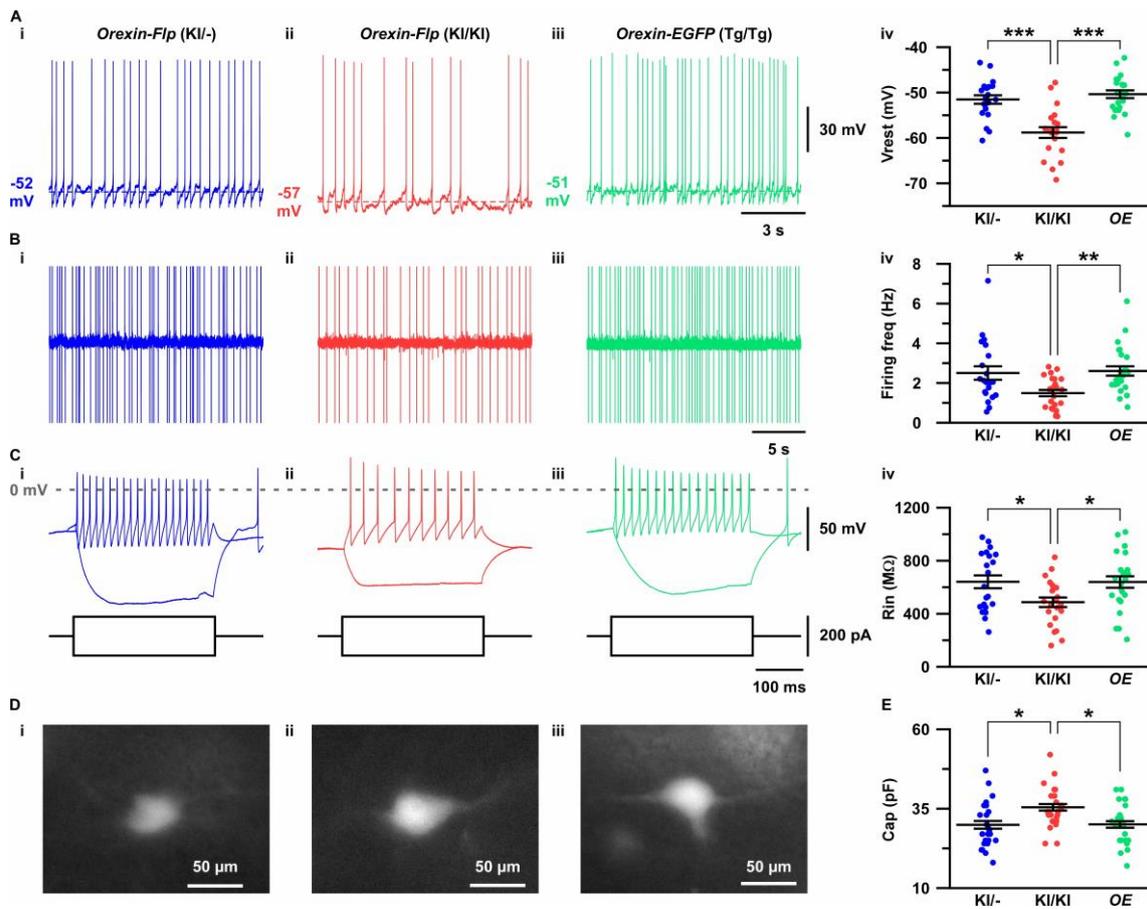
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2 **Figure 2:** Gene expression control using *OF* mice to target different cell types within the
 3 same brain region. A, Schematic showing Cre (left) and Flp (right)
 4 recombinase-dependent gene expression control. B and E, The breeding scheme for
 5 *orexin-Flp; MCH-Cre* and *orexin-Flp; Gad67-Cre* bigenic mice, respectively. C and F,
 6 Schematic drawings showing micro-injection of the AAV cocktail into the LHA of bigenic

- 1 mice. D and G, Representative coronal brain sections showing the segregated
- 2 expression of tdTomato and hrGFP in a Cre and Flp recombinase-dependent manner,
- 3 respectively.

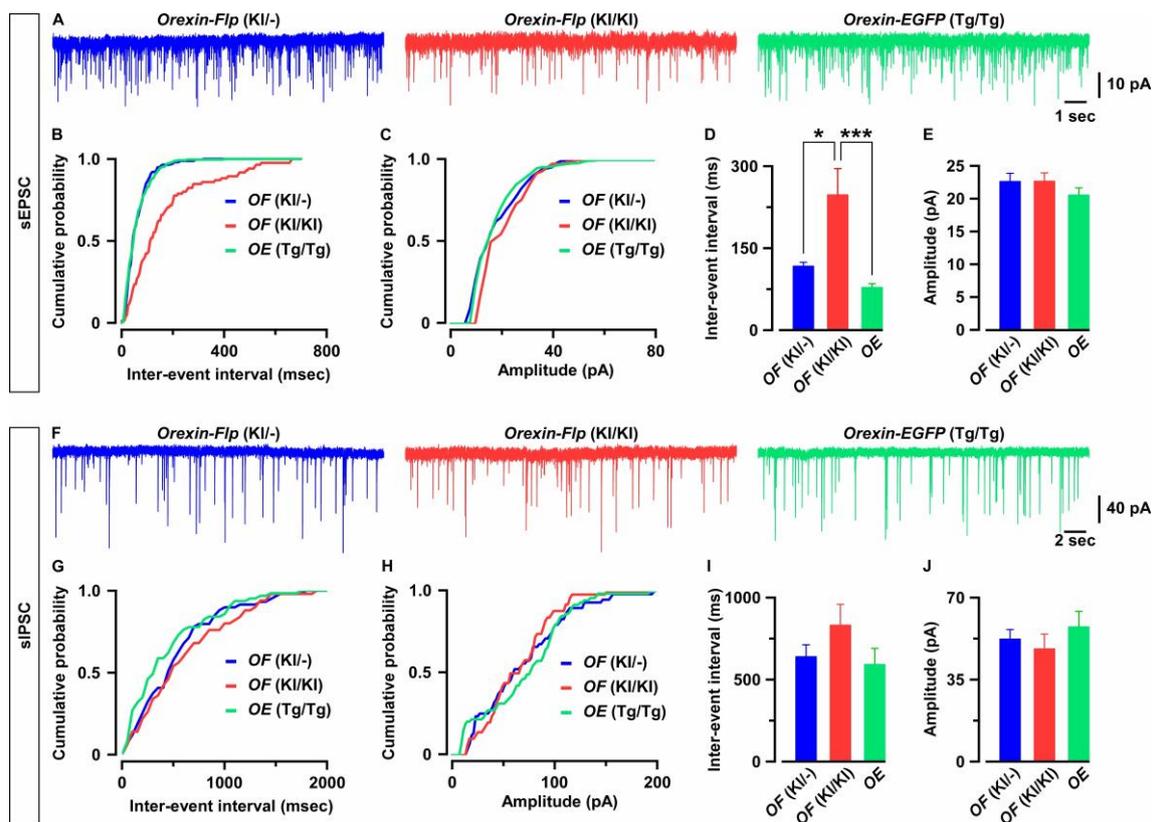


- 1 **Figure 3:** Immunohistochemical confirmation of *OF* mice. A and B, Representative
- 2 coronal brain sections showing the expression of EGFP (green), orexin (red) and
- 3 dynorphin (blue).

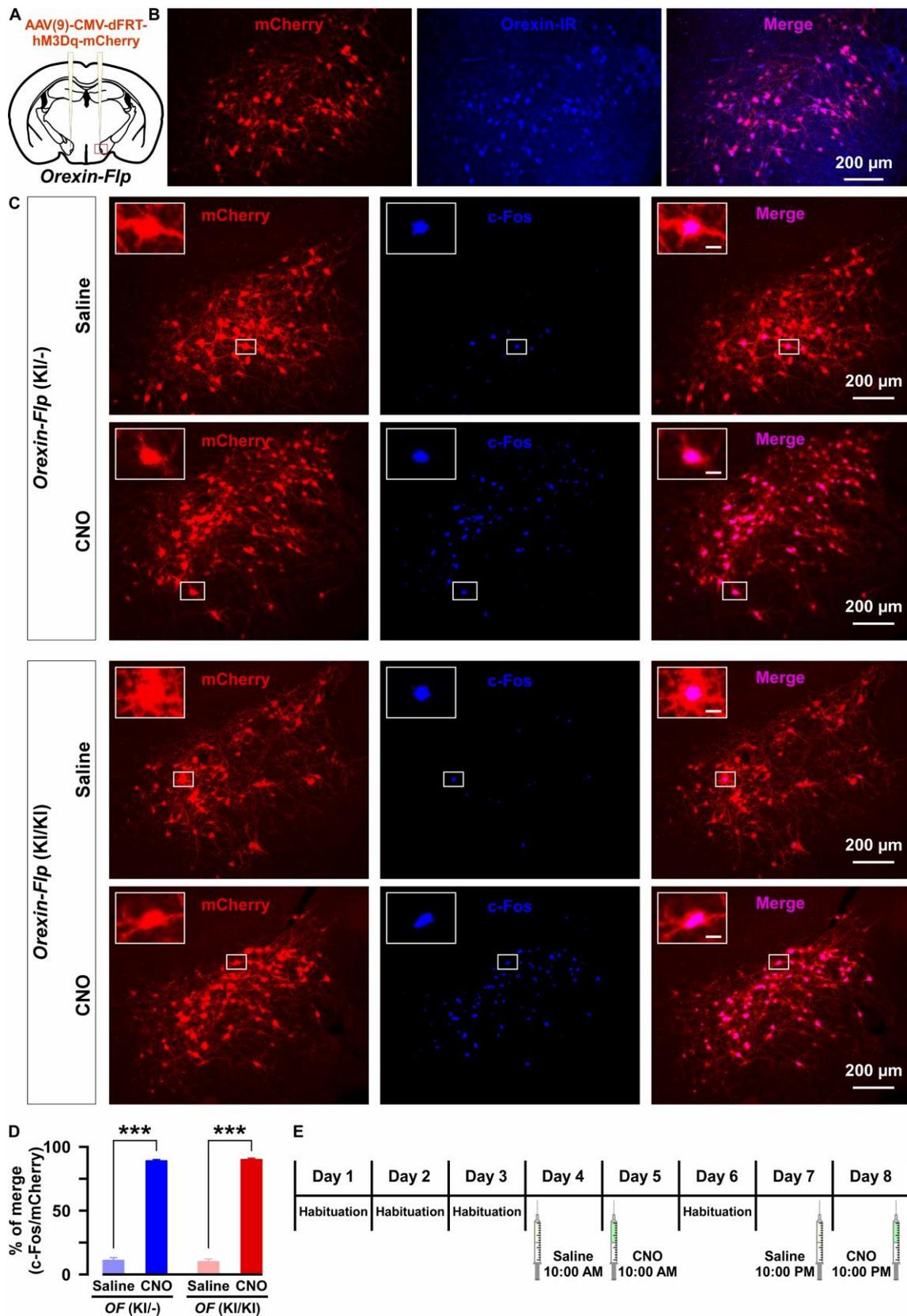


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Figure 4: Electrophysiological properties of orexin neurons with/without orexin. A-C show representative traces recorded from *OF (KI/-)* (i), *OF (KI/KI)* (ii) and *OE* (iii) mice. A, Membrane potential in whole-cell current clamp recordings. B, Spontaneous firing in cell-attached recordings. C, Step current injection-induced membrane potential changes. Panel iv is a summary of the data in panel i to iii. D, Representative images showing EGFP expression in acute coronal brain slices during electrophysiological recording. E, Cell capacitance from whole-cell current clamp recording. The *p* values were determined using one-way ANOVA test followed by a post-hoc Tukey analysis. Data represent the mean \pm SEM.



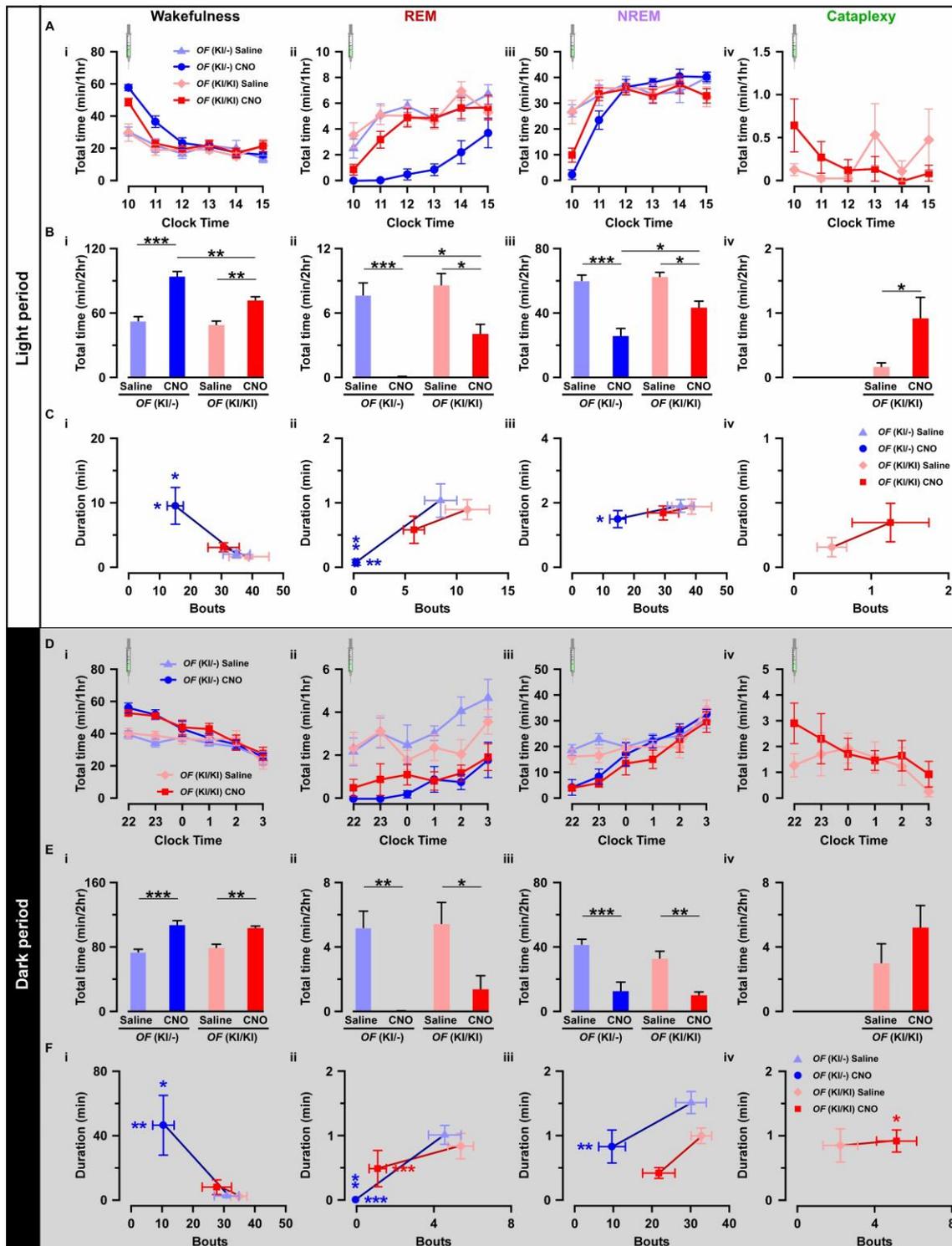
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2 **Figure 5:** Orexin neurons receive fewer glutamatergic inputs in the absence of orexin. A,
3 Representative sEPSC traces recorded from EGFP-expressing neurons at a holding
4 potential of -60 mV. B-C, Cumulative probability plot for the representative traces shown
5 in A. Bar diagrams in D and E summarize the sEPSC data. D, Inter-event interval. E,
6 amplitude (n = 25-29). F, Representative sIPSC traces recorded from EGFP-expressing
7 neurons at a holding potential of -60 mV. G-H, Cumulative probability plot for the
8 representative traces shown in F. Bar diagrams in I and J summarize the sIPSC data. G,
9 Inter-event interval. H, amplitude (n = 17-22). The p values were calculated by one-way
10 ANOVA followed by a post-hoc Tukey test. Data represent the mean ± SEM.



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2 **Figure 6:** Selective chemogenetic activation of orexin neurons. A, Intracranial injection

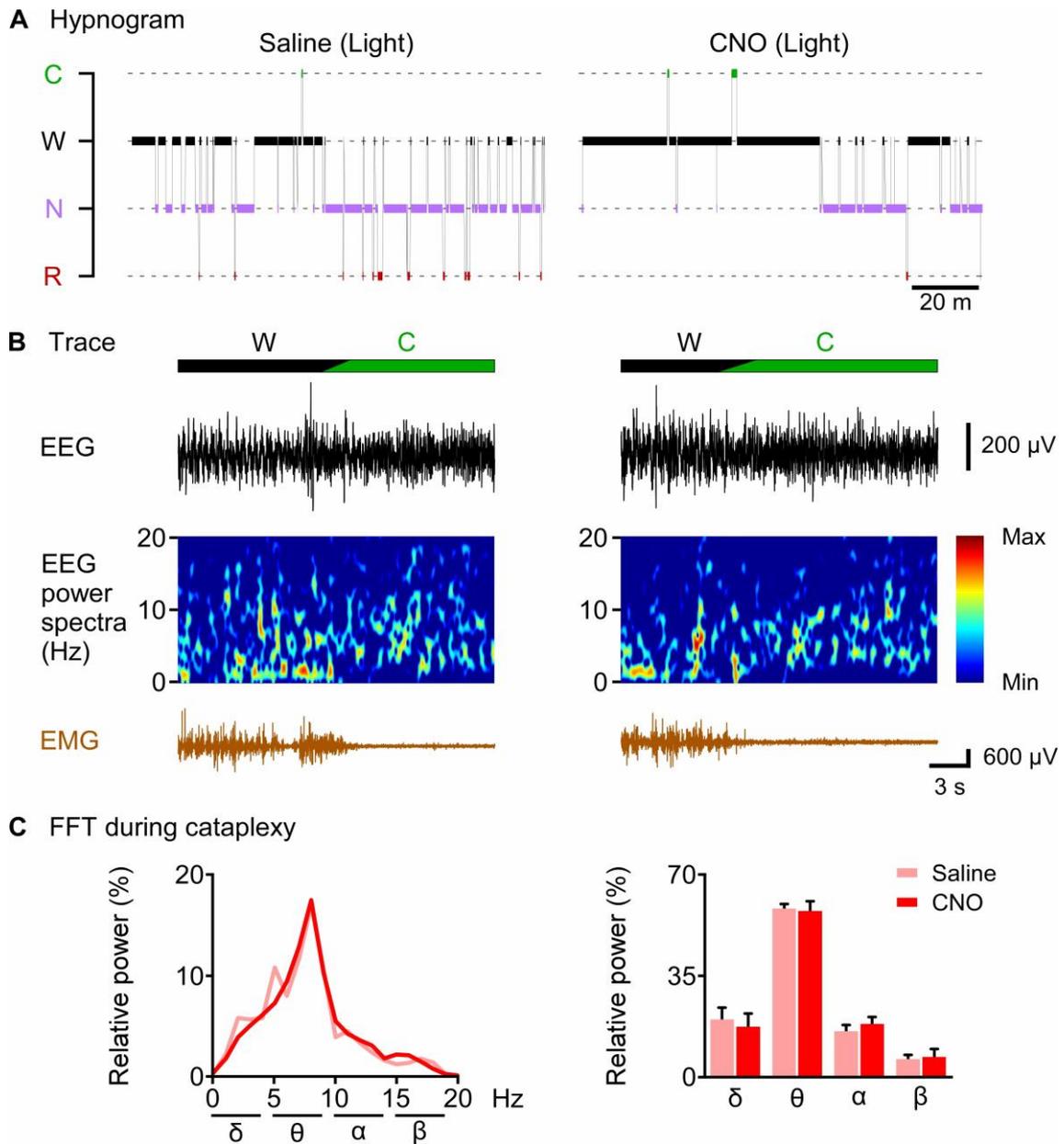
1 of AAV in *OF* mice to achieve Flp-dependent expression of hM3Dq-mCherry fusion
2 protein in orexin neurons. B, Representative images showing the expression of mCherry
3 in orexin-immunoreactive neurons in heterozygous *OF* (KI/-) mice. C, c-Fos expression
4 after i.p. administration of either saline or CNO in both *OF* (KI/-) and *OF* (KI/KI) mice. D,
5 Summary of the immunostaining data shown in C. CNO administration can significantly
6 increase neuronal activity in both heterozygous and homozygous mice (n = 3 mice per
7 group). E, Schematic showing the schedule of i.p. administration during sleep recording.
8 The *p* values were determined by a two-tailed student's *t*-test; data represent the mean ±
9 SEM.



1

2 **Figure 7:** Chemogenetic activation of orexin neurons resulted in altered
 3 sleep/wakefulness based on the availability of orexin. A, Line graph with symbols
 4 showing the time spent in wakefulness (i), REM sleep (ii), NREM sleep (iii), and
 5 cataplexy (iv) during each hour for the 6 hrs following CNO or saline

1 administration during light period. B, Bar graph showing the 2-hr average of the
2 total time spent in wakefulness (i), REM sleep (ii), NREM sleep (iii), and cataplexy
3 (iv) following CNO or saline injection during the light period. C, Scatter plot
4 showing the averaged bout and duration in wakefulness (i), REM sleep (ii),
5 NREM sleep (iii), and cataplexy (iv). The data in D-F are shown similar to the
6 representation in A-C, respectively, recorded during the dark period (*OF* (KI/-): n
7 = 9 mice and *OF* (KI/KI): n = 8 mice). Data represent the mean \pm SEM in both the
8 line and bar graph. The *p* values were determined by either two-way ANOVA
9 followed by a post-hoc Bonferroni test or paired student's *t*-test (cataplexy).



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2 **Figure 8:** CNO-induced cataplexy was not different from naïve cataplexy. A,

3 hypnogram of 2 hours after saline or CNO administration. B, typical traces

4 showing EEG signal, EEG power spectrum and EMG of cataplexy episode. C,

5 line graph (left) and bar graph (right) showing relative power spectrum of EEG

6 during cataplexy episode in the light period (n = 8 mice). Data represent the mean

7 \pm SEM in both the line and bar graph.

1

24 hours	REM	Cataplexy	NREM	Wake
Total time (min)	96.0±5.7	18.7±6.3	584.6±19.1	740.7±18.3
Duration (sec)	1429.1±129.7	550.9±140.6	1951.1±168.5	2620.7±177.5
bouts	111.4±8.6	17.7±5.4	450.4±37.4	463.6±36.4
Light period				
Total time (min)	57.3±3.8	2.1±0.8	355.1±13.5	305.5±14.6
Duration (sec)	626.1±77.4	110.0±40.5	1134.7±93.6	1068.0±70.7
bouts	74.0±7.6	2.0±0.7	239.7±17.0	235.0±17.7
Dark period				
Total time (min)	38.7±4.8	16.6±5.6	229.5±12.4	435.2±11.3
Duration (sec)	803.0±83.6	440.9±111.3	816.4±87.5	1552.7±123.7
bouts	37.4±4.3	15.7±4.9	210.7±21.3	228.6±20.4

2

3 **Table 1:** Vigilance states in *OF* (KI/KI) mice. The table shows total time spent in
4 each state in minutes, duration of state in seconds and number of episode (bouts)
5 observed in either 24 hr or in the light or dark period in *OF* (KI/KI) mice (n = 7
6 mice). Values are represented as mean ± SEM.