Altered sleep–wake characteristics and lack of arousal response to H3 receptor antagonist in histamine H1 receptor knockout mice

Zhi-Li Huang†*, Takatoshi Mochizuki‡*, Wei-Min Qu*, Zong-Yuan Hong*, Takeshi Watanabe§, Yoshihiro Urade*, and Osamu Hayashi*†

†Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Osaka 565-0874, Japan; ‡National Key Laboratory of Medical Neurobiology, Shanghai Medical College of Fudan University, Shanghai 200032, People’s Republic of China; §Department of Neurology, Beth Israel Deaconess Medical Center, Boston, MA 02115; and †Unit for Immune Surveillance Research, Research Center for Allergy and Immunology, RIKEN Institute, Tsurumi-ku, Yokohama 230-0045, Japan

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Histaminergic neurons play an important role in the regulation of sleep–wake behavior through histamine H1 receptors (H1R). Blockade of the histamine H2 receptor (H2R) is proposed to induce wakefulness by regulating the release of various wake-related transmitters, not only histamine. In the present study, we characterized sleep–wake cycles of H1R knockout (KO) mice and their arousal responses to an H3R antagonist. Under baseline conditions, H1R KO mice showed sleep–wake cycles essentially identical to those of WT mice but with fewer incidents of brief awakening (<16-s epoch), prolonged durations of non-rapid eye movement (NREM) sleep episodes, a decreased number of state transitions between NREM sleep and wakefulness, and a shorter latency for initiating NREM sleep after an i.p. injection of saline. The H3R antagonist pyrilamine mimicked these effects in WT mice. When an H3R antagonist, ciproxifan, was administered i.p., wakefulness increased in WT mice in a dose-dependent manner but did not increase at all in H1R KO mice. In vivo microdialysis revealed that the i.p. application of ciproxifan increased histamine release from the frontal cortex in both genotypes of mice. These results indicate that H1R is involved in the regulation of behavioral state transitions from NREM sleep to wakefulness and that the arousal effect of the H3R antagonist completely depends on the activation of histaminergic systems through H3R.

brief awakening | ciproxifan | microdialysis | pyrilamine | wakefulness

H histaminergic neurons reside in the tuberomammillary nucleus (TMN) in the posterior hypothalamus and send widespread projections to various brain areas (see ref. 1 for review). A growing body of evidence has implicated histamine as a crucial player in mediating wakefulness in mammals. It has been reported that histaminergic neurons discharge tonically and specifically during wakefulness (2), that the central release of histamine exhibits circadian variation associated with wakefulness (3), and that arousal is provoked by the enhancement of histaminergic transmission with thioperamide (4) or orexin (5), as well as with prostaglandin E2 or an agonist of prostaglandin E2 receptor subtype EP4 (6). In contrast, sleep is promoted by the inhibition of histidine decarboxylase (HDC), a key enzyme for histamine biosynthesis (7), by the hyperpolarization of the TMN with GABAergic agonists (8) and by the inhibition of the TMN through increased GABA release evoked by an adenosine A2A receptor agonist (9).

Three distinct subtypes of histamine receptors, H1 receptor (H1R), H2 receptor, and H3 receptor (H3R), exist in the brain and exhibit well-defined distribution patterns (10, 11). H1R and H2 receptor are postsynaptic receptors coupled to Gα11 and Gs protein, respectively, and are widely distributed in the brain. H3R is a presynaptic autoreceptor coupled to Gi/o protein, located on histaminergic and other cell somata, dendrites, and axons (varicosities), and provides negative feedback to restrict histamine synthesis and release (1). H1R also acts as an inhibitory hetero-receptor, presynaptically modulating the release of noradrenaline (12, 13), serotonin (14), dopamine (15), and acetylcholine (16, 17). H3R is proposed to play an important role in the control of sleep–wake behavior (18) by regulating various wake-related transmitters including histamine.

Clinical use of H1R antagonists promotes sleepiness (19). Slow-wave sleep is induced in cats by microinjections of the selective H1R antagonist pyrilamine into the preoptic area (20) or the dorsal pontine tegmentum (21). These results suggest that the H1R mediates the waking effect of histamine. H1R knockout (KO) mice have been generated (22) and used as a unique tool to clarify the contribution of H1R to the induction of wakefulness or the suppression of sleep induced by various stimulants. For example, we previously demonstrated that orexin induced wakefulness in WT mice but not in H1R KO mice, suggesting a functional connection of the histaminergic pathways to orexin-induced wakefulness (5). However, the characteristics of the sleep–wake behavior of H1R KO mice remain to be clarified. In this study, we obtained data showing that H1R KO mice have alterations in the regulation of state transitions from non-rapid eye movement (non-REM or NREM) sleep to wakefulness. Using the KO mice, we also demonstrate that the arousal effect of an H3R antagonist completely depends on the activation of the histaminergic system through H1R.

Results

Reduction of Brief Awakenings and Prolonged Durations of NREM Sleep Episodes in H1R KO Mice in Spontaneous Sleep–Wakefulness Cycles. Under baseline conditions, both H1R KO and WT mice exhibited a clear-cut circadian sleep–wake rhythm, with larger amounts of sleep during the light period than during the dark period, as reported in ref. 5. Time-course changes (Fig. 1A), as well as total amounts (data not shown) in the light/dark phase, of sleep and wakefulness were identical between the groups, suggesting that the loss of H1R did not affect the quantitative control of the sleep–wake cycle.

However, the KO mice exhibited fewer state transitions as compared with their WT littermates. As shown in Fig. 1B, brief awakening, defined as a period of wakefulness <16 sec during NREM sleep episodes (25), occurred in a series of NREM sleep

Conflict of interest statement: No conflicts declared.

Abbreviations: EEG, electroencephalogram; EMG, electromyogram; FrCx, frontal cortex; H1R, histamine H1 receptor; H2R, histamine H2 receptor; H3R, histamine H3 receptor; HDC, histidine decarboxylase; KO, knockout; REM, rapid eye movement; NREM, non-REM; PLSD, probable least-squares difference; TMN, tuberomammillary nucleus; VPLo, ventrolateral preoptic nucleus.

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episodes. In representative hypnograms for 90 min from the onset of the light period (Fig. 1C), NREM sleep was interrupted by numerous brief awakenings in the WT mice, whereas a notably smaller number of interruptions occurred in the H1R KO mice. To better understand the whole profile of wake behavior in the H1R KO mice, we determined the wake bout distribution as a function of bout duration (Fig. 2A). Compared with WT mice, H1R KO mice lost 40% and 50% of wake bouts <16 sec, namely, brief awakening, in light and dark periods, respectively. In contrast, H1R KO mice had a nearly normal number of the long wake bouts. Thus, the loss of brief awakening was a clear deficit in the wake behavior of the H1R KO mice.

The fewer brief awakening episodes in the H1R KO mice resulted in prolonged durations of NREM and REM sleep episodes as compared with those of the WT mice (Table 1). The number of NREM sleep episodes in the H1R KO mice during the light period was 219 ± 11, being 25% reduced as compared with the number of episodes for the WT mice. Because the total amount of NREM sleep was nearly the same between the genotypes, the mean duration of NREM sleep episodes in the H1R KO mice was prolonged by 36%. The episodes of REM sleep in the H1R KO mice were also prolonged during the light period, with episode lengthening of 31%. During the dark period, there was essentially no change in episode quantity or duration between the genotypes.

As a result, the number of state transitions from NREM to wakefulness was reduced by 36% in the light phase (Fig. 2B). Transitions from wakefulness to NREM were consequently reduced, but neither the number of transitions from NREM to REM nor that from REM to wakefulness changed in the light phase. Similar tendencies of fewer transitions from NREM to wakefulness were observed in the dark phase, but there was no statistical difference between the two genotypes. These results indicate that H1R KO mice differed from WT mice in the regulation of state transitions but only in the regulation of state transitions between NREM sleep and wakefulness and not between the total amount of sleep and wakefulness.

Right-Shift of Electroencephalogram (EEG) Power Spectra During NREM Sleep in H1R KO Mice. We determined EEG power spectra of these mice (Fig. 2C). Compared with WT mice, the H1R KO animals had relatively lower delta (0.5–1.75 Hz) activity, although the difference was not statistically significant; yet they did display a significant increase in the frequency range of 3.25–5.25 Hz and 3.75–5.0 Hz (corresponding to the upper delta through lower theta range of the EEG frequency) during the light and dark period, respectively. The peak of the power density curve was shifted to the right from 1.25 Hz in WT mice to 2.75 Hz in the KO mice during the light period and from 1.75 Hz in WT mice to 3 Hz in the KO mice during the dark period. EEG power distribution during REM sleep was unchanged in both genotypes.

Reduction in Brief Awakenings in WT Mice Treated with H1R Antagonist. To further confirm the relevance of H1R to the occurrence of brief awakening, we injected i.p. pyrilamine, a brain-penetrating, selective H1R antagonist, at a dose of 5 mg/kg of body weight into WT mice at 1000 hours during the light period.
and calculated the average number of brief awakenings over the ensuing 2-h time units. This dose of pyrilamine significantly decreased the number of brief awakening episodes by 38%, 40%, and 40% in the first, second, and third time units, respectively, after the injection as compared with that for the saline-injected controls (Fig. 3A) but did not increase the amounts of NREM and REM sleep (Fig. 3B). The number of brief awakenings returned to the same as that for the controls 6 h after the injection. We calculated the stage transition numbers for 6 h after pyrilamine administration and found that this H1R antagonist decreased the transitions from NREM sleep to wakefulness by 26% and from wakefulness to NREM sleep by 25% compared with those for the vehicle controls. Similar to the results for H1R KO mice, blockade of H1R did not influence the transition from NREM to REM sleep nor that from REM sleep to wakefulness (Fig. 3C). Pyrilamine did not further decrease the number of brief awakening in H1R KO mice (data not shown). These findings indicate that pharmacological blockade of H1R in WT mice could mimic the reduction in brief awakening and state transitions between NREM sleep and wakefulness observed in H1R KO mice and that the effective duration of pyrilamine action was 6 h if given at 5 mg/kg i.p.

**Table 1. Number and duration of sleep bouts under baseline conditions**

<table>
<thead>
<tr>
<th>Sleep</th>
<th>Mice</th>
<th>No. of bouts</th>
<th>Duration, sec</th>
<th>Difference, %</th>
<th>No. of bouts</th>
<th>Duration, sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>NREM</td>
<td>WT</td>
<td>292 ± 19</td>
<td>76 ± 6</td>
<td>—</td>
<td>131 ± 9</td>
<td>69 ± 8</td>
</tr>
<tr>
<td></td>
<td>H1R KO</td>
<td>219 ± 11**</td>
<td>103 ± 5**</td>
<td>−25</td>
<td>120 ± 12</td>
<td>85 ± 7</td>
</tr>
<tr>
<td>REM</td>
<td>WT</td>
<td>51 ± 3</td>
<td>69 ± 4</td>
<td>—</td>
<td>15 ± 1</td>
<td>67 ± 6</td>
</tr>
<tr>
<td></td>
<td>H1R KO</td>
<td>67 ± 7*</td>
<td>60 ± 4</td>
<td>+31</td>
<td>23 ± 3</td>
<td>58 ± 6</td>
</tr>
</tbody>
</table>

The % differences are with respect to WT mice. Each value represents the mean ± SEM of 10–12 mice. *(P < 0.05; **P < 0.01 vs. WT mice.)*

**Fig. 3.** Pharmacological blockade of H1R in WT mice mimics the fewer brief awakenings (<16 sec) and state transitions and shorter latency to NREM sleep after saline injection in H1R KO mice. (A) Pyrilamine (5 mg/kg, i.p.) reduces the number of brief awakenings in WT mice over 6 h. (B) This dose of pyrilamine does not increase the amounts of NREM and REM sleep. (C) The pyrilamine injection reduces the number of NREM to wakefulness (N→W) transitions. (D) H1R KO mice have a shorter NREM sleep latency than WT mice after the saline injection, and pyrilamine shortens the sleep latency in WT mice. Each value represents the mean ± SEM of six or seven mice. *(P < 0.05, compared with the vehicle injection in WT mice and as assessed by ANOVA followed by Fisher’s LSD test.)*

**Fig. 4.** Shorter Latency to NREM Sleep in H1R KO and Pyrilamine-Treated WT Mice After Stimulation by Saline Injection. The i.p. injection of saline vehicle uncovered an interesting feature of sleep onset in H1R KO mice in that they exhibited a significantly shorter NREM sleep latency, which is defined as the time from saline injection to the appearance of the first NREM sleep episode lasting for at least 20 sec. The latency to NREM sleep in the saline-injected H1R KO mice was 28 min, significantly shorter than the 40-min latency observed in WT mice. Moreover, pyrilamine administration to WT mice shortened the latency to NREM sleep by 60%, from 38 min to 13 min in the vehicle-treated group (Fig. 3D). Pyrilamine did not further shorten the latency to the NREM sleep in H1R KO mice (data not shown). The short sleep latency for both the H1R KO mice and the pyrilamine-injected WT mice clearly indicates that H1R was necessary for arousal maintenance.

**Ciproxifan Induced Wakefulness in WT Mice but Not in H1R KO Mice.** We next investigated the contribution of H1R to the arousal effects of ciproxifan, a highly potent histamine H1R antagonist (24). Ciproxifan administration at 0800 hours to WT mice at 3 mg/kg rapidly increased the wake time by 1.5- and 1.8-fold at the first and the second hour, respectively, when compared with the wake time of the vehicle control (Fig. 4A). This enhancement of wakefulness was concomitant with decreases in NREM and REM sleep (data not shown). There was no further disruption of sleep architecture during the subsequent period. However, H1R KO mice did not exhibit any significant changes in time spent in wakefulness or NREM or REM sleep within 24 h after ciproxifan had been given at 3 mg/kg, as compared with the vehicle-treated group (Fig. 4A).

The total time spent in wakefulness and NREM and REM sleep for 2 h postadministration was also determined. Ciproxifan given to WT mice at 0.3, 1, or 3 mg/kg increased the total amount of wakefulness during that 2-h period by 1.2-, 1.3-, or 1.6-fold, respectively. The increase in wakefulness was concomitant with the reduction in NREM and REM sleep. In contrast, no difference was observed in H1R KO mice in the amounts of wakefulness and NREM and REM sleep in response to vehicle versus ciproxifan administration (Fig. 4B). These results clearly indicate that H1R was essential to the arousal effect of the H3 antagonist.

**Ciproxifan Increased Histamine Release in Anesthetized WT and H1R KO Mice.** To examine the effect of ciproxifan on the release of histamine in both WT and H1R KO mice, we assayed histamine release every 20 min for 4 h after the i.p. injection of ciproxifan by using an *in vivo* microdialysis technique. In WT mice, ciproxifan dose dependently induced a significant increase in histamine release from the frontal cortex (FrCx), with a maximal elevation of 1.7-, 2.2-, and 2.5-fold over the baseline in the groups given the drug at doses of 0.3, 1, and 3 mg/kg, respectively (Fig. 5A). In H1R KO mice, ciproxifan produced similar rapid and persistent increases in histamine release (Fig. 5B). Ciproxifan at
doses of 0.3, 1, and 3 mg/kg increased the release with a maximal elevation of 1.7-, 2.5-, and 3.1-fold, respectively, over the baseline.

The calculated amount of histamine released from the FrCx for 2 h after ciproxifan injection increased in a dose-dependent manner in both WT and H1R KO mice, although the concentration of histamine recovered in the dialysates was slightly lower in the H1R KO mice than in the WT mice (Fig. 5C). These results clearly demonstrate that blockade of H3R by ciproxifan increased histamine release in both genotypes and that a constitutive deficiency of H1R did not essentially affect the histamine release evoked by ciproxifan.

Discussion

In the present study, we clearly demonstrated that H1R KO mice have fewer brief awakening episodes than WT mice, although they showed equal amounts of NREM and REM sleep (Fig. 1). This report reveals the physiological function of H1R, not in regulating the basal amount of sleep–wake but in controlling the behavioral state transition from NREM sleep to wakefulness.

Although the physiological significance of brief awakening is not understood yet, the phenomenon is considered to be a parameter of sleep fragmentation and light sleep, as first reported in prion protein KO mice (23). The prion protein KO mice exhibited an almost 2-fold increase in the number of brief awakening episodes, which was positively correlated with sleep fragmentation and light sleep and negatively correlated with the slow-wave activity. Contrary to the prion protein KO mice, the H1R KO mice showed fewer episodes of brief awakening, fewer state transitions from NREM sleep to wakefulness, prolonged durations of NREM sleep episodes, and increased EEG power density in the frequency range of 3.25–5.25 Hz during NREM sleep (Fig. 2), showing a more consolidated sleep pattern than that of WT mice. The injection of pyrilamine into WT mice also decreased the hourly number of brief awakenings and state transitions (Fig. 3). These findings strongly suggest the relevance of H1R to the manifestation of brief awakening and to accelerating the state transition from NREM sleep to wakefulness.

Total amounts of sleep and wakefulness were unchanged between H1R KO and WT mice (Fig. 1), although previous pharmacological studies demonstrated that the blockade of the histaminergic system in the CNS induced sleep. For example, the depletion of endogenous histamine by injection of 6-fluoromethylhistidine, an HDC inhibitor, decreased the amount of nocturnal wakefulness in rats by 10% (7). Microinjection of pyrilamine into the preoptic area in cats increased deep slow-wave sleep by 40% (20). These differences in the effects of the inhibition of the histaminergic system on the total amounts of sleep and wakefulness may result from the species differences among these three mammals. In the case of the H1R KO mice, the constitutive H1R deficiency may be compensated by other waking systems. In fact, the turnover rate of serotonin was increased by 20% in the cerebral cortex and hippocampus of H1R KO mice.

Fig. 4. Sleep–wake states after an i.p. injection of ciproxifan into WT and H1R KO mice. (A) Time-course changes of wakefulness in the 3 mg/kg ciproxifan treatment group, with each circle representing the hourly mean amounts of wakefulness in WT and H1R KO mice. (B) Total time spent in wakefulness and NREM and REM sleep for 2 h after an i.p. injection of ciproxifan at 0.3, 1, or 3 mg/kg in WT and H1R KO mice. Each value represents the mean ± SEM of six or seven mice. One or two asterisks indicate that results are significantly different (P < 0.05 and P < 0.01, respectively) from the vehicle-injected group, as assessed by one-way ANOVA followed by Fisher’s PLSD test.

Fig. 5. Histamine release from the FrCx of WT (A) and H1R KO (B) mice and total amount of released histamine for 2 h (C) after an i.p. injection of ciproxifan at 0.3, 1, or 3 mg/kg. Each value represents the mean ± SEM of eight or nine mice. One or two asterisks indicate results are significantly different (P < 0.05 and P < 0.01, respectively) from the control, as assessed by two-way ANOVA followed by Fisher’s PLSD test.
KO mice (25). Such chronic activation of the serotonergic waking system may compensate for the loss of H1R arousal effect in H1R KO mice under basal conditions. The sleep circadian patterns and amounts of sleep and wakefulness under baseline conditions were essentially unchanged from normal in mice gene-manipulated for orexin (26), prostaglandin D2 receptor (27), or adenosine A1 and A2A receptors (28, 29) or in HDC KO mice (30). Therefore, sleep and wakefulness may be considered to be regulated by multiple neuronal and humoral systems and to be efficiently compensated by each other.

Similar to the H1R KO mice shown in this report, HDC KO mice exhibited no major difference in the daily amount of spontaneous wakefulness and NREM sleep (30). However, HDC KO mice, lacking histamine, exhibited an increase in REM sleep, whereas H1R KO mice did not show any changes in their REM sleep. These results suggest that H1 receptor and/or H1R may be involved in the REM sleep regulation. On the other hand, the changes in the brief awakening and stage transitions from NREM sleep to wakefulness found in H1R KO mice have not been evaluated in HDC KO mice (30). Both H1R KO mice and HDC KO mice displayed a shorter latency to NREM sleep than that of WT mice when they were exposed to i.p. injection stimulation (Fig. 3D) or environmental changes (30), indicating that the histamine/H1R system plays a key role in maintaining the wakefulness state when animals are faced with such stressful situations.

Ciproxifan, an antagonist for H1 autoreceptors, induced sustained increases in cortical histamine release in both WT and H1R KO mice (Fig. 5), indicating that H1R is not involved in the cortical histamine release induced by ciproxifan. H1R is proposed to be important for autoinhibition of TMN by inhibiting histamine release and regulating the release of other neurotransmitters such as noradrenaline, serotonin, and acetylcholine (1). As shown here, ciproxifan induced wakefulness in WT mice in a dose-dependent manner but not in H1R KO mice (Fig. 4), strongly indicating that the increased histaminergic transmission by ciproxifan promoted wakefulness through H1R. These results are consistent with the findings that ciproxifan markedly enhanced cerebral histamine neuron activity in vivo and increased the level of a histamine metabolite, telemethylhistamine (24, 31), and that the arousal effect of ciproxan at a single dose of 1 mg/kg was not observed in HDC KO mice (30).

The effect of ciproxifan on the enhancement of histamine release in anesthetized mice (Fig. 5) was observed to be longer than that on the arousal duration of freely moving mice (Fig. 4). In freely moving animals, ciproxifan activates the histamine release to induce wakefulness, during which sleep substances such as adenosine (32) and prostaglandin D2 (33) accumulate in the brain to activate the sleep center in the ventrolateral preoptic nucleus (VLPO). Because the sleep-active VLPO sends inhibitory fibers to the TMN (34), the activation of VLPO leads to suppression of the histamine release to shorten the duration of wakefulness. Thus, the accumulation of sleep substances during wakefulness induces sleep, and the histamine release is decreased to lessen the wakefulness. However, under anesthesia, wakefulness was not induced; although the cortical histamine concentration was increased by ciproxifan. Therefore, sleep substances were not accumulated under anesthesia such that the high level of the histamine release was maintained for a longer period than that of wakefulness in freely moving animals.

The direct mutual inhibition between the VLPO, sleep center, and the arousal systems, including the TMN, forms a classic flip-flop switch, which produces sharp transitions in state but is relatively unstable (35). Because histamine does not inhibit VLPO neurons in vitro (36), this phenomenon should not happen if H1R acted on VLPO neurons directly (i.e., reducing the power of either side of a flip-flop switch makes the switch unstable in both states). An H1R antagonist or KO stabilizes the switch because it blocks the effect of histamine at a cortical or basal forebrain level (21, 37), where it may block arousal. This blockade, downstream from the switch, may prevent waking influences from the cortex, which ordinarily may inhibit the VLPO. The reduction in this inhibition may strengthen the switch, thus curbing its instability to decrease brief waking and consolidate NREM sleep.

In conclusion, H1R plays a critical role in the organization of sleep, specifically, the regulation of the transient state shift from NREM sleep to wakefulness and in the maintenance of wakefulness and is essential to the arousal effects of the H1R antagonist ciproxifan.

Materials and Methods

Animals. Male inbred (C57BL/6J strain) WT and H1R KO mice (22), weighing 22–26 g (11–13 weeks old), were maintained at Oriental Bioservice (Kyoto, Japan) and used in these experiments. The animals were housed in an insulated and soundproof recording room that was maintained at an ambient temperature of 22 ± 0.5°C with a relative humidity of 60 ± 2% and that was on an automatically controlled 12-h light/12-h dark cycle (light on at 0800 hours, illumination intensity ~100 lux). The animals had free access to food and water. Experimental protocols were approved by the Animal Care Committee of Osaka Bioscience Institute. Every effort was made to minimize the number of animals used and any pain and discomfort experienced by the subjects.

Polygraphic Recordings and Vigilance State Analysis. Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were chronically implanted with EEG and electromyogram (EMG) electrodes for polysomnographic recordings. The implant consisted of two stainless steel screws (1 mm in diameter) that were inserted through the skull of the cortex (anteroposterior, +1.0 mm; left-right, −1.5 mm from bregma or lambda) according to the atlas of Franklin and Paxinos (38) and that served as EEG electrodes. Two insulated stainless steel, Teflon-coated wires bilaterally placed into both trapezius muscles served as EMG electrodes. All electrodes were attached to a microconnector and fixed to the skull with dental cement.

The EEG and EMG recordings were carried out by means of a slip ring designed so that behavioral movement of the mice would not be restricted. After a 10-day recovery period, the mice were housed individually in transparent barrels and habituated to the recording cable for 3–4 days before polygraphic recording. For the study of spontaneous sleep–wakefulness cycles, each animal was recorded for 24 h beginning at 0800 hours, the onset of the light period. The animals then entered the pharmacological phase of the study, in which sleep–wakefulness parameters were recorded for 48 h. The data collected during the first 24 h also served as baseline comparison data for the second experimental day.

First, cortical EEG and EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz) and then digitized at a sampling rate of 128 Hz and recorded by using SLEEPSIGN (Kissei Comtec, Nagano, Japan) as described in refs. 27 and 29. When complete, polygraphic recordings were automatically scored offline by 4-sec epochs as wake, REM, and NREM sleep by SLEEPSIGN according to standard criteria (5, 29). As a final step, defined sleep–wake stages were examined visually and corrected if necessary.

Pharmacological Treatments. Ciproxifan, cyclopropyl 4-[(1H-imidazol-4-yl)propoxy]phenyl ketone (Bioprojet, Paris), was dissolved in sterile saline immediately before use and administered i.p. at 0800 hours on the experimental day at a dose of 0.3, 1, or 3 mg/kg. For baseline data, mice were injected i.p. with vehicle (saline) only (20 ml/kg body weight). Pyrilamine (5
mg/kg, i.p.; Sigma–Aldrich) and vehicle (saline) were injected into WT mice at 1000 hours. We used separate groups of mice for each dose.

**Microdialysis Procedure.** Because it is technically difficult to monitor histamine release in freely moving mice, the animals were anesthetized with urethane (1.8 g/kg, i.p.), and a microdialysis probe (CMA/12, membrane length of 2 mm; CMA/Microdialysis, Stockholm) was stereotaxically inserted into the FrCx (anteroposterior, 1.8 mm; left-right, 0.8 mm; depth, 2.2 mm) according to the atlas of Franklin and Paxinos (38). The probe was perfused with artificial cerebrospinal fluid at a flow rate of 2 μl/min. Ciprofloxin was diluted in saline to the required concentrations and injected i.p. Three hours after insertion of the microdialysis probes, dialysates were continuously collected from the FrCx in anesthetized mice at 20-min intervals (40 μl each) for 1 h before ciprofloxin injection and until 4 h after the administration. The dialysates were kept at −20°C until they were assayed for histamine by HPLC–fluorometry (39). Histamine output was observed to be stable after implantation of the probe. Thus, the mean value of histamine output found during the next 1 h was defined as the basal output, and the subsequent fractions were expressed as percentages of this value.

**Statistical Analysis.** All results were expressed as means ± SEM. Time-course changes in the amounts of sleep–wake and brief awakenings, as well as histograms of sleep–wake bouts, were compared between WT and KO mice by using two-way, repeated-measures ANOVA followed by the posthoc Fisher’s probable least-squares difference (PLSD) test or two-tailed Student’s t-tests. Comparisons of sleep/wake amounts in light/dark phases, number and duration of sleep/wake bouts in light/dark phases, and sleep latency between WT and KO mice were made by using a nonpaired, two-tailed Student’s t test. For the microdialysis data, two-way ANOVA followed by a posthoc Fisher’s PLSD test was used to determine whether the difference between groups was statistically significant. In all cases, P < 0.05 was taken as the level of significance.

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