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Enhanced Histaminergic Neurotransmission and Sleep-Wake Alterations, a Study in Histamine H3-Receptor Knock-Out Mice

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Long-term abolition of a brain arousal system impairs wakefulness (W), but little is known about the consequences of long-term enhancement. The brain histaminergic arousal system is under the negative control of H3-autoreceptors whose deletion results in permanent enhancement of histamine (HA) turnover. In order to determine the consequences of enhancement of the histaminergic system, we compared the cortical EEG and sleep-wake states of H3-receptor knockout (H3R−/−) and wild-type mouse littermates. We found that H3R−/− mice had rich phenotypes. On the one hand, they showed clear signs of enhanced HA neurotransmission and vigilance, i.e., a higher EEG θ power during spontaneous W and a greater extent of W or sleep restriction during behavioral tasks, including environmental change, locomotion, and motivation tests. On the other hand, during the baseline dark period, they displayed deficient W and signs of sleep deterioration, such as pronounced sleep fragmentation and reduced cortical slow activity during slow wave sleep (SWS), most likely due to a desensitization of postsynaptic histaminergic receptors as a result of constant HA release. Ciproxifan (H3-receptor inverse agonist) enhanced W in wild-type mice, but not in H3R−/− mice, indicating a functional deletion of H3-receptors, whereas triprolidine (postsynaptic H1-receptor antagonist) or α-fluoromethylhistidine (HA-synthesis inhibitor) caused a greater SWS increase in H3R−/− than in wild-type mice, consistent with enhanced HA neurotransmission. These sleep-wake characteristics and the obesity phenotypes previously reported in this animal model suggest that chronic enhancement of histaminergic neurotransmission eventually compromises the arousal system, leading to sleep-wake, behavioral, and metabolic disorders similar to those caused by voluntary sleep restriction in humans.

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INTRODUCTION

During wakefulness (W), the cerebral cortex is maintained in an activated state by a large subcortical ascending network made up of several arousal systems. It is well known that long-term abolition of an arousal system impairs wakefulness (reviewed in Moruzzi, 1972; Lin, 2000; Jones, 2005), but, curiously, little is known about the sleep-wake consequences of long-term enhancement, which can lead to insomnia or, conversely, a wake deficit

after exhausting the arousal systems. It can also be associated with voluntary sleep restriction in humans. Such sleep-wake disorders result in a deterioration of brain and body functions (Spiegel et al, 1999; 2004; Revel et al, 2009). To gain insights into wake regulation under pathophysiological conditions, it is essential to study animal models with chronic enhancement of the arousal systems.

The widespread projecting histamine (HA) neurons constitute a major wake-promoting system (reviewed in Lin, 2000; Lin et al, 2011b). Located in the tuberomammillary nucleus and adjacent areas of the posterior hypothalamus (Panula et al, 1984; Watanabe et al, 1984; Lin et al, 1986; 1993), they exhibit the most wake-selective discharge pattern so far identified in the brain (Sakai et al, 1990; Vanni-Mercier et al, 2003; Takahashi et al, 2006). HA modulates the neuronal activity of target structures through H1−, H2−, H3−, and perhaps H4-receptors. Activation of postsynaptic H1-receptors elicits cell depolarization and tonic neuronal discharge, leading to general brain arousal, while activation of postsynaptic H2-receptors increases neuronal excitability and discharge rate and therefore could promote cortical arousal (McCormick,

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1992; Brown *et al*, 2001; Haas *et al*, 2008). Finally, H3-receptors on both HA and non-HA neurons play a role as auto- and hetero-receptors (Arrang *et al*, 1983; Schwartz *et al*, 1991) and are involved in sleep-wake control through negative feedback on HA neurons (Lin *et al*, 1990; 2011a; Vanni-Mercier *et al*, 2003). Drugs impairing HA neurotransmission increase slow wave sleep (SWS), whereas those causing acute enhancement of transmission, such as H3-receptor inverse agonists, promote waking (Lin *et al*, 1988; Monti, 1993; Lin *et al*, 1996; 2008; Passani *et al*, 2004; Parmentier *et al*, 2007).

Knockout (KO) mice lacking HA synthesis, and thus HA neurotransmission, are characterized by an impaired cortical EEG, somnolence, and a wake deficit when high vigilance is required, e.g., lights-off or environmental change (Parmentier *et al*, 2002). Thus, the use of a permanently HA-deficient model has demonstrated the major role of this arousal system in maintaining the brain awake when faced with behavioral challenges.

The present study was designed to determine the consequences of permanent enhancement of HA neurotransmission using H3-receptor KO mice. In this genotype, deletion of the H3-receptor gene abolishes negative feedback on HA neurons, causing constantly high HA turnover and neurotransmission (Takahashi *et al*, 2002; Toyota *et al*, 2002). We compared the cortical EEG and sleep-wake parameters of the KO mice with those of wild-type littermates under baseline conditions and after behavioral or pharmacological tests and revealed rich and apparently 'contradictory' phenotypes providing new insights into wake regulation under conditions of chronic activation of the brain arousal systems.

MATERIALS AND METHODS

Animals and Genotype Identification

All experiments followed the European Ethics Community Directive (86/609/EEC), and every effort was made to minimize the number of animals used and any pain and discomfort. The general protocol of chronic sleep-wake recording in mice was approved by the Service of Animal Protection, *Préfecture du Rhône*, France (N° 69388200).

Histamine (HA) H3-receptor knockout (H3R KO or H3R $-/-$) mice were descendants of the mouse strain generated by Takahashi *et al.* (2002) and kept on the C57BL/6J genomic background. They were transferred to our laboratory animal facility after seven backcrosses. To ensure genetic congeniality of experimental animals used for the present study, male H3R $-/-$ mice were backcrossed with female wild-type (H3R $+/+$ or WT) mice, and the generated H3R $+/-$ mice were crossed between themselves resulting in both heterozygotes and homozygotes. Only littermate homozygote H3R $+/+$ and H3R $-/-$ mice ($n=15$ pairs) were used in this study to ensure

that any detected phenotype resulted from the deletion of H3-receptor gene rather than the genetic heterogeneity between individual animals.

At the age of 4 weeks, tail biopsies were performed and mice were genotyped using PCR with a kinase phosphoglycerate primer: WT Sens: 5′-TGT CCC CGA GCC CTG TGA GCC TGC-3′; WT Antisens: 5′-GGT CGA AGC GCC AAG CCC CAT TGC-3′; KO sens: 5′-GGA GTC CTG AGG CGC AAG TTC CCT AG-3′; KO Antisens: 5′-CCA AAG AAC GGA GCC GGT TGG CGC C-3′. The expected products were 280 bps and 361 bps for KO and WT respectively. These two sets of primers were included concomitantly and PCR was performed using 1 cycle of 15 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 62 °C, and 1 min at 72 °C, finished by one cycle at 72 °C for 10 min. The whole reaction mix was then fractionated on a 2% agarose gel, and the PCR product was visualized by ethidium bromide staining (Figure 1).

Surgery

Only male mice were used in this study. At the age of 12 weeks and with a body weight of 23–28 g, the animals were chronically implanted with four cortical electrodes (gold-plated tinned copper wire, Ø = 0.4 mm; Filotex, Draveil, France) and three muscle electrodes (fluorocarbon-coated gold-plated stainless steel wire, Ø = 0.03 mm; Cooner Wire, Chatworth, CA), under deep isoflurane anesthesia (2%, 200 ml/min in oxygen mixed with gas anesthesia machine, TEM, France), to record electroencephalograms (EEG) and electromyograms (EMG) and to monitor their sleep-wake cycles. All electrodes were previously soldered to a multi-channel electrical connector and each was separately insulated with a covering of heat-shrinkable polyolefin/polyester tubing. The cortical electrodes were inserted into the dura through two pairs of holes (Ø = 0.5 mm) made in the skull, located, respectively, in the frontal (1 mm lateral and anterior to the bregma) and parietal (1 mm lateral to the midline at the midpoint between the bregma and lambda) cortices. The EMG electrodes were inserted into the neck muscles. Finally, the electrode assembly was anchored and fixed to the skull with Super-Bond (Sun Medical Co., Shiga, Japan) and dental cement. This implantation allowed stable polygraphic recordings to be made for more than 5 months.

Polygraphic Recording and Data Acquisition and Analysis

After surgery, the animals were housed individually in transparent barrels (Ø 20 cm, height 30 cm) in an insulated sound-proof recording room maintained at an ambient temperature of 22 ± 1 °C and on a 12 h light/dark cycle (lights on at 7 a.m.), food and water was available *ad libitum*. After a recovery period of one week, the mice were
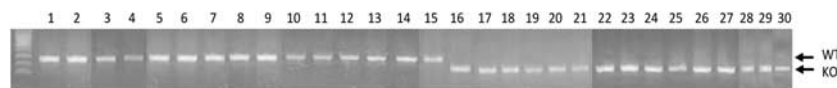
Figure 1 PCR genotyping of mouse tail DNA showing the presence of H3-receptor gene (361 bps) in the wild type (WT) mice (animals 1–15) and its absence (replaced by a 280 bps allele) in their knockout (KO) littermates (animals 16–30).

habituated to the recording cable for one more week before starting polygraphic recordings.

Cortical EEG (ipsilateral frontoparietal leads) and EMG signals were amplified, digitized with a resolution of 256 and 128 Hz, respectively, and computed on a CED 1401 Plus (Cambridge Electronic Design, UK). Using Spike2 (Cambridge Electronic Design) script and with the assistance of spectral analysis using the fast Fourier transform, polygraphic records were visually scored by 30-sec epochs for wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) according to previously described criteria validated for mice (Valatx, 1971; Valatx and Bugat, 1974; Parmentier et al, 2002; Anaclet et al, 2009). In order to evaluate the contrast of sleep-wake states between light and dark phases and as a criterion of sleep-wake circadian rhythms, we used the light/dark ratio for sleep stages (dark/light for W), obtained by 8 h sleep-wake amounts before lights-off, divided by those after lights-off.

Cortical EEG power spectrum analysis was performed on recordings during the dark phase period between 7 and 10 p.m. and during the light phase between 11 and 2 p.m. in baseline conditions. To avoid any variation caused by the positioning of the cortical electrodes, the cortical EEG used for power spectral density analysis was captured from frontoparietal leads and set with reference to the bregma, lambda, and midline in all mice. EEG power spectra were computed for consecutive 4-sec epochs within the frequency range of 0.5–60 Hz using a fast Fourier transform routine. The data were collapsed in 0.5 Hz bins. On the basis of visual and spectral analysis, epochs containing artefacts occurring during active waking (with large movements) were visually identified and omitted from the spectral analysis when the threshold value in the 0–1 Hz band was exceeded. The power densities obtained for each state were summed over the frequency band of 0.5–60 Hz (total power). To standardize the data, all power spectral densities at the different frequency ranges, i.e., 0.5–3.5 Hz (δ); 3.5–5.5 Hz (low θ); 5.5–9 (fast θ); 9–20 Hz (spindle or α); and 20–60 Hz (β and γ), were expressed as a percentage relative to the total power (eg, power in the δ range/power in the 0.5–60 Hz range) of the same epochs. To evaluate contrast in the cortical EEG between SWS and W or PS, we used an EEG power ratio determined by the averaged cortical EEG total power density during SWS divided by that during either W or PS.

Experimental Procedures

In each experiment, recordings were simultaneously made from an equal number (usually in batches of three) of H3R −/− and H3R +/+ mice. The mice were submitted to the following experimental procedures.

Spontaneous Cortical EEG and Sleep-Wake Cycle

After recovery from surgery and habituation to the recording cables, each mouse (15 pairs) was subjected to a recording session of two continuous days, beginning at 7 a.m. During these periods, the animals were left undisturbed to obtain baseline parameters on the cortical EEG, sleep-wake cycle and circadian rhythm. The data from the two sets of mice were then compared.

Sleep-Wake Cycle After Behavioral Tests

Three tests were used to characterize the behavioral phenotypes of WT and H3R −/− littermates. Sleep-wake recordings were maintained during and 12 h after the tests, which were performed in a random sequence:

1) A new environment, with mice transferred for 4 h from their habitual transparent barrel to an opaque rectangular box (21 × 30 cm, height 20 cm, with open field). In this test, the ability of the two genotypes to remain awake after this environmental change was tested. Each mouse was subjected to this test four times separated by an interval of 14–20 days, twice at 2 p.m. when the animals would normally be sleeping for ∼80% of the time (defined as 'sleep period'; n = 22 from 11 pairs of mice), and twice at 6 p.m. when they would normally be awake a majority of the time ('awake period'; n = 18 from nine pairs of mice).

2) A locomotion (wheel) test, which is a motor challenge to study the ability of mice to enhance W and cortical activation through locomotion. This test consisted of placing mice, for 4 h, on a wheel (Ø = 13 cm, 5.5 cm wide) which was composed of two circles, connected by regular-spaced bars (length, 5.5 cm; distance between bars, 9 mm). The circles and bars were made up of stainless steel wires (Ø = 1.5 mm). The hub of the wheel was screwed to a transparent Plexiglas box (10 cm long, 7 cm wide, 28 cm high) which served to support the wheel and to prevent mice from getting off the wheel. The turning resistance of the wheel was set in such a way that the mice were able, after a few seconds of habituation, to either stay immobilized and fall asleep or to run over the regularly spaced bars to turn it and as a result to remain awake. This locomotion test was essentially voluntary even though the mice had to stay on the wheel during the test. The wheels were equipped with a magnetic detector, allowing quantification and computerization of the wheel turn number and time spent turning every 30 s by using a homemade data acquisition system. For the test, mice were placed on the wheel, which together with the plexiglas box was placed in each mouse's home barrel to keep them in their familiar environment. The mice stayed on the wheel for 4 h, with food and water available ad libitum. Each mouse was subjected to this test twice separated by an interval of 5–15 days, at 2 p.m. (n = 24 from 12 pairs of mice) and at 6 p.m. (n = 26 from 13 pairs of mice). Sleep-wake stages during their stay in the new environment or on the wheel were compared with the baseline recordings for the same group and between genotypes.

3) A test of motivation using palatable food, which consisted of introducing a piece of hard nougat and caramel and a grain of corn, suspended at a height difficult to reach for mice. The ability of the two genotypes to remain awake and catch the tasty food allowed the evaluation of wakefulness driven by motivation. All WT and KO mice were habituated two times to the test in order to avoid neophobic behavior and to determine their interest in the food. The food was then placed at the bottom of their barrel. Only mice which immediately tried to eat the food and consumed it within 2 h were included in the final test. This was the case for the great majority of the mice.

Each mouse was subjected to this test twice, separated by an interval of 14–20 days, twice at 2 p.m. and twice at 7 p.m.

($n = 16$ from 8 pairs of mice). The control of this test consisted of sleep-wake recordings after applying the system without food. Sleep-wake stages during the presence of tasty food were compared with those during control of the same group and between the genotypes.

Cortical EEG and the Sleep-Wake Cycle After Pharmacological Administration

To compare the effects of drugs acting on the histaminergic system, the two mouse genotypes received intraperitoneal injections of the following agents with an interval of at least 7 days between injections, with subsequent recordings for 12 or 24 h. Drug doses were expressed as compound (salt) weight. They were dissolved in 0.1–0.3 ml of saline, fresh before each administration. Mice were injected either with saline alone or containing 1) cyclopropyl-(4-(3-(1 H-imidazol-4-yl) propyloxy) phenyl) ketone (ciproxifan; 1 mg/kg; Bioprojet, Paris, France; $n = 10$), a potent HA H3-receptor inverse agonist/antagonist (Ligneau *et al*, 1998); administrations were performed at 10 a.m. when the mice were normally sleeping most of the time ('sleep period') to detect an awakening effect; 2) triprolidine (5 mg/kg; ICN, Ohio, USA; $n = 14$), a HA H1-receptor antagonist (Yanai *et al*, 1990; McCormick and Williamson, 1991); injections were given at 8 p.m. when the mice would normally be awake most of the time ('awake period') to detect a sedative effect; and 3) (S)-α-fluoromethylhistidine (α-FMH; 50 mg/kg; Merck, Sharp & Dohme; $n = 18$), a specific inhibitor of histidine decarboxylase, the HA-synthesizing enzyme. Injections were given at 4 p.m., in order to reach a significant and important decrease in brain histamine at lights-off (7 p.m.) and in view of the latency of drug action shown previously by Garbarg *et al*, (1980) and Maeyama *et al*. (1982).

Statistical Analysis

All experimental data was firstly tested for their value distribution using Shapiro-Wilk normality test. When the data was non-normally distributed, such as that of body weight and cortical EEG, nonparametric Mann Whitney tests were used. Two-way ANOVA for repeated measures and unpaired Student's *t*-test were used to evaluate the differences between genotypes when the data was normally distributed. This concerned all comparisons of baseline sleep-wake quantitative data between the WT and KO mice. Regarding the comparison between control data (baseline recordings or saline injection) and those after behavioral challenges or pharmacological tests, we applied split plot two-way ANOVA for repeated measures using genotype (H3R +/+ *vs* H3R −/−) and experimental condition (control *vs* treatment) as factors. These tests were followed, if the p values were significant, by *post hoc* Bonferroni test, with individual animals served as their own control. The impact of genotype on the behavioral or pharmacological effects was further evaluated by direct comparisons of the net sleep-wake changes between the two genotypes using unpaired Student's *t*-test. All data are expressed as the mean ± SEM. All tests used were two-tailed with a significance level of alpha set at 0.05.

RESULTS

Genotyping and General Observations

All wild-type (WT or H3R +/+) mice displayed a strong PCR signal, corresponding to a 361 base pair band (animals 1–15), whereas all H3-receptor gene disrupted (H3R KO or H3R −/−) littermates showed a signal corresponding to a 280 base pair band (animals 16–30), indicating the deletion of the histamine (HA) H3R gene (Figure 1).

Like WT mice, H3R −/− mice appeared to develop normally. No obvious abnormalities were detected in general morphology, fertility, spontaneous movement or other behavior under baseline conditions. The number of littermates appeared normal (6–7 per litter). At post-natal 70 ± 7 days and before surgical implantation, body weight was similar (25.9 ± 0.5 g *vs* 24.5 ± 0.3 g in H3R +/+ mice; $n = 15$ for each genotype, $p = 0.1198$ nonparametric Mann Whitney test). Nevertheless, a difference in body weight appeared with age, the respective weights at 36 weeks were 31.6 ± 0.8 g in H3R −/− mice *vs* 29.4 ± 0.4 g in WT mice ($n = 15$ for each genotype, $p = 0.0250$ nonparametric Mann Whitney test). This body weight difference between genotypes became more noticeable over 45 weeks.

Baseline Sleep-Wake Cycle and Cortical EEG in H3R −/− Mice

In baseline conditions, the H3R KO mice exhibited a circadian sleep-wake rhythm quite characteristic of C57BL/6 J mice, with a larger amount of W during the dark than during the light period (Figure 2a–c). Their sleep-wake amount appeared quite similar to that of the WT counterpart during the light phase, even though there was a tendency for less PS. Immediately following lights-off, however, the KO mice showed major differences in sleep-wake. A detailed analysis revealed several specific phenotypes:

A significant decrease in W after lights-off, during darkness and over 24 h. While there was no significant difference between genotypes in terms of total amount of W, SWS and PS during the light phase (Figures 2b and c), the hourly analysis revealed a sharp and significant decrease in W immediately after lights-off [(F(df1 = 1; df2 = 23) = 2.44, $P = 0.0002$, two way ANOVA for repeated measures followed by *post hoc* Bonferroni tests between genotypes) This effect lasted for more than 4 h (between 19 and 23 h: 153 ± 3 *vs* 184 ± 5 min in H3R +/+, $p = 0.0083$, unpaired Student's *t*-test, Figure 2b, *boxed areas*) and was accompanied by a simultaneous and significant increase in SWS and PS. These effects also led to a significant decrease in W and an increase in SWS during darkness and over 24 h. No significant change was found, however, for PS during the same periods (Figure 2b and c).

A reduced dark/light ratio for W and light/dark ratio for all sleep stages. The above sleep-wake changes during darkness also resulted in reduced light/dark (or dark/light for W) ratios for all sleep-wake states as compared with those of WT littermates (Figure 2b), indicating disturbed circadian sleep-wake rhythms in the KO mice.
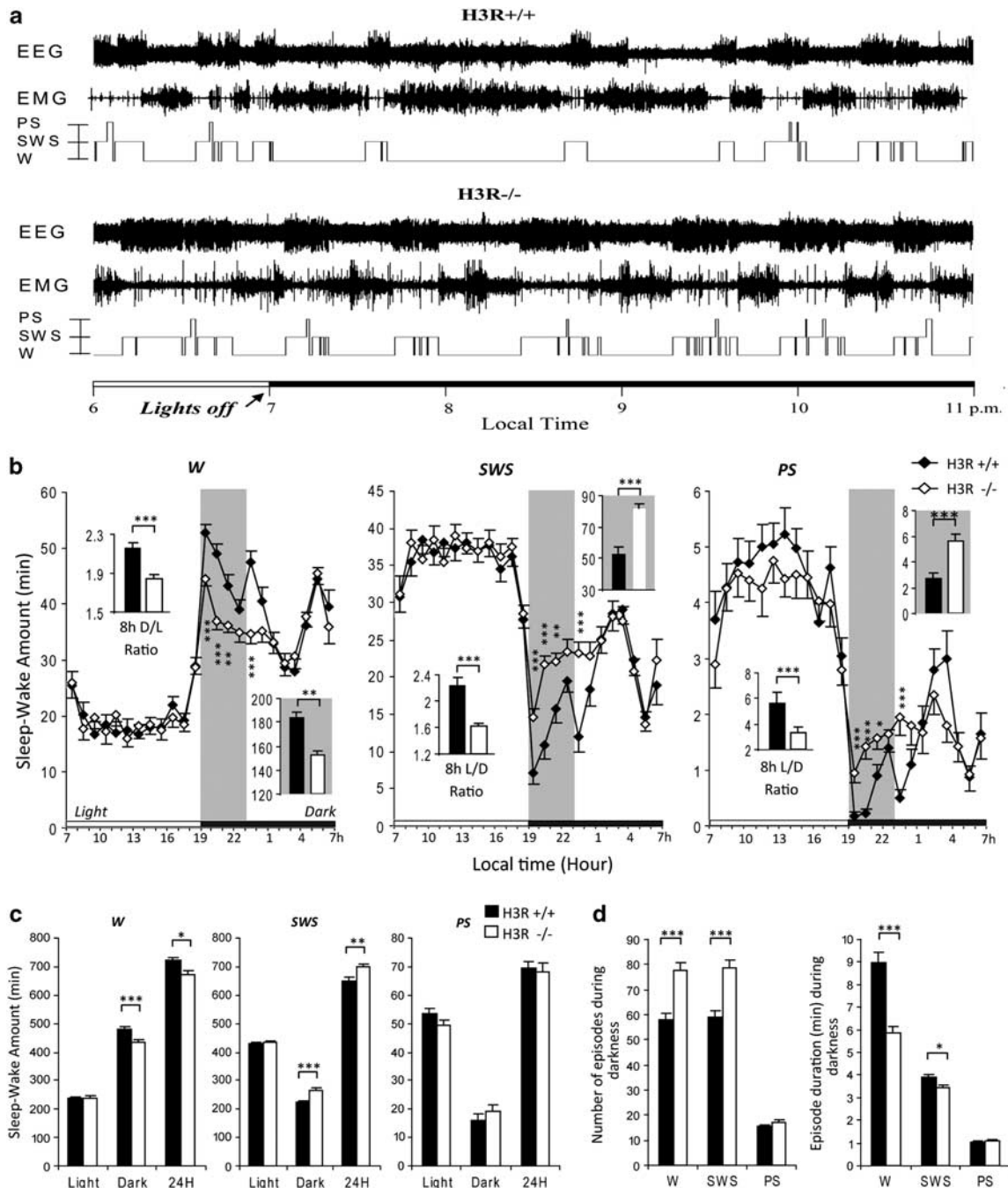
Figure 2 Comparison of spontaneous sleep-wake parameters in H3-receptor (H3R) +/+ and -/- littermates. H3R +/+, filled symbols and columns; H3R -/-, unfilled symbols and columns. a, Examples of polygraphic recordings and corresponding hypnograms showing the spontaneous sleep-wake cycle before and after lights-off. b, mean hourly values (± SEM in min) of the sleep-wake states. The gray areas correspond to the period between 7 and 11 p.m., and the total values for each sleep-wake state during this period are indicated in the bars with background situated in the right side of each figure. The left side non-background inserted histograms correspond to the light/dark or dark/light (L/D or D/L) ratio of sleep-wake amount before and after lights-off. They were obtained by 8 h sleep-wake amounts before lights off, divided by those after lights-off or verse versa. c, Means ± SEM (in min) of the sleep-wake stages for the 12 h light and dark and 24 h periods. d, mean values ± SEM of episode number and duration of each sleep-wake state during the darkness. Note that, compared with H3R +/+ mice, H3R -/- mice exhibited 1) a deficit of wakefulness (W) immediately after the lights-off (a), during darkness (a, b) and over 24 h (c) at the expense of slow wave sleep (SWS); 2) a pronounced sleep fragmentation during the darkness (a, d). Other abbreviations: PS, Paradoxical Sleep; EEG, electroencephalogram; EMG, electromyogram. n = 30, corresponding to 2 × 24 h recordings for 15 animals of each genotype. $^{\circ}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two way ANOVA for repeated measures (b except for the bar graphics) and unpaired Student's t-test (c, d and bar graphics in b, two-tails).

A pronounced sleep fragmentation during darkness. Like the unchanged total sleep-wake amounts during the light phase, the number of episodes and their duration of each sleep-wake state during this period remained unmodified in H3R -/- mice. In sharp contrast, they developed a pronounced sleep fragmentation immediately
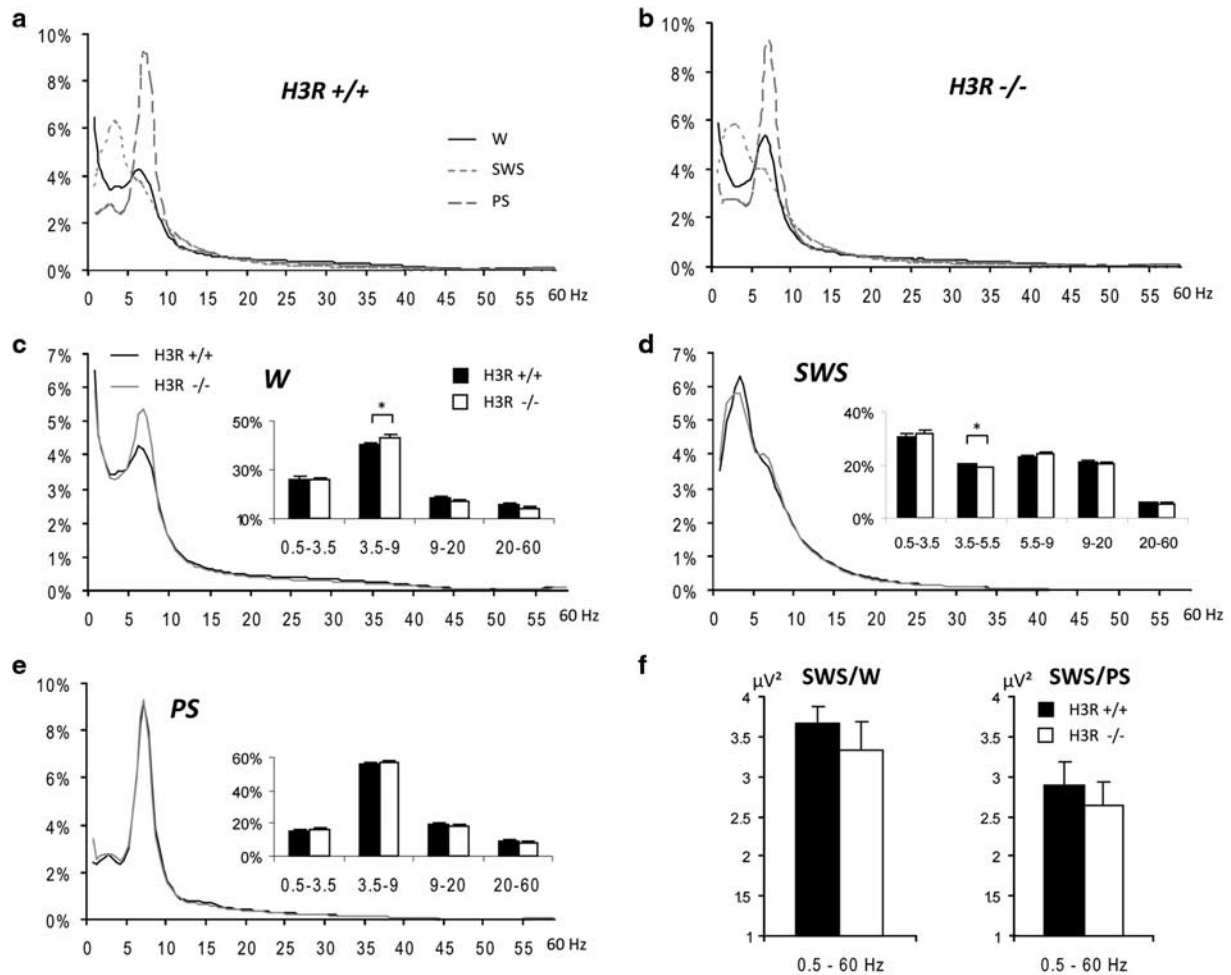
Figure 3 Mean spectral distribution of cortical EEG power density in baseline sleep-wake states in H3R +/+ and H3R −/− mice. The data were obtained from 16 samples of 8 pairs of littermates by pooling consecutive 4 s epochs during the period of 7–10 p.m. (darkness) using the fast Fourier transform routine within the frequency range of 0.5–60 Hz. a–b, Mean percentage power values in each 0.4 Hz frequency bin. Note the state-dependent profiles of cortical EEG spectra across wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) in H3R +/+ (a) and H3R −/− (b) mice. c–e, Mean percentage of cortical EEG power spectrum density (curves) and cortical EEG power band density (bars) in H3R +/+ (black line or filled bars) and H3R −/− (gray line or unfilled bars) mice. Note that the H3 −/− mice show an increase in theta band (3.5–9 Hz) during W and a decrease in slow activity in 3.5–5.5 Hz range during SWS. f, Cortical EEG SWS/W and SWS/PS power ratio (0.5–60 Hz) in H3R +/+ (filled bars) and H3R −/− (unfilled bars) mice. *p < 0.05, nonparametric Mann Whitney test (two tails).

after lights-off, manifested as, for both W and SWS, a marked increase in the episode number and a decrease in the episode duration during the whole dark phase, e.g., the mean episode number and duration of W were $78 \pm 3 \times 6 \pm 0.3$ min in the KO mice vs $58 \pm 3 \times 9 \pm 0.5$ min in WT mice ($P < 0.001$, unpaired Student's t-test, Figure 2d). The duration and number of PS episodes remained unchanged during the same period.

An increase in θ rhythm during W and a decrease in slow activity during SWS after lights-off. From the fronto-parietal leads, the cortical EEG of both sets of animals manifested changes across the behavioral states and signs characteristic of C57BL6J mice, i.e., with dominant presence of θ frequencies, notably during PS and W (Figure 3a). When the cortical EEG power spectrum analysis was performed at 11 to 2 p.m. (Sleep period), no difference was found between genotypes ($n = 8$ pairs of

mice). Firstly, the distribution of their mean cortical EEG power spectral density was not significantly modified during either W, SWS or PS. The morphology of their EEG spectra was virtually the same as that of their WT littermates. Secondly, analysis of the mean power density in each cortical frequency band during W, SWS and PS revealed no significant difference between the WT and H3R −/− mice. Finally, their cortical EEG power ratio between SWS and or PS was unchanged: EEG SWS/W: 3.9 ± 0.3 in KO vs 3.4 ± 0.1 in WT, $p = 0.100$; EEG SWS/PS: 2.8 ± 0.2 in WT vs 3.1 ± 0.3 in KO, $p = 0.390$, $n = 8$ pairs of animals in both cases using nonparametric Mann Whitney test.

In contrast, after lights-off (between 7 and 10 p.m.) and simultaneously with the quantitative changes, H3R −/− mice showed several cortical EEG phenotypes compared to their WT littermates: i) an increase in the power spectral density in the θ rhythm during W. This increase, limited to the 3–9 Hz frequency range, was seen only during W

(Figure 3c). ii) a decrease in the power spectral density of slow activity during SWS. This decrease, limited to the frequency range of 3.5–5.5 Hz, was seen only during SWS (Figure 3d). iii) the power spectral density in the θ rhythm during PS remained identical between the genotypes (Figure 3e). There was no change in the other bands of the cortical EEG during any of the behavioral states. The cortical EEG SWS/W or SWS/PS power ratio tended to decrease in the H3R −/− mice owing to the increased θ power during W and its decrease during SWS. These changes were, however, not statistically significant with the number of samples used (Figure 3f) ($n = 8$ pairs of mice, nonparametric Mann Whitney test).

All the above phenotypes were identified during darkness. Such a clear association with darkness is also seen with other models affecting HA (Parmentier et al, 2002, Haas et al, 2008). Although the mechanisms involved remain to be determined, HA is most likely linked with circadian rhythm control and brain clock gene expressions (Abe et al, 2004; Hong et al, 2006).

Effects of Behavioral Challenges on Sleep-Wake Parameters

A new environment. When placed in a new cage, both genotypes appeared to be interested in the new environment, as manifested by a large amount of spontaneous locomotion and exploratory behavior, such as ambulation and rearing. When tested between 2–6 p.m., both of them responded to the new environment with an extended W associated with a significant decrease in SWS and PS, as compared with their own baseline values. However, no significant difference was found between genotypes when their net sleep-wake changes were compared (data nonshown). For W, for example, $+62 \pm 5$ in WT *vs* $+52 \pm 4$ min in KO, $n = 22$ from 11 pairs of mice [$F(1,40) = 2.358$, $p = 0.13$ using a two way ANOVA followed by *post hoc* Bonferroni tests using Genotype and Experimental condition as factors].

Conversely, when placed in a new cage between 6–10 p.m., both WT and H3R −/− mice responded to the environmental change with significant sleep-wake changes and with greater effects seen in H3R −/− mice (Figures 4a and b) as attested by two way ANOVA followed by *post hoc* Bonferroni tests using Genotype and Experimental condition as factors. Indeed, there are significant interactions between the two factors: $F(1, 40) = 5.284$, 4.431 and 8.137 and $p = 0.027$, 0.042 and 0.007, respectively for W, SWS and PS. Bonferroni tests confirmed significant sleep-wake changes within the genotype in the new environment (Figure 4b). Comparisons of the net sleep-wake changes between phenotypes using unpaired Student's *t*-tests also confirmed significantly greater effects in the KO than in WT mice. Indeed, their increase in W was 40% superior to that of WT mice, or $+73 \pm 5$ *vs* $+52 \pm 8$ min ($n = 22$ from 11 pairs of mice, $p = 0.027$, Figure 4c). Their decrease in SWS or PS during the 4 h stay is also significantly greater than that of the WT littermates (Figures 4b and c).

A locomotion (wheel) test. The transfer of the mice to the wheel during 4 h elicited, at any of the tested times, a great amount of running activity in both genotypes even though they had the possibility to find a position to stay immobilized and fall asleep. During the 2–6 p.m. test, this running activity was accompanied by a significant increase in W and decrease in SWS and PS in both genotypes as compared respectively with their own baseline amount. No significant differences between genotypes were found, however, in terms of their net motor and sleep-wake changes. For W, for example, $+88 \pm 10$ in WT *vs* $+70 \pm 9$ min in KO, $n = 26$ from 13 pairs of mice, [$F(1, 48) = 1.927$, $p = 0.17$ using two way ANOVA followed by *post hoc* Bonferroni tests using Genotype and Experimental condition as factors].

During the 6–10 p.m. test, the running activity of the mice was also accompanied by significant sleep-wake changes in both genotypes with, however, greater effects seen in H3R −/− than in WT mice (Figures 5a and c), as demonstrated firstly by two way ANOVA showing significant interactions between the Genotype and Experimental condition, for W, for example, $F(1, 48) = 4.541$, $p = 0.038$. Significant increase in W and decrease in SWS were evidenced using *post hoc* Bonferroni tests within genotype (Figure 5c) and greater sleep-wake changes were further confirmed by direct comparison of the net sleep-wake changes between genotypes showing a greater extension of W in the KO mice (ie, 39% more, or $+68 \pm 6$ min *vs* $+49 \pm 7$ min in WT mice, $n = 26$ from 13 pairs of mice, $p = 0.038$, unpaired Student's *t*-test), associated with a larger decrease in SWS and PS (Figure 5d). In H3R −/− mice, interestingly, whatever their wake amount, either inferior (2–6 p.m. test, 144 ± 8 in KO *vs* 162 ± 9 min in WT) or superior (6–10 p.m. test, Figure 5c) to that of the WT littermates, their wheel turning remained at a lower level than that of the WT mice (see example in Figure 5b), in line with an overall decrease in locomotion or activity, identified previously in H3R KO mice (Toyota et al, 2002; Nuutinen et al, 2011). This dissociation between vigilance state and locomotion indicates that the extended W seen in the KO mice is not due to an enhanced locomotion.

Test of motivation-driven wakefulness. Placement of palatable food (a piece of nougat and caramel and a grain of corn, suspended at a height almost out of reach) in the mouse barrel, elicited, in either genotype, a behavioral activation, manifested as an increased locomotion and numerous attempts to climb, catch and eat the food. The H3R −/− mice appeared to be more active than the WT littermates as seen from our EMG recordings.

During the 2–6 p.m. test, i.e., within the sleep period, the presence of palatable food resulted in a significant increase in W over the whole 4 h period in the two genotypes, mainly at the expense of SWS (Figure 6a and b). There are significant interactions between Genotype and Experimental condition for all the sleep-wake states. For W, for example, $F(1, 30) = 12.81$, $p = 0.001$ (two way ANOVA). *Post hoc* Bonferroni tests confirmed significant increase in W and decrease in SWS within genotypes (Figure 6b). The increase in W driven by the motivation test was greater in H3R −/− than in H3R +/+ mice, associated with a more pronounced decrease in SWS and PS (Figure 6b), observations further confirmed by comparisons of the net sleep-
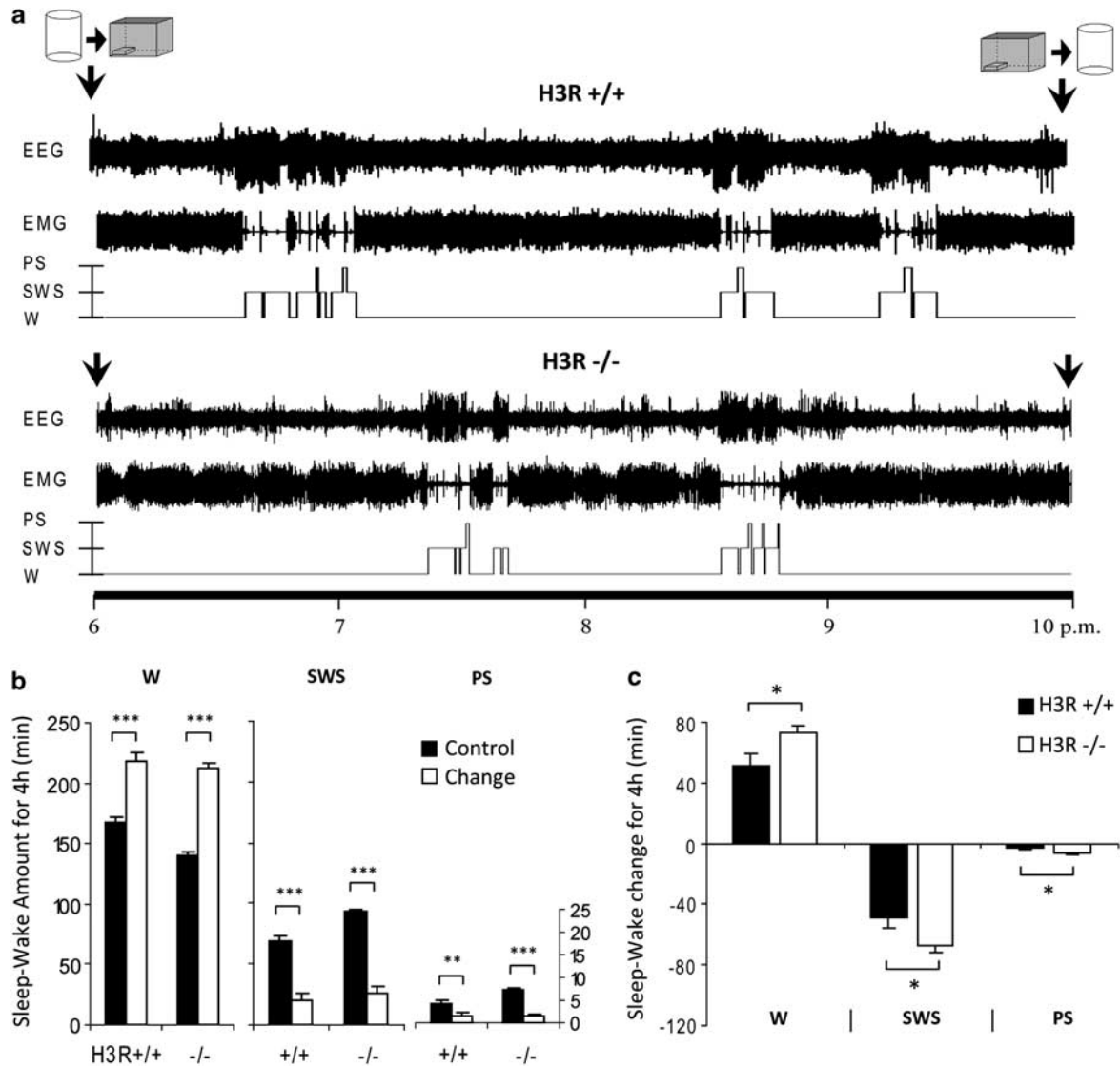
Figure 4 Effects of an environmental change on the sleep-wake states in H3R +/+ and H3R −/− mice. a, examples of polygraphic recording and corresponding hypnograms showing the effects of an environmental change (6–10 p.m.) on cortical EEG, EMG and sleep-wake cycle. b, mean values (±SEM in min) of each sleep-wake stage during the 4 h stay in the new environment, compared with the own baseline values of each genotype. c, mean net sleep-wake changes (±SEM) during the test, relative to the baseline values of each genotype. Note 1) a significant increase in waking (W) and decrease in slow wave sleep (SWS) and paradoxical sleep (PS) in both genotypes compared with their own baseline values (b); 2) a larger increase in W and greater decrease in SWS and in PS in H3R −/− than in H3R +/+ mice (c). n = 22 from 11 pairs of animals, °p > 0.05, *p < 0.05; **p < 0.01; ***p < 0.001, post hoc Bonferroni test after significance in two-way ANOVA for repeated measures for b and unpaired Student's t-test for c (two-tails).

wake changes between the two genotypes (Figure 6c). Thus, the net increase in W during 4 h was 40 ± 4 min in the KO mice vs 16 ± 6 min in the WT mice (n = 16 from 8 pairs of mice, p = 0.0012, Student's t-test), i.e., > 2.5 fold the increase seen with WT mice. The decrease in SWS in KO mice was also > 2 fold that of WT mice (Figure 6c). Finally, this behavioral test had no effect on PS in WT mice, but caused a significant decrease in PS in the KO mice, indicating again a greater impact of the motivation test on H3R −/− than on WT mice (Figures 6b and c).

When tested between 6–10 p.m., during the awake period, both mouse genotypes responded actively to the presence of tasty food with increased W and decreased SWS and PS. The

elicited sleep-wake changes were, however, not significantly different between genotypes. For W, for example, +17 ± 7 in WT vs +14 ± 8 min in KO, n = 16 from 8 pairs of mice [F(1, 30) = 0.083, p = 0.77 using two way ANOVA followed by post hoc Bonferroni tests using Genotype and Experimental condition as factors].

The extended wakefulness exhibited during the three behavioral tasks in H3 −/− mice is also qualified here as sleep restriction, distinct from sleep deprivation in which sleep is not allowed but similar to a voluntary sleep restriction such as that driven by personal, professional or social motivations in humans (Spiegel et al, 1999, 2004).
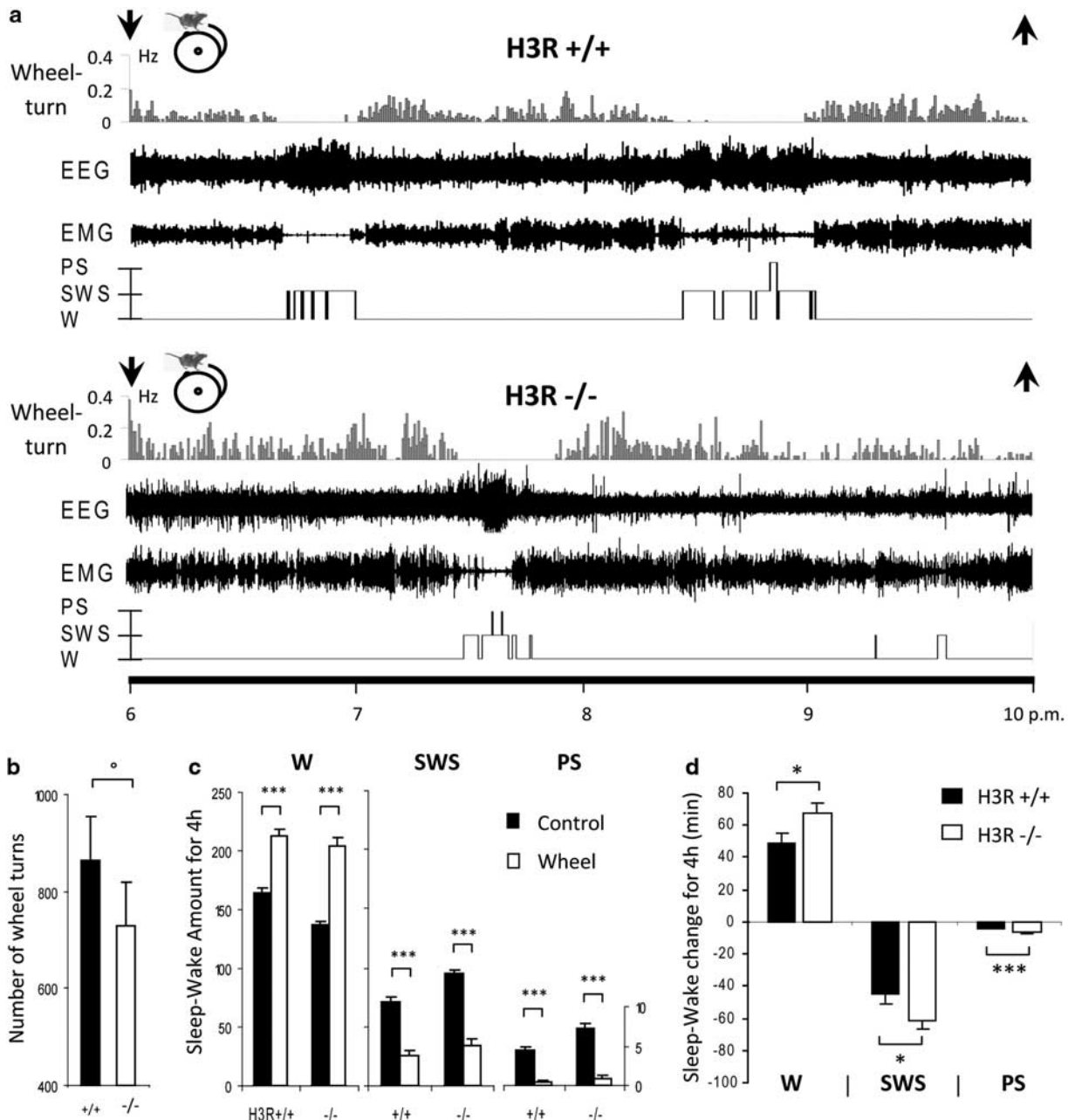
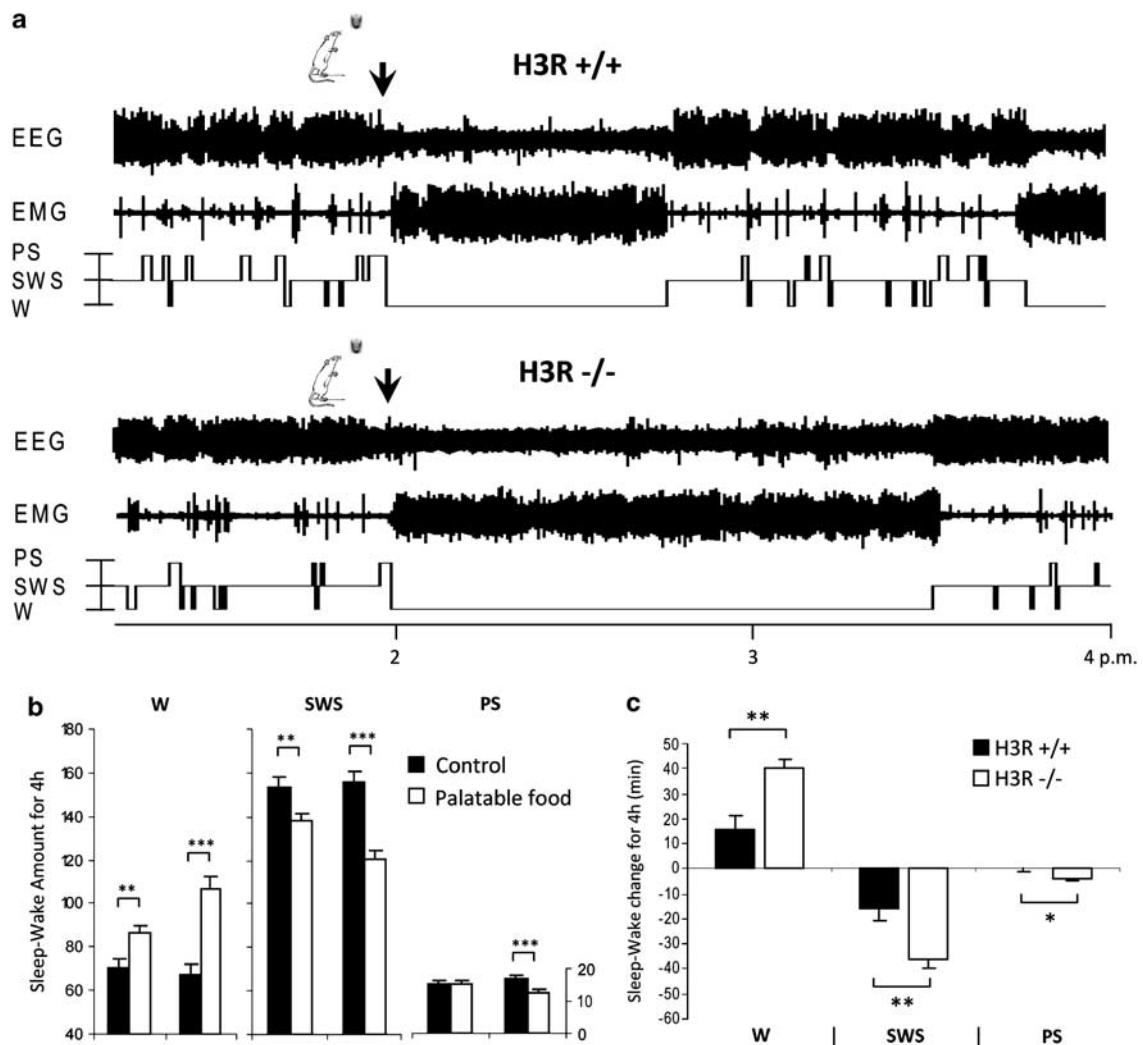**Figure 5** Effects of a wheel test on the sleep-wake states in H3R +/+ and H3R −/− mice. a, Examples of polygraphic recording and corresponding hypnograms illustrating the effects of the wheel test (6–10 p.m.) on cortical EEG, EMG and sleep-wake cycle. b, effect of the wheel test on the locomotion (number of wheel turns). c, quantitative variations of the sleep-wake states. Mean values (± SEM in min) of each sleep-wake stage during their 4 h stay on the wheel, compared with the own baseline values of each genotype. d, mean net sleep-wake changes (± SEM) during the test, relative to the baseline values of each genotype. Note 1) non significant difference between genotypes in terms of locomotion on the wheels (b); 2) a significant increase in waking (W) and decrease in slow wave sleep (SWS) and paradoxical sleep (PS) in each genotype compared with its own baseline values (c); 3) a greater increase in W and larger decrease in SWS and PS in H3R −/− than in H3R +/+ mice (d), n = 26 from 13 pairs of animals. °p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, post hoc Bonferroni test after significance in two-way ANOVA for repeated measures for c and unpaired Student's t-test for b and d (two-tails).

Drug Actions on the Sleep-Wake Cycle

Ciproxifan. Two way ANOVA shows a significant interaction between Genotype and Experimental condition (ie, saline vs ciproxifan) for W and SWS during 4 h after intraperitoneal injection of ciproxifan (1 mg/kg, n = 10, at 10 a.m. during the light phase), a potent H3-receptor inverse

agonist/antagonist (Ligneau et al, 1998): e.g., F(1, 18) = 10.67; p = 0.004 for W. In H3R +/+ mice, ciproxifan caused a delayed appearance of SWS (65 ± 10 min instead of 19 ± 5 min seen with vehicle injection in the same animal, the same as below, p = 0.012) and PS (115 ± 15 min instead of 39 ± 6 min, p = 0.0009, paired t-tests) leading to a significant increase in time spent in W (126 ± 11 min
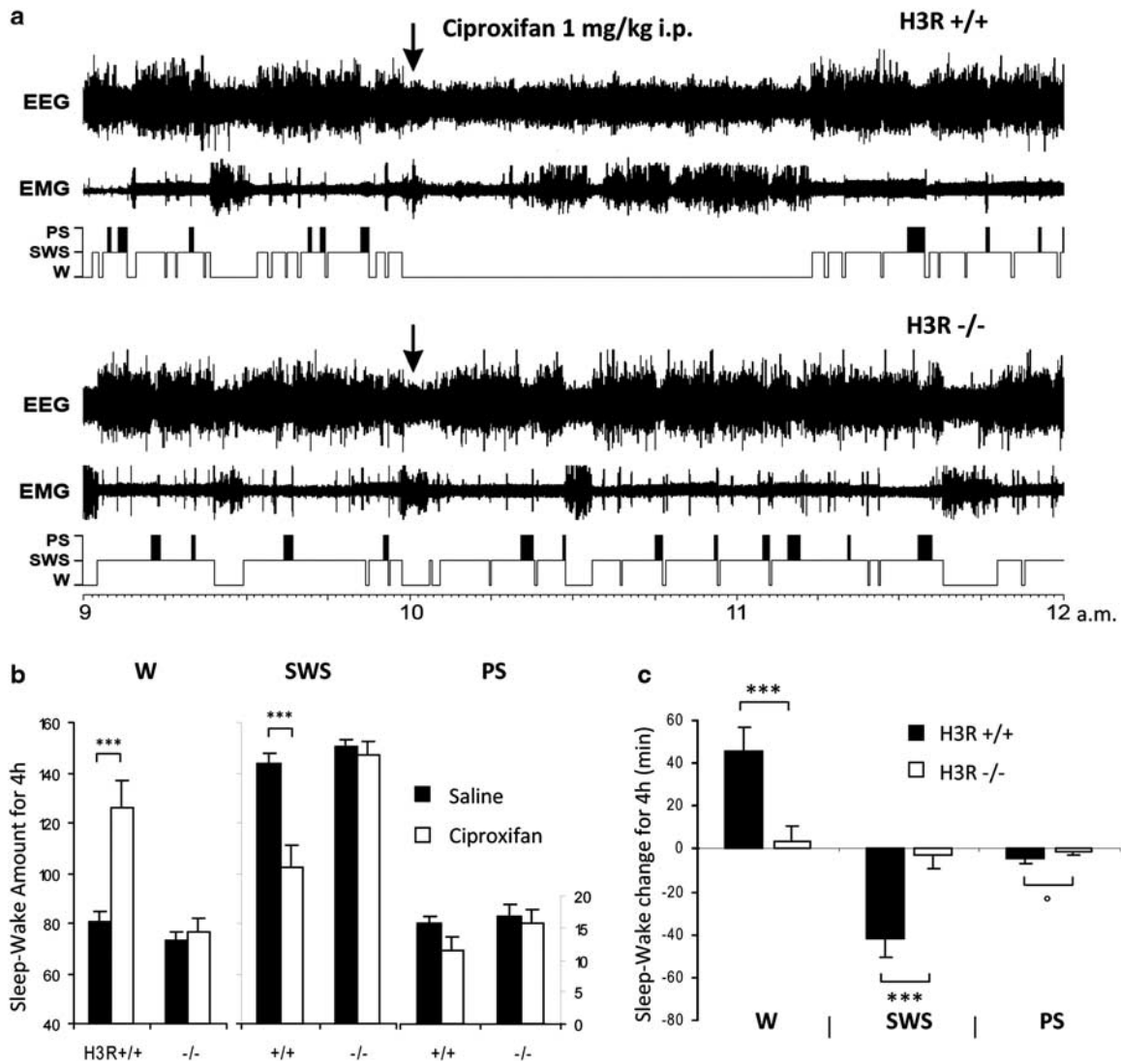
Figure 6 Effects of a motivation test using palatable food on the sleep-wake states in H3R +/+ and H3R −/− mice. a, Examples of polygraphic recording and corresponding hypnograms showing the effects of the motivation test (2–6 p.m.) on the cortical EEG, EMG and sleep-wake cycle. b, mean values (± SEM in min) of each sleep-wake stage during the 4 h in which the palatable food was presence, compared with the own baseline values of each genotype. c, mean net sleep-wake changes (± SEM in min) during the text, relative to the baseline values of each genotype. Note 1) a significant increase in waking (W) and decrease in slow wave sleep (SWS) in both genotypes and a decrease in paradoxical sleep (PS) only in H3R −/− mice, compared with their own baseline values (a, b); 2) a markedly greater effects on all sleep-wake states in H3R −/− than in H3R +/+ mice (c). $n = 16$ from 8 pairs of animals. $°p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *post hoc* Bonferroni test after significance in two-way ANOVA for repeated measures for b and unpaired Student's *t*-test for c (two-tails).

instead of 80 ± 4 min during the first 4 h recordings after dosing, $p < 0.001$, Bonferroni tests, Figure 7b) associated with a significant decrease in SWS and a non significant decrease in PS (Figure 7b).

In contrast, the same injection of ciproxifan in H3R −/− mice had no effect on either the latency to sleep (SWS or PS) or time spent in W, SWS and PS as compared with vehicle injection in the same animals (eg 77 ± 5 min spent in W vs 73 ± 4 min in control, $n = 10$, $p > 0.05$, Bonferroni tests, Figure 7b). The absence of effect of ciproxifan on W and SWS in the H3R −/− mice was further confirmed, by comparison of the net changes of W and SWS during the 4 h after ciproxifan dosing between the two genotypes (Figure 7c). At the dose used, ciproxifan had no clear effect

on the amount of PS during 4 h in either genotype (Figure 7b and c). These data unambiguously indicate the functional invalidation of H3-receptor and, together with PCR-genotyping, validate our animal model.

Triprolidine. Two way ANOVA indicates a significant effect of genotype on SWS 4 h after intraperitoneal injection of triprolidine (5 mg/kg, at 8 p.m. dark phase), a HA H1-receptor antagonist (Yanai *et al*, 1990; McCormick and Williamson, 1991): $F(1, 100) = 13.83$ $p = 0.0003$. In the WT mice, the effects lasted nearly 4 h with notably a significant increase in SWS at the second hour post injection ($n = 14$, Figure 8b). However, the total sleep-wake amounts during

Figure 7 Effects of ciproxifan a H3-receptor inverse agonist, on the sleep-wake states in H3R +/+ and H3R −/− mice. a, Examples of polygraphic recording and corresponding hypnograms showing the effects of intraperitoneal injection of ciproxifan on cortical EEG and EMG and sleep-wake cycle. b, Mean values (±SEM in min) of each sleep-wake stage after saline or ciproxifan injection in both genotypes. c, mean net sleep-wake changes (±SEM in min) relative to the baseline values of each genotype. Note in H3R +/+ mice, but not in H3R −/− mice, that ciproxifan elicited a significant increase in waking (W) and decrease in slow wave sleep (SWS), $n = 10$ for each genotype, °$p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, post hoc Bonferroni test after significance in two-way ANOVA for repeated measures for b and unpaired Student's t-test for c (two-tails).

the 4h post injection were not significantly different between vehicle and drug injections (Figures 8a and b), indicating a rapid recovery within 4h. PS remained unchanged after dosing.

In H3R −/− mice, the same injection of triprolidine ($n = 14$) constantly increased SWS at the expense of W during the 4h post injection, as shown by the hourly cumulated SWS increase over the vehicle data (Figures 8a and b). This led to a significant increase in SWS for the whole 4h period, an effect significantly different from that seen in the WT mice (Bonferroni tests, Figure 8b), Thus, triprolidine induced a greater and longer lasting effect in the KO than in WT mice, as further confirmed by direct comparisons of the net sleep-wake changes showing a significant difference for both W and SWS between genotypes. PS remained unchanged (Figure 8c).

(S)-α-fluoromethylhistidine (α-FMH). As previously reported in mice (Parmentier et al, 2002), rats (Kiyono et al, 1985; Monti et al, 1988) and cats (Lin et al, 1988), intraperitoneal injection of α-FMH (a specific inhibitor of histidine decarboxylase, the HA-synthesizing enzyme, 50 mg/kg) in WT mice at 4 p.m. resulted in a progressively developing and significant decrease in W and an increase in both SWS and PS ($n = 18$, Figures 9a and b). The effects became significant at the 8th h after dosing for W and 5th h for SWS and PS and were maintained for 6–10 more hours for all the sleep-wake states.

Compared with those in WT mice, the effects of α-FMH in H3R −/− mice were characterized by 1) a more prompt onset: W and SWS were significantly changed as early as the 5th and 4th h post injection; 2) larger changes of W and SWS, starting at 14 h post dosing even though the difference
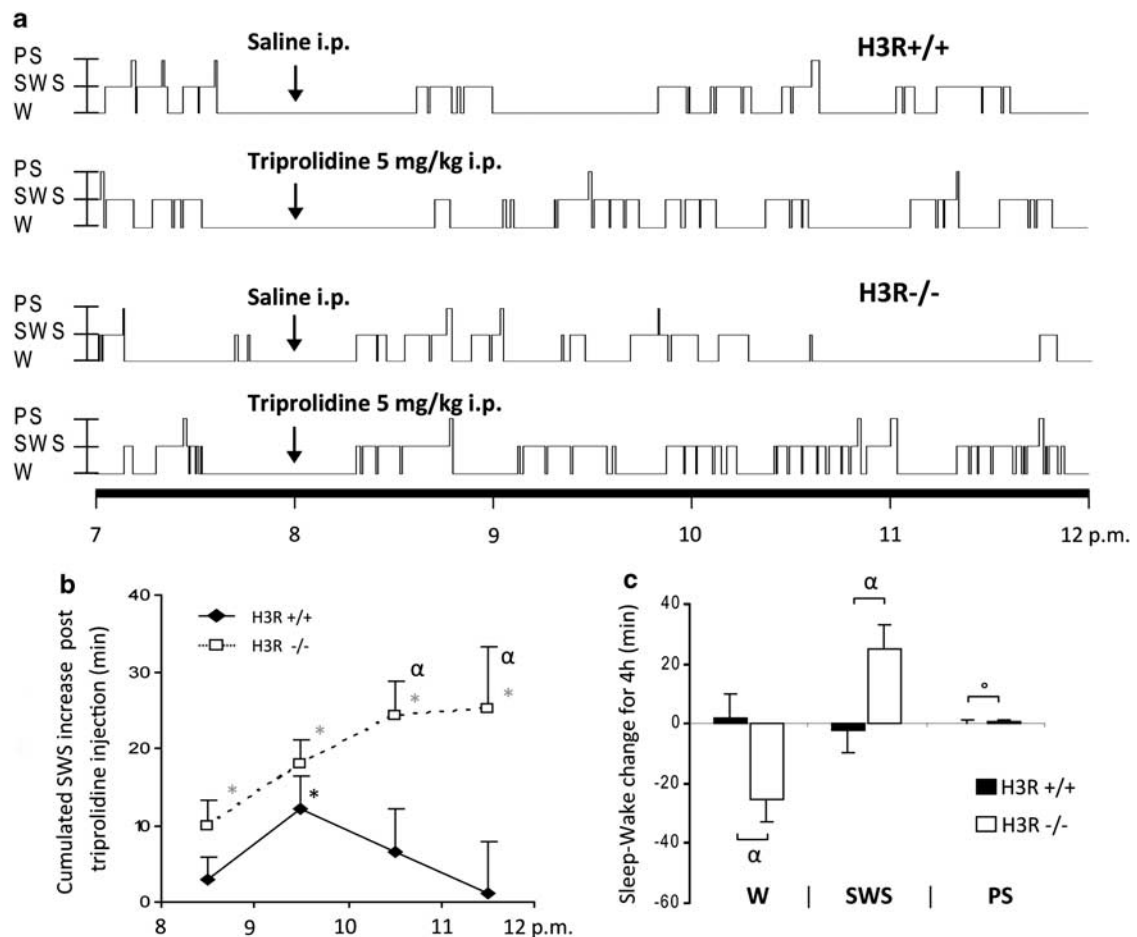
Figure 8 Effects of triprolidine, a H1-receptor antagonist, on the sleep-wake states in H3R +/+ and H3R −/− mice. a, Examples of hypnograms showing the effects of intraperitoneal injection of triprolidine on the sleep-wake cycle. b, hourly cumulated increase in slow wave sleep (SWS) post triprolidine injection, relative to saline injection (baseline 0), comparison using post hoc Bonferroni test after significance in a two-way ANOVA for repeated measures c, mean net sleep-wake changes (±SEM in min) relative to the baseline values of each genotype obtained with saline injection, comparison using unpaired Student's t-test (two-tails). Note that tiprolidine induced an increase in SWS and decrease in W in both genotypes (a), but the effects are significantly greater in H3R −/− than in H3R+/+ mice in that 1) the SWS increase was evidenced each hour during 4 h in the KO mice instead of only the second hour in the WT mice (b); 2) there is a significant difference between genotypes in terms of W and SWS changes (c). n = 14 for each genotype, °p > 0.05; *p < 0.05; **p < 0.01 compared with values of saline injection in the same animals; $^{\alpha}$p < 0.05 compared with values of H3 +/+ mice.

remained non-significant at this time parameter (Figure 7a) but became pronounced and significant at 24 h post dosing (ANOVA, Figure 7b) and 3) a longer-lasting action on the sleep-wake states. Thus, for the 24 h period post-dosing, two way ANOVA indicates a significant interaction between Genotype and Experimental condition (ie, saline vs α-FMH) for W: F(1, 33) = 6.112, p = 0.019 and SWS: F(1, 33) = 6.108, p = 0.019. Moreover, post hoc Bonferroni tests within genotypes indicate that the effects on W and SWS remained significantly modified in H3R −/− mice compared with their own control whereas those in the WT mice had returned to control levels (Figure 9b). Finally, as in other behavioral and pharmacological tests, the impact of the genotype on the effects of α-FMH is further confirmed by a significant difference between the two genotypes regarding the effects on both W and SWS during 24 h post dosing (Figure 9c).

DISCUSSION

The present study was designed to determine the sleep-wake consequences of long-term increased histamine (HA) neurotransmission using wild-type (WT) and H3-receptor knockout (H3R −/−) mouse littermates, a promising experimental model for sleep-wake disorders. We found that H3R −/− mice were rich in phenotypes, characterized notably by a wide-range of sleep-wake alterations. On the one hand, immediately after lights-off, they showed deficient wakefulness (W) and signs of sleep deterioration, such as pronounced sleep fragmentation and reduced cortical slow activity during slow wave sleep (SWS). On the other hand, they displayed signs of enhanced vigilance, i.e., increased cortical EEG θ power during spontaneous W and an exaggerated increase in W in response to behavioral challenges, including an environmental change, locomotion
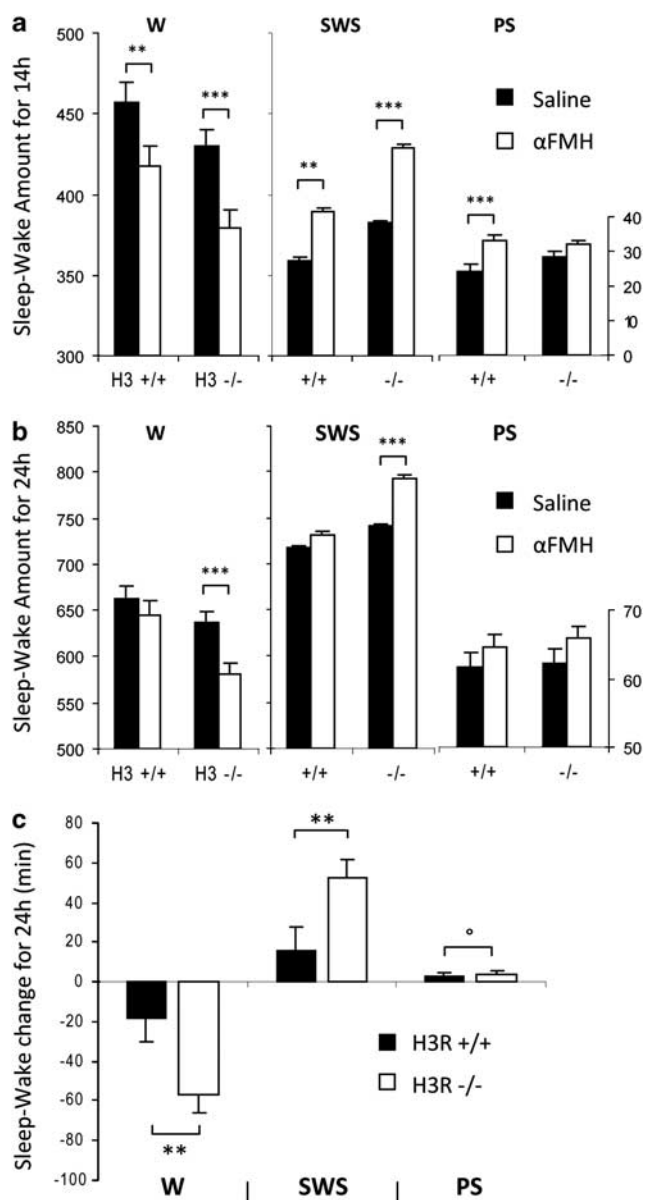
Figure 9 Effects of α-FMH, an inhibitor of histamine synthesis, on the sleep-wake states in H3R +/+ and H3R −/− mice. A,B, mean values (±SEM in min) of each sleep-wake stage during 14 h (a) or 24 h (b) after injection of saline or α-FMH (50 mg/kg, i.p., at 4 p.m.). c, mean net sleep-wake changes during 24 h post α-FMH injection (±SEM in min), relative to the baseline values of each genotype obtained with saline injection. Note that 1) α-FMH induced an increase in SWS and decrease in W in both genotypes during 14 h post injection (a, comparison using paired t-test, two-tails). The effects appear larger in H3R −/− mice than in H3R +/+ mice, but the difference is not yet statistically significant at this time parameter (a, two way ANOVA); 2) at 24 h post-injection (b), the difference becomes significant between genotypes: the decrease in W and increase in SWS remain significant in HR3 −/− mice but not in H3R +/+ mice (b, post hoc Bonferroni tests after significance in two-way ANOVA for repeated measures) and that 3) there is a significant difference between genotypes in terms of the net changes of W and SWS (c, unpaired Student's t-test, two-tails). n = 18 for each genotype, °p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001.

and motivation tests. This is the first study, to the best of our knowledge, in which the sleep-wake phenotypes of H3R −/− mice were clearly identified. The questions raised

here are how the deletion of the H3-receptor gene resulted in such apparently 'contradictory' phenotypes, how they can be interpreted in light of the altered HA neurotransmission of this mouse model and whether they are consequences of long-term enhancement of the HA arousal system.

Wake Deficiency in H3-Receptor Knockout Mice, Consequence of Decreased Histamine Cell Activity and Desensitization of Postsynaptic Histamine Receptors?

HA neurons are currently thought to promote W. H3-receptor is widespread distributed in the brain on both HA and non-HA neurons and plays a role of auto- and hetero-receptor. As auto-receptor, it controls the activity of HA neurons and the release and synthesis of HA through negative feedback (Reviewed in Schwartz et al, 1991; Haas et al, 2008. See Figure 10a). In H3R −/− mice, abolition of negative feedback results in enhanced brain HA turnover, manifested as a decrease in HA and a general increase in tele-methylhistamine (t-MHA), the direct metabolite of the amine (Takahashi et al, 2002; Toyota et al, 2002). One would therefore expect such an enhancement to promote waking, while, in contrast, we found that H3-KO mice exhibited under baseline conditions marked wake deficiency, sleep fragmentation and impaired EEG slow activity during SWS, phenotypes reminiscent of those seen with histidine-decarboxylase (HDC) −/− mice (Parmentier et al, 2002) which lack HA synthesis and neurotransmission.

First, could the decrease in HA, seen in H3R −/− mice (Takahashi et al, 2002; Toyota et al, 2002), cause such wake deficient phenotypes? A down-regulation of HA synthesis or/and an exhausted stock resulted from the over-release of HA could then be responsible. However, this interpretation appears to be unlikely because the decreased HA is accompanied by a marked increase in t-MHA in many brain regions – characteristics of an overuse of the amine and an up-regulated HA turnover rather than a down regulation. In support of this, we found that presynaptic inhibition of the HA synthesis by α-FMH (specific inhibitor of HDC) or postsynaptic blockade using triprolidine (H1-receptor antagonist), both induced a greater and longer-lasting increase in SWS in the KO than the WT mice, suggesting an up-regulated synthesis and release of HA in H3R −/− mice.

Another hypothesis would be that this wake deficiency results from a chronic alteration of other neural systems mediated by H3-heteroreceptors which control the release and synthesis of some neuropeptides and neurotransmitters such as serotonin, dopamine, noradrenalin and acetylcholine. Like HA, these neurotransmitters are also components of the brain ascending activating systems controlling different behavioral and cognitive aspects of wakefulness. Nevertheless, the brain levels of the afore mentioned mono-amines are unchanged in H3R −/− mice (Toyota et al, 2002; Nuutinen et al, 2011) in contrast to a clearly altered HA turnover. They could not, therefore, be accountable for the observed wake deficiency. It remains, however, to determine whether the cholinergic transmission is altered in H3R −/− mice. The cholinergic system constitutes a privileged target for HA neurons and the
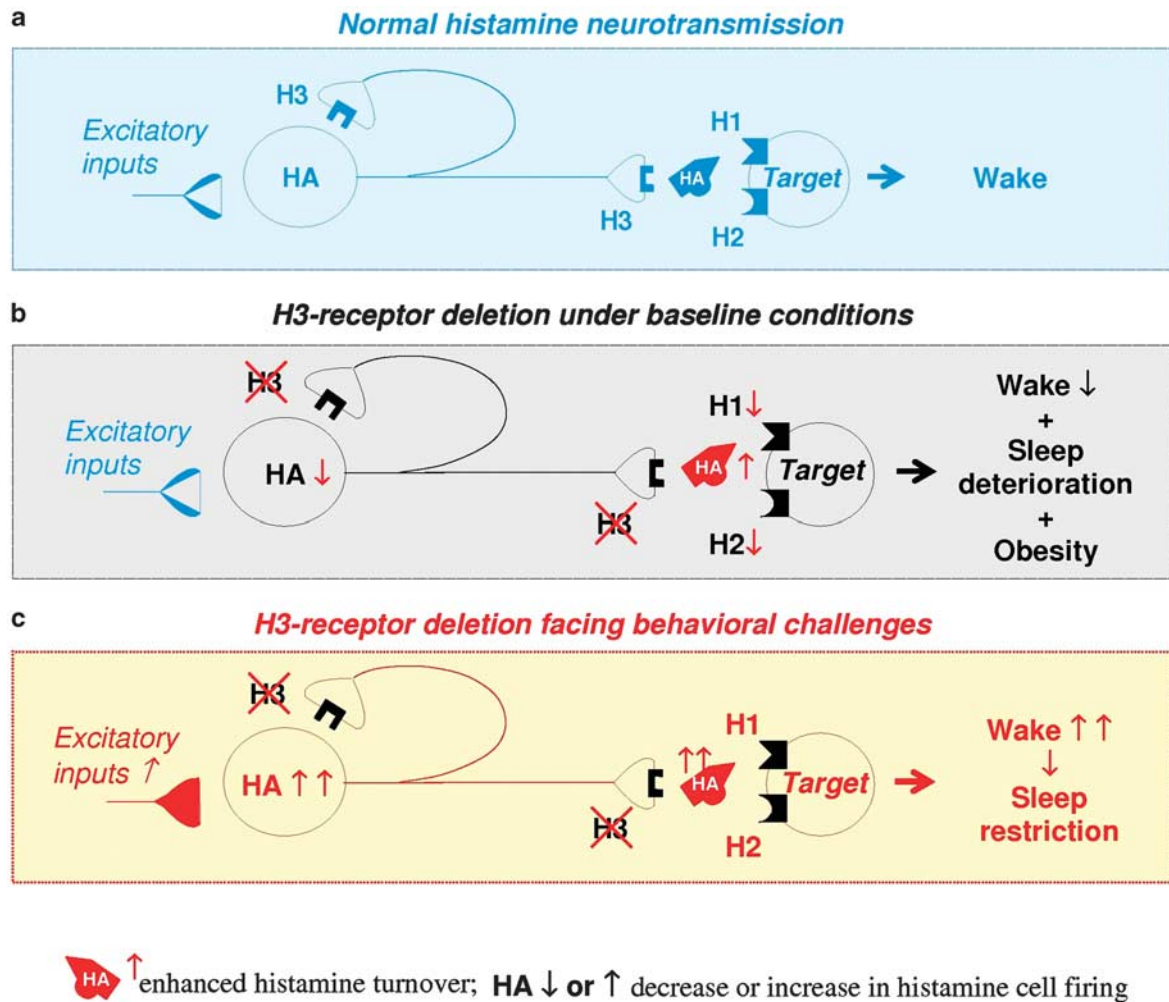
Figure 10 Schematic representation of our hypothesis on the consequences of H3-receptor deletion on histamine neurotransmission, sleep-wake states and metabolic outputs in H3-receptor knockout mice. Only presynaptic H3-autoreceptor is considered here. a, normal histamine (HA) neurotransmission promoting wake. Postsynaptic H1 and H2 receptors are responsible for the arousal effect of HA whereas presynaptic H3-receptors are involved by a negative feedback on HA neurons. b. Under baseline conditions, the constantly-enhanced HA neurotransmission in H3-receptor knockout mice would finally produce by homeostatic processes a decrease in HA neuronal activity and desensitization of H1 and H2 postsynaptic receptors, causing wake deficit, sleep deterioration and phenotypes of obesity. c. Facing behavioral challenges and consequently enhanced excitatory inputs, the absence of negative feedback results in an over activity of HA neurons which would overcome the postsynaptic defect, causing exaggerated wake responses and thus sleep restriction. Blue, black or red color signifies respectively normal, impaired or enhanced activity. ↓ down regulation; ↑ up regulation.

interaction between the two systems plays a major role in the brain network responsible for cortical arousal and cognition (Lin *et al*, 1996; Reviewed in Lin, 2000; Passani *et al*, 2004). The cholinergic system certainly could be involved in the procognitive effect of H3-receptor inverse agonists/antagonists (Reviewed in Lin *et al*, 2011a) but does not seem to be directly responsible for the sleep-wake phenotypes seen in H3R − / − mice because H3-receptor mediated arousal appears clearly HA-dependent. Indeed, ciproxifan, a H3-receptor inverse agonist/antagonist enhanced waking in WT mice but had no effect in either H3R − / − mice as demonstrated in the present study, or in HDC − / − or H1R − / − mice (Parmentier *et al*, 2002; 2007). Similarly, thioperamide, another inverse agonist had no effect in another H3R − / − mouse strain (Toyota *et al*, 2002).

We therefore suggest that acute blockade of H3-receptors and removal of negative feedback would cause an over

activity of HA neurons (Schwartz *et al*, 1991; Vanni-Merci et al. 2003; Lin *et al*, 2011a) whereas chronic loss of HA auto-regulation would, through homeostatic processes (Turrigiano, 2008), lead to a decrease in HA neuronal firing, contributing to the wake deficit of H3R − / − mice. Moreover, due to the constant and intense release of HA, postsynaptic receptors would finally lose their responsiveness to HA. Because H1- and H2-receptors are responsible for histaminergic arousal (Schwartz *et al*, 1991; Lin, 2000; Brown *et al*, 2001; Haas *et al*, 2008; Figure 10a), their desensitization would result in deficient arousal under baseline conditions (Figure 10b), phenotypes similar to, or even more severe than, those of KO mice lacking either HA neurotransmission or postsynaptic receptors (Inoue *et al*, 1996; Parmentier *et al*, 2002; Masaki *et al*, 2004; Masaki and Yoshimatsu, 2006).

This desensitization appears to occur towards agonist, i.e., endogenous HA itself and apparently not towards

antagonist. Repeated receptor activation by agonists would instead cause high sensitization to antagonists as suggested in this study by a larger SWS-increasing effect of triprolidine in H3R − / − mice. In support of such a desensitization hypothesis, the H1-receptor is down-regulated in H3R − / − mice as indicated by a decrease in binding sites in the hypothalamus (Takahashi *et al*, 2002; Tokita *et al*, 2006). Further studies are needed to determine if this down regulation extends to all postsynaptic HA-receptors and other brain areas. Investigations combined with functional approaches are necessary to assess whether this desensitization occurs at the level of gene transcript or/ and receptor affinity and excitability.

Increased Vigilance in H3-Receptor Knockout Mice and Enhanced Histamine Neuronal Activity and Neurotransmission Facing Behavioral Challenges

A decreased HA neuronal activity and desensitization of postsynaptic HA receptors seemed pronounced in that H3R − / − mice exhibited an impaired maintenance of brain arousal under baseline conditions. This situation was reversed, however, when facing behavioral challenges which increased excitatory inputs to HA neurons (Figure 10c). In fact, H3R − / − mice responded to behavioral challenges with an exaggerated increase in wakefulness. They showed a more extensive wakefulness in a new environment and were more alert on a wheel even though their running activity was below the normal level. Moreover, they were more motivated, making greater efforts to obtain difficult-to-reach palatable food and, as a result, remained highly awake: > 2.5 fold of the wake increase of WT littermates. This occurred at times when they were normally most sleepy. The extended wakefulness demonstrated here facing behavioral tasks is reminiscent of human voluntary sleep restriction. Finally, although quantitatively deficient after lights-off, their waking state during darkness was associated with a higher θ rhythm. This indicates higher cortical activation and alertness, which would allow H3R − / − mice to respond to behavioral challenges with sustained waking. The fact that H3R − / − mice show enhanced spatial learning and memory (Rizk *et al*, 2004) also indicates their improved behavioral performance.

These signs of enhanced vigilance contrasting with the somnolence and wake deficiency of HDC − / − mice in a new environment (Parmentier *et al*, 2002) are consistent with the proposed role for HA in promoting wakefulness and the functions of H3-receptors in HA neurotransmission. Pharmacological enhancement of histaminergic neuronal activity by acute blockade of H3-receptors using inverse agonists, prolongs wake duration, enhances cortical fast activities (and θ rhythm in mice) during waking and improves cognitive outputs in several species (Lin *et al*, 1990, 2008, 2011a; Monti, 1993; Ligneau *et al*, 1998, 2006, 2007; Barbier *et al*, 2004; Bonaventure *et al*, 2007; Parmentier *et al*, 2007; Esbenshade *et al*, 2008; Hajos *et al*, 2008; Guo *et al*, 2009).

Thus, these data further support the role of HA in wake maintenance during behavioral challenges. Even though HA's over-release would, under baseline conditions, damage histaminergic neuronal activity and neurotransmission leading to wake defect, under behavioral stimuli which enhance excitatory inputs to HA neurons (Sakai *et al*, 1990; Haas *et al*, 2008), the absence of negative feedback would, nevertheless, allow these neurons to fire in an exaggerated manner, thus overcoming the postsynaptic defect and ultimately leading to improved vigilance and behavioral performance (Figure 10c). Additional experimental data in H3R − / − mice, notably HA release *in vivo* and HA neuronal activity during behavioral tasks, would certainly be very valuable for the validation of this hypothesis (Figure 10).

H3-receptor Knockout Mouse, a Murine Model of Sleep Restriction?

The sleep-wake characteristics demonstrated here and the previously reported obesity phenotypes of H3R − / − mice, e.g., increased food intake and decreased glucose tolerance/insulin sensitivity (Takahashi *et al*, 2002) are very similar to the metabolic and sleep-wake consequences seen with voluntary sleep restriction in humans, an increasing phenomenon in our society with major impact on public health (Spiegel *et al*, 1999, 2004).

This leads us to suggest H3R − / − mice as an animal model of voluntary sleep restriction. Hence, in consistence with the proposed role for HA and when facing diverse tasks or behavioral challenges (motivation, eg), the absence of negative feedback on HA neurons results in a response with overly extended waking akin to voluntary sleep restriction in humans (Figure 10c). However, as a 'price to pay' for such a 'high performance', constantly intensified brain arousal would lead to exhaustion of the HA arousal system, i.e., impaired cell activity and neurotransmission under baseline conditions, causing wake deficit, sleep deterioration and disturbances leading to increased adiposity and obesity (Takahashi *et al*, 2002)(Figure 10c). Other specific behavioral studies are needed in order to assess whether such a long-term enhancement of the HA system produces any cognitive output relevant to human voluntary sleep restriction.

Whereas this model remains to be further characterized using inducible KO mice in which the immediate effects and time course following H3-receptor deletion could be evaluated, H3R − / − mice already appear as a useful experimental model for determining the relationship between sleep restriction and metabolism. It would also allow investigation of the impact of sleep therapy on metabolic disorders, such as obesity, a major problem of public health.

Taken together, we suggest that chronic enhancement of an arousal system, like the histaminergic system, would eventually produce dysfunction of the arousal system *per se*, leading not only to baseline wake deficiency and sleep restriction facing behavioral tasks, but also to behavioral and metabolic disorders similar to the consequences of voluntary sleep restriction in humans. It is expected that normalizing the arousal systems would restore sleep-wake quantity and quality, thus improving the metabolic parameters and the affected functions.

DISCLOSURE

The authors declare no conflict of interest.

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