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Contribution of 5-HT₂ Receptor Subtypes to Sleep–Wakefulness and Respiratory Control, and Functional Adaptations in Knock-Out Mice Lacking 5-HT_{2A} Receptors

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Serotonin (5-hydroxytryptamine; 5-HT) plays key roles in sleep–wakefulness regulation. Evidence indicates that 5-HT₂ receptors are involved mainly in non-rapid eye movement sleep (NREMS) regulation and respiratory control. Here, we investigated the relative contribution of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor subtypes to NREMS and breathing during sleep, using 5-HT₂ subtype-selective ligands in wild-type (5-HT_{2A}^{+/+}) and knock-out (5-HT_{2A}^{-/-}) mice that do not express 5-HT_{2A} receptors. Acute blockade of 5-HT_{2A} receptors induced an increase in NREMS in 5-HT_{2A}^{+/+} mice, but not 5-HT_{2A}^{-/-} mutants, which spontaneously expressed less NREMS than wild-type animals. In 5-HT_{2A}^{+/+} mice, 5-HT_{2B} receptor blockade produced a reduction of NREMS, whereas receptor activation induced an increase in this sleep stage. These effects were less pronounced in 5-HT_{2A}^{-/-} mice, indicating a lower sensitivity of 5-HT_{2B} receptors in mutants, with no change in 5-HT_{2B} mRNA. Blockade of 5-HT_{2C} receptors had no effect on NREMS in both strains. In addition, an increase in EEG power density after sleep deprivation was observed in 5-HT_{2A}^{+/+} mice but not in 5-HT_{2A}^{-/-} mice. Whole-body plethysmographic recordings indicated that 5-HT_{2A} receptor blockade in 5-HT_{2A}^{+/+} mice reduced NREMS apneas and bradypneas that occurred after sighs. In contrast, in 5-HT_{2A}^{-/-} mutants, NREMS apneas were not modified, and bradypnea after sighs were more pronounced. Our results demonstrate that 5-HT exerts a 5-HT_{2B}-mediated facilitation of NREMS, and an influence respectively inhibitory on NREMS and facilitatory on sleep apnea generation, via 5-HT_{2A} receptors. Moreover, 5-HT_{2A} gene knock-out leads to functional compensations yielding adaptive changes opposite to those caused by pharmacological blockade of 5-HT_{2A} receptors in 5-HT_{2A}^{+/+} mice.

Key words: serotonin; sleep; mice; 5-HT₂ receptors; sleep deprivation; apnea; respiration; serotonergic; depression; knock-out mice; EEG

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) has long been known to be a key regulator of the vigilance states (Jouvet, 1969). Notably, midbrain 5-HT neurons are part of the system that opposes rapid eye movement sleep (REMS) expression (for review, see Pace-Schott and Hobson, 2002). Investigations using receptor-specific pharmacological treatments and knock-out mice have demonstrated that 5-HT_{1A} and 5-HT_{1B} receptors are involved in the regulation of REMS (Boutrel et al., 1999, 2002; Adrien et al., 2004). In contrast, it has been reported that 5-HT₂ receptors play

an inhibitory role in non-REMS (NREMS) expression. Indeed, the wide-spectrum antagonist at 5-HT₂ receptors, ritanserin (Leysen et al., 1985), substantially increases NREMS in humans (Idzikowski et al., 1991) and in rats (Dugovic et al., 1989). 5-HT₂ receptors belong to the superfamily of G-protein-coupled receptors and comprise three subtypes: 5-HT_{2A} and 5-HT_{2C} receptors, which have a widespread distribution in the CNS, and 5-HT_{2B} receptors, which have a restricted expression pattern (Leysen, 2004). Thus far, the respective contribution of each 5-HT₂ receptor subtype in NREMS regulation has been scarcely described (Frank et al., 2002; Kantor et al., 2004).

A large body of evidence indicates that the 5-HT_{2A} receptor subtype is involved in the physiopathology of psychiatric disorders associated with sleep disturbances, such as depression (Benca, 2000). Indeed, antidepressants with 5-HT₂ receptor antagonistic properties alleviate insomnia in depressed patients (Thase, 2000). In addition, a polymorphism in the promoter region of the 5-HT_{2A} receptor gene appeared to be associated with mood disorders (Bonnier et al., 2002). Therefore, the study of mice with a mutation disrupting the expression of the 5-HT_{2A} receptor gene (5-HT_{2A}^{-/-}) (Fiorica-Howells et al., 2002) might

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provide insights into the contribution of these receptors to the sleep abnormalities found in psychiatric disorders.

The sleep apnea syndrome is a prevalent disease (Young, 1993) to which 5-HT₂ receptors could contribute. Indeed, serotonergic neurons with carbon dioxide chemosensitivity play a crucial role in maintaining eupneic breathing (Richerson, 2004), and the reduction in 5-HT delivery to the respiratory motor system during sleep might facilitate sleep apneas (Veasey, 2003).

To investigate the specific role of the 5-HT₂ receptor family in the modulation of sleep and respiratory control, we analyzed the sleep–wakefulness regulations and respiratory parameters in wild-type (5-HT_{2A}^{+/+}) and 5-HT_{2A}^{-/-} mice after treatment with 5-HT₂ receptor subtype-selective drugs. In addition, because of the deficit in sleep homeostasis (Benca, 2000) and the abnormalities in 5-HT_{2A} receptor function observed in depressed patients, we investigated the effects of sleep deprivation in 5-HT_{2A}^{-/-} mutants compared with 5-HT_{2A}^{+/+} mice. Finally, because we found alterations in 5-HT_{2B} receptor-mediated effects in 5-HT_{2A}^{-/-} mice, possible changes of the expression of 5-HT₂ receptor subtypes in the mutants were also examined at mRNA level in brain structures involved in sleep control.

Materials and Methods

All of the procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive number 87-848, October 19, 1987, Ministère de l'agriculture et de la forêt, Service vétérinaire de la santé et de la protection animale, permissions 75-116 to M.H. and 75-125 to J.A.).

Animals. Heterozygous mice with a targeted disruption of the promoter region of the 5-HT_{2A} receptor encoding gene (5-HT_{2A}^{+/-}) (12 generations backcrossed; 129 Sv/Ev Taconic background) (Fiorica-Howells et al., 2002) were bred at Unité Mixte de Recherche 677, Institut National de la Santé et de la Recherche Médicale/Université Pierre et Marie Curie unit animal facility and were mated together. Homozygous 5-HT_{2A}^{-/-} and 5-HT_{2A}^{+/+} littermates were selected by genotyping using PCR analyses of tissue samples from the tails (Fiorica-Howells et al., 2002). Males and females were separated at weaning (postnatal day 21) and housed six per cage under standard conditions (12 h light/dark cycle; lights on at 7:00 A.M.; 22 ± 1°C ambient temperature; 60% relative humidity; food and water *ad libitum*). For all experiments, data were collected from adult males.

Implantation of electrodes for polygraphic sleep–wakefulness monitoring. At 2–3 months of age, male mice of the 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} genotypes were implanted under ketamine/xylazine anesthesia (100 and 5 mg/kg, *i.p.*, respectively) with the classical set of electrodes (made of enameled nichrome wire, 150 μm in diameter) for polygraphic sleep monitoring (Boutrel et al., 1999). Briefly, EEG electrodes were inserted through the skull onto the dura over the right cerebral cortex (2 mm lateral and 4 mm posterior to the bregma) and over the cerebellum (at midline, 2 mm posterior to λ), electro-oculogram (EOG) electrodes were positioned subcutaneously on each side of the orbit, and EMG electrodes were inserted into the neck muscles. In some animals, electrodes were also placed under the skin at the level of the sternum and the left forepaw (D1), to record electrocardiogram (ECG) in plethysmographic experiments (see below). All electrodes were anchored to the skull with superbond and acrylic cement and soldered to a mini-connector also embedded in cement. After completion of surgery, animals were housed in individual cages (20 × 20 × 30 cm) and maintained under standard laboratory conditions. They were allowed 10–14 d to recover, during which they were habituated to the recording conditions (*i.e.*, one animal per cage), with the home cage being the recording cage.

Recording of sleep–wakefulness cycles. After the animals had been connected to the recording cable for 2 d, polygraphic recording of the spontaneous sleep–wakefulness states was performed during 48 h, beginning at 7:00 P.M. (*i.e.*, at the onset of the dark period) (Boutrel et al., 1999). On the third day, mice were awakened at 10:00 A.M., and left to sleep again,

to evaluate their induced sleep latency (see below). The signals were fed into an Embla device (Medcare, Reykjavik, Iceland) for differential amplification, sampled at 100 Hz (EEG, EOG, pressure signal in plethysmographic experiments; see below) or 200 Hz (EMG, ECG), and recorded with the software Somnologica (Medcare).

Pharmacological treatments. BW 723C86 [methyl-5-(2-thienylmethoxy)-1-*H*-indole-3-ethanamine hydrochloride] (10–20 mg/kg) (Kennett et al., 1997a), SB 242084 [6-chloro-5-methyl-1-[[2-(2-methylpyrid-3-yloxy)pyrid-5-yl] carbamoyl] indoline dihydrochloride] (0.5, 1, 2.5 mg/kg) and SB 206553 [*N*-3-pyridinyl-3,5-dihydro-5-methyl-benzo(1,2-*b*:4,5-*b'*)dipyrrole-1(2*H*)carboxamide hydrochloride] (2.5 mg/kg) (Kennett et al., 1997b) were dissolved in saline (0.9% NaCl, *w/v*). MDL 100907 [*R*-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol] (1, 2, 5 mg/kg) (Padich et al., 1996) was dissolved in a 5.5% glucose solution containing 0.005% acetic acid (*v/v*). SB 215505 [6-chloro-5-methyl-1-5-quinolylcarbamoyl-indoline] (0.5, 1, 2.5 mg/kg) (Kantor et al., 2004) was dissolved in 0.9% NaCl containing 10% dimethylsulfoxide (*v/v*). In all cases, a volume of 0.1 ml was injected intraperitoneally at 10:00 A.M., except for BW 723C86, which was injected subcutaneously. For baseline data, mice were injected with vehicle, intraperitoneally or subcutaneously, as appropriate. A washout period of at least 7 d was allowed between two consecutive treatments. Drugs were purchased from Sigma (St. Louis, MO) except for MDL 100907, which was provided by Hoechst Marion Roussel (Bridgewater, NJ). The pK values of the drugs are, respectively on 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors: MDL 100907: 9.1, 6.1, 6.95; BW 723C86: 6.7, 9.0, 7.0; SB 206553: 5.8, 8.3, 8.5; SB 242084: 6.8, 7.0, 9.0; and SB 215505: 6.8, 8.3, 7.7 (Reavill et al., 1999; Leysen, 2004). 5-HT₂ antagonists had no effect on core temperature [see the study by Morishima and Shibano (1995) for 5-HT_{2A/C} ligands and the present study for 2.5 mg/kg of the 5-HT_{2B} antagonist SB 215505 in 12 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice; data not shown].

Sleep deprivation. Mice were subjected to 6 h of sleep deprivation starting at light onset (7:00 A.M.). For this purpose, animals were continuously observed, and various objects (pencil, pieces of paper, plastic tubes) were introduced into the cage to arouse the mouse as soon as it was engaged in a sleeping posture (Tobler et al., 1997; Lena et al., 2004). At the end of the deprivation period, the animals were left to sleep freely while being recorded for 18 h (from 1:00 P.M. to 7:00 A.M. on the next day).

Plethysmography. Respiration was monitored with a two-chamber, constant-flow, plethysmograph (Jacky, 1978) equipped with a pressure transducer (DP45; Validyne, Northridge, CA) and a swivel for combined polygraphic recordings. The outflow gas concentrations of oxygen and carbon dioxide were continuously monitored using a Capnomac Ultima (Datex, Helsinki, Finland) and maintained within the physiological range. All signals were recorded with the same Embla device, as described above. Mice were placed into the plethysmograph during 24 h, starting at 6:00 P.M., and recordings were collected from 10:00 A.M. to 4:00 P.M. the next day. Injections were always performed at 10:00 A.M., as in the sleep studies. Analyses were done with custom Matlab (MathWorks, Natick, MA) routines (Lena et al., 2004). Sigh is defined as respiratory cycles of amplitude greater than two times the average baseline amplitude for the 30 preceding cycles (~10 s). Apnea is defined as cessation of breathing during at least twice the average respiratory cycle duration in NREMS. The traces of each computer-detected sigh and apnea were visually inspected to discard artifacts. Only sighs and apneas occurring during NREMS were analyzed. The heart rate was obtained from inter-beat R–R intervals in the ECG recorded in D1.

mRNA expression. RNA preparation and first-stranded cDNA synthesis. Mice were killed by decapitation, and the entire brain was removed. The brain was dissected into five regions: raphe area, hypothalamus, striatum, hippocampus, and anterior cortex. Samples were frozen with liquid nitrogen and stored at –80°C until use. Total RNA extraction was done using the RNeasy Mini or Midi kit, according to the instructions of the manufacturer, including removal of genomic DNA by DNase treatment (Qiagen, Courtaboeuf, France). RNA integrity was checked by agarose gel electrophoresis, and RNA concentrations were determined by spectrophotometric measurements. First-stranded cDNA synthesis (1 μg of total RNA per 20 μl reaction) was performed with the SuperScript III

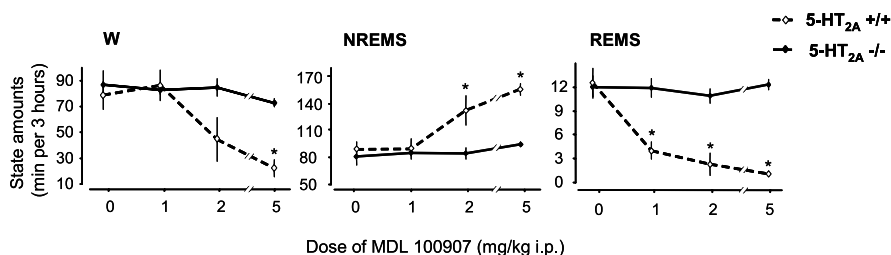


Figure 1. Effects of acute treatment with the 5-HT_{2A} receptor antagonist MDL 100907 at various doses (abscissa) on amounts of vigilance states in 5-HT_{2A}^{+/+} (dotted line) and 5-HT_{2A}^{-/-} (solid line) mice. Data (mean ± SEM of 4–8 animals) are expressed as minutes during the first 3 postinjection hours. At the time of injection, there were no significant differences in amounts of vigilance states between 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice. **p* < 0.05, significantly different from vehicle (0 on abscissa); *post hoc* Bonferroni’s–Dunn’s test. Error bars represent SEM.

Reverse Transcriptase and random primers, as recommended by the manufacturer (Invitrogen, Cergy Pontoise, France).

Quantitative TaqMan RT-PCR. PCR amplification in triplicate for each sample was performed on the ABI Prism 7300 apparatus (Applied Biosystems, Courtaboeuf, France) by using TaqMan Universal PCR Master Mix (Applied Biosystems) and the Assays-on-Demand Gene Expression probes (Applied Biosystems) for target genes: 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptor genes, and reporter genes encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyltransferase (HPRT). The polymerase activation step at 95°C for 10 min was followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The validity of the results was checked by running appropriate negative controls (substitution of cDNA with water in PCR amplification or omission of reverse transcriptase in the cDNA synthesis).

Data analysis. The 2^{-ΔΔCt} method (Livak and Schmittgen, 2001) was used to analyze the relative change in gene expression in 5-HT_{2A}^{-/-} compared with 5-HT_{2A}^{+/+} animals (RQ Study Software version 1.2; Applied Biosystems). All of the data were analyzed using both GAPDH and HPRT as references.

Quantitative polygraphic analyses and statistics. Polygraphic recordings were scored visually every 15 s epoch as wakefulness (W), NREMS, or REMS, following classical criteria (Tobler et al., 1997; Lena et al., 2004), using the Somnologica software. We also scored events in NREMS corresponding to a 5–15 s drop of at least 50% of the EEG power with a spectral content mostly in the delta band. These events were scored as microarousals (MAs) (Lena et al., 2004). They are distinct from single episodes in NREMS, in which the EEG spectral content increases in the theta band with an EMG activation and which were thus scored as “short awakenings” and counted as W. Fragmentation of NREMS by MAs was quantified by the number of events per minute of NREMS. NREMS bouts duration ignored MAs.

For analysis of the spontaneous sleep–wakefulness patterns, the amounts of vigilance states for each animal were calculated for every hour throughout 48 h and summed over 3 and 12 h.

The sleep latencies were calculated as follows: the spontaneous REMS latency (SL) is the time interval between each sleep onset and the next REMS episode, averaged throughout 48 h, and the induced REMS latency (IL) is the time elapsing from sleep onset after the animal had been awakened, to the first REMS episode.

For sleep amounts, the mean values across animals were expressed as minutes ± SEM for each genotype, and significance was tested by performing a two-way ANOVA with the factors time and genotype and repeated measures over time. In case of significance (*p* < 0.05), the ANOVA was followed by the Bonferroni’s/Dunn’s test for comparisons of means. For sleep latencies and respiratory variables, the mean values were expressed as minutes ± SEM for each genotype, and significance was tested by performing a Student’s *t* test, paired or unpaired, as appropriate.

For pharmacological experiments, the effects of each dose of each drug tested for each state of vigilance were analyzed for 1 or 3 h after injection. For a given treatment, each animal was referred to its own baseline represented by the data obtained after injection of the vehicle. Statistical

analyses were performed using two-way ANOVA for factors dose and group with repeated measure over dose, and, in case of significance (*p* < 0.05), the *F* test was followed by the Bonferroni’s/Dunn’s test for comparisons of means.

Power spectra analysis and statistics. The EEG signal was processed for power spectra analysis (Franken et al., 1998; Lena et al., 2004). 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice presented an EEG profile similar to that reported for their 129 Sv/Ev genetic background (Franken et al., 1998) (supplemental Fig. B, available at www.jneurosci.org as supplemental material). EEG power in the delta (0.5–4.99 Hz) and theta (5–9.99 Hz) band was extracted from a spectrogram obtained by averaging the power spectrum density over 512 samples overlapping

periods and using a Hanning window.

The absence of qualitative changes in EEG spectrum after pharmacological treatments was verified by two-way ANOVA with repeated measures over treatments (vehicle vs the highest dose of drug used) and EEG power in the delta and theta band for each vigilance. No effect could be evidenced for the four drugs studied (BW 723C86, MDL 100907, SB 206553, and SB 242084).

For sleep deprivation, the absolute values of EEG power in the delta band during NREMS was analyzed by a three-way ANOVA performed to compare genotypes, with repeated measure over time and conditions (baseline vs sleep deprivation).

Results

5-HT_{2A} receptor blockade by MDL 100907 increases NREMS amounts

In 5-HT_{2A}^{+/+} animals, the 5-HT_{2A} receptor antagonist MDL 100907 (at 2–5 mg/kg, i.p.), induced a significant dose-dependent increase in NREMS amounts during the first 3 h after injection ($F_{(3,19)} = 7.7$; *p* < 0.01). The drug also caused a decrease in W and REMS amounts during the same period ($F_{(3,19)} = 5.3$, *p* < 0.01; $F_{(3,19)} = 12.4$, *p* < 0.001, respectively) (Fig. 1). As expected, no effect of MDL 100907 was observed on the states of vigilance in 5-HT_{2A}^{-/-} animals [Fig. 1, genotype/treatment interactions for NREMS ($F_{(3,37)} = 4.1$; *p* < 0.05) and REMS ($F_{(3,37)} = 7.0$; *p* < 0.001)].

5-HT_{2A}^{-/-} mutants express decreased NREMS amounts

Under baseline conditions, 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice exhibited the typical polyphasic structure of vigilance states found in rodents and a diurnal rhythm of sleep and W, with larger amounts of sleep during the light period classically observed in nocturnal species (Fig. 2). However, marked differences in the amounts of sleep and W were observed between the two genotypes. 5-HT_{2A}^{-/-} mice exhibited significantly increased W ($F_{(1,126)} = 15.2$; *p* < 0.01) and reduced NREMS ($F_{(1,126)} = 12$; *p* < 0.01) amounts throughout the entire circadian period compared with 5-HT_{2A}^{+/+} mice (Fig. 2). The enhancement of W in 5-HT_{2A}^{-/-} mice was accompanied by an increase in the mean episode duration of this stage for the dark period [(in minutes, mean ± SEM) 5-HT_{2A}^{-/-}, 4.5 ± 0.4 vs 5-HT_{2A}^{+/+}, 3.2 ± 0.4; *n* = 10; *p* < 0.05]. There was also a mild difference between the duration of NREMS bouts (24 h: 5-HT_{2A}^{-/-}, 3.1 ± 0.2 vs 5-HT_{2A}^{+/+}, 3.6 ± 0.2; *n* = 10; *p* < 0.05), but no significant difference was found in the fragmentation of NREMS by MAs in 5-HT_{2A}^{-/-} compared with 5-HT_{2A}^{+/+} mice [(in MA per minute of NREMS, mean ± SEM) 5-HT_{2A}^{-/-}, 0.25 ± 0.03 (*n* = 10) vs 5-HT_{2A}^{+/+}, 0.28 ± 0.03 (*n* = 10); *p* = 0.54].

The amounts of REMS were similar in both groups. However, mild changes in REMS were noted in the mutants. The SL and the IL (after awakening at 10:00 A.M.; see Materials and Methods) were shorter in 5-HT_{2A}^{-/-} than in 5-HT_{2A}^{+/+} mice [(in minutes) 5-HT_{2A}^{-/-}, 5.11 ± 0.32 and 13.67 ± 1.70 for SL and IL, respectively; 5-HT_{2A}^{+/+}, 8.31 ± 1.55 and 28.05 ± 3.49 for SL and IL, respectively; *n* = 10–12 in each group; *p* < 0.01]. 5-HT_{2A}^{-/-} mice also presented a higher frequency of EEG theta rhythm during REMS compared with 5-HT_{2A}^{+/+} animals [peak of theta (in hertz), 7.7 ± 0.1 vs 7.3 ± 0.2; *n* = 10; *p* < 0.05]. Interestingly such modifications are also observed in animal models of depression (Adrien et al., 1991; Benca, 2000; Dugovic et al., 2000).

Sleep deprivation does not enhance delta power at recovery in 5-HT_{2A}^{-/-} mutants

Because 5-HT_{2A}^{-/-} mutants exhibited reduced NREMS, we examined how they would respond to an increase of sleep pressure produced by a sleep deprivation. After 6 h of sleep deprivation (starting at the onset of the light phase), no significant modification of the amounts of any vigilance state was observed in either genotype. However, the EEG power density in the delta frequency range, a marker of NREMS homeostasis (Borbely and Achermann, 1999), was significantly increased above baseline values during 8 h of recovery in 5-HT_{2A}^{+/+} ($F_{(3,32)} = 5.8$; *p* < 0.01), whereas it was unchanged in 5-HT_{2A}^{-/-} ($F_{(3,32)} = 1.0$; *p* = 0.4) mice (Fig. 3). In contrast, during the same time, the duration of NREMS bouts was not modified in 5-HT_{2A}^{+/+} mice [(in minutes, mean ± SEM) baseline, 3.2 ± 0.4; recovery, 3.3 ± 0.4; *n* = 9; *p* = 0.83] and increased in 5-HT_{2A}^{-/-} mutants (baseline, 2.6 ± 0.2; recovery, 3.1 ± 0.3; *n* = 9; *p* < 0.05). Finally, NREMS fragmentation was decreased during the recovery period for mice of both genotypes [(in MA per minute of NREMS, mean ± SEM) 5-HT_{2A}^{+/+}: baseline, 0.19 ± 0.02; recovery, 0.11 ± 0.01; *n* = 9; *p* < 0.05; 5-HT_{2A}^{-/-}: baseline, 0.15 ± 0.03; recovery, 0.09 ± 0.02; *n* = 9; *p* < 0.05; with no significant difference between strains, ANOVA genotype/treatment: $F_{(1,112)} = 0.42$; *p* = 0.52].

NREMS is affected by 5-HT_{2B}, but not 5-HT_{2C}, receptor ligands

Because 5-HT_{2A}^{-/-} mice exhibited a phenotype opposite of that obtained after blockade of 5-HT_{2A} receptors in 5-HT_{2A}^{+/+} animals, we then examined the role of the other subtypes (5-HT_{2B} and 5-HT_{2C}) of the 5-HT₂ receptor family.

In 5-HT_{2A}^{+/+} animals, acute blockade of 5-HT_{2B/2C} receptors by SB 206553 (2.5 mg/kg, i.p.) induced a short lasting reduction of NREMS and REMS and an increase in W ($F_{(1,12)} = 7.8$, *p* < 0.05; $F_{(1,12)} = 10.8$, *p* < 0.01; $F_{(1,12)} = 8.4$, *p* < 0.05, respectively) (Fig. 4). Thus, 5-HT_{2A} and 5-HT_{2B/2C} receptor blockade produced opposite effects on NREMS. To identify which receptor subtype was involved in this action, we then performed selective blockade of either 5-HT_{2C} or 5-HT_{2B} receptors. Blockade of only 5-HT_{2C} receptors by SB 242084 (0.5, 1.0, 2.5 mg/kg, i.p.) decreased REMS ($F_{(3,15)} = 5.0$; *p* < 0.05) but failed to modify NREMS and W (Fig. 5). In contrast, acute blockade of only 5-HT_{2B} receptors by SB 215505 (0.5, 1.0, 2.5 mg/kg, i.p.) induced a reduction of NREMS and REMS and an increase in W during the first hour after injection ($F_{(3,28)} = 8.4$, *p* < 0.001; $F_{(3,28)} =$

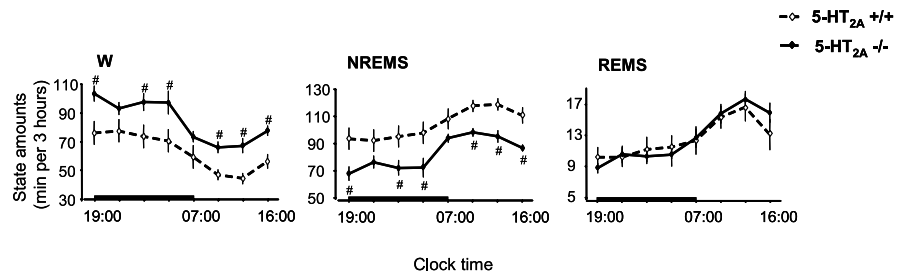


Figure 2. Amounts of W, NREMS, and REMS across the light/dark cycle in 5-HT_{2A}^{+/+} (dotted line) and 5-HT_{2A}^{-/-} (solid line) mice. Data (mean ± SEM of 10 animals per group) are expressed as minutes per 3 h during 24 h. The black bar on the abscissa represents the dark period of the light/dark cycle. #*p* < 0.05, significantly different from 5-HT_{2A}^{+/+} mice; *post hoc* Bonferroni's–Dunn's test. Error bars represent SEM.

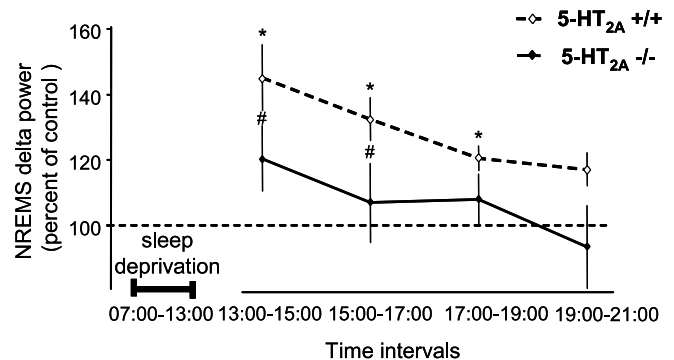


Figure 3. Effects of 6 h of sleep deprivation starting at light onset on delta power, during four consecutive 2 h intervals of the recovery period in 5-HT_{2A}^{+/+} (dotted line) and 5-HT_{2A}^{-/-} (solid line) mice. Statistical analysis was performed on EEG power values, and data (mean ± SEM of 9 animals in each group) are plotted as a percentage of the paired values obtained under control conditions. **p* < 0.05, significantly different from respective control value; *post hoc* Bonferroni's–Dunn's test. #*p* < 0.05, significantly different from 5-HT_{2A}^{+/+} value; *post hoc* Bonferroni's–Dunn's test. Error bars represent SEM.

12.0, *p* < 0.0001; $F_{(3,28)} = 10.9$, *p* < 0.0001, respectively) (Fig. 6A). Reciprocally, acute activation of 5-HT_{2B} receptors by BW 723C86 (10–20 mg/kg, s.c.) induced an increase in NREMS amounts and a decrease in W and REMS ($F_{(2,24)} = 10.6$, *p* < 0.001; $F_{(2,24)} = 5.3$, *p* < 0.05; $F_{(2,24)} = 20.6$, *p* < 0.0001, respectively) (Fig. 6B).

In 5-HT_{2A}^{-/-} mutants, 5-HT_{2C} receptor blockade by SB 242084 (0.5–2.5 mg/kg, i.p.) did not affect W and NREMS amounts, as also found in 5-HT_{2A}^{+/+} mice (Fig. 5). However, the dose–response curve of REMS inhibition by this antagonist ($F_{(3,16)} = 6.5$; *p* < 0.01) was shifted to the left, suggesting an increased influence of 5-HT_{2C}-mediated regulation of REMS in the mutants (genotype/dose, $F_{(3,18)} = 4.0$; *p* < 0.05). In contrast, the action on NREMS of the 5-HT_{2B/2C} or 5-HT_{2B} receptor antagonists, and of the 5-HT_{2B} receptor agonist, was respectively abolished, or reduced compared with 5-HT_{2A}^{+/+} mice (BW 723C86: genotype/dose $F_{(3,42)} = 5.9$, *p* < 0.01; SB 215505: genotype/dose, $F_{(3,42)} = 5.0$, *p* < 0.01) (Figs. 4, 6). Indeed, an enhancement of NREMS ($F_{(1,10)} = 5.0$; *p* < 0.05) was obtained only with the highest dose of BW 723C86 (20 mg/kg, s.c.) (Fig. 6B). This indicates a reduced influence of 5-HT_{2B} receptors over NREMS regulatory system in 5-HT_{2A}^{-/-} compared with 5-HT_{2A}^{+/+} mice.

5-HT₂ mRNA expression in CNS

To test whether the changes in sensitivity to 5-HT₂ receptor ligands in 5-HT_{2A}^{-/-} mice were attributable to changes in recep-

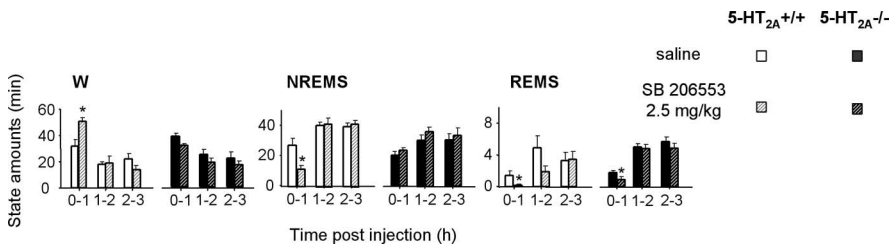


Figure 4. Effects of acute treatment with the 5-HT_{2B/2C} receptor antagonist SB 206553 (2.5 mg/kg, i.p.; hatched bars) on amounts of vigilance states in 5-HT_{2A}^{+/+} (white bars) and 5-HT_{2A}^{-/-} (black bars) mice. Data (mean ± SEM of 7 animals per group) are expressed as minutes per hour during each of the first 3 postinjection hours. **p* < 0.05, significantly different from paired-saline-treated animals; *post hoc* Bonferroni's–Dunn's test. Error bars represent SEM.

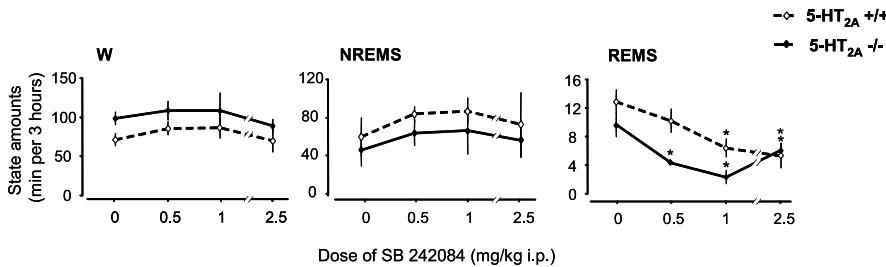


Figure 5. Effects of acute treatment with the 5-HT_{2C} receptor antagonist SB 242084 at various doses (abscissa) on amounts of vigilance states in 5-HT_{2A}^{+/+} (dotted line) and 5-HT_{2A}^{-/-} (solid line) mice. Data (mean ± SEM of 4–6 animals) are expressed as minutes during the first 3 postinjection hours. **p* < 0.05, significantly different from vehicle (0 on abscissa); *post hoc* Bonferroni's–Dunn's test. Error bars represent SEM.

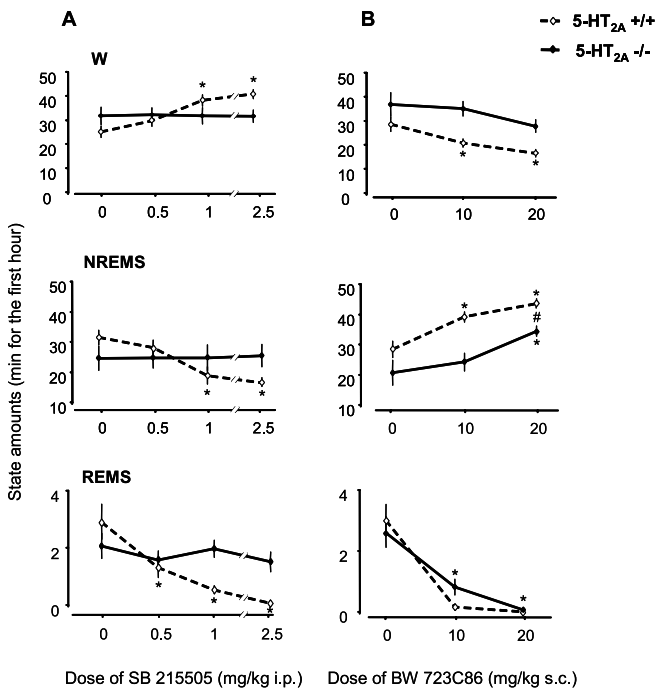


Figure 6. *A, B*, Effects of acute treatment with the 5-HT_{2B} receptor antagonist SB 215505 (*A*) or the 5-HT_{2B} receptor agonist BW 723C86 (*B*) at various doses on amounts of vigilance states in 5-HT_{2A}^{+/+} (dotted line) and 5-HT_{2A}^{-/-} (solid line) mice. Data (mean ± SEM of 5–11 animals per group) are expressed as minutes during the first postinjection hour. **p* < 0.05, significantly different from vehicle (0 on abscissa); #*p* < 0.05, significantly different from respective 5-HT_{2A}^{+/+} value; *post hoc* Bonferroni's–Dunn's test. Error bars represent SEM.

tor expression, we measured the levels of mRNA encoding 5-HT₂ receptor subtypes in several brain regions: hypothalamus, raphe area, striatum, hippocampus, and anterior cortex. The levels of mRNA analyzed by relative quantitative gene expression were

600-fold less for 5-HT_{2B} and twofold less for 5-HT_{2C} receptors than those for 5-HT_{2A} receptors (data not shown). Compared with 5-HT_{2A}^{+/+} mice, 5-HT_{2B} and 5-HT_{2C} mRNA expression was not modified in 5-HT_{2A}^{-/-} mice neither in the hypothalamus (Fig. 7) nor in other areas investigated (data not shown).

As observed previously in intestines of 5-HT_{2A}^{-/-} mice (Fiorica-Howells et al., 2002), residual expression of 5-HT_{2A} mRNA could still be detected in the brain (Fig. 7).

Implication of 5-HT₂ receptors in sigh-related bradypnea and apnea generation

Apneas and sighs were studied during NREMS. Two classes of apneas could be distinguished: those preceded by a sigh within the previous 10 s and those occurring during steady breathing. These events occurred at a similar frequency in 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice (Table 1). Acute blockade of 5-HT_{2A} receptors by MDL 100907 (2 mg/kg, i.p.) in 5-HT_{2A}^{+/+} mice had no effect on the number of sighs but induced a reduction in the index of apneas after sighs, during 3 h after injection of the 5-HT_{2A} receptor antagonist (Table 1).

We then analyzed the time course of ventilation after sighs (Fig. 8*A–C*). The postsigh average respiratory frequency had a biphasic pattern with an early pause immediately after the sigh and a delayed (3–10 s) decrease in respiratory rate (Fig. 8*A, B*). Sigh-related apneas were found in these two phases (Fig. 8*C*). Acute blockade of 5-HT_{2A} receptors by MDL 100907 in 5-HT_{2A}^{+/+} mice was found to suppress the delayed bradypnea after sighs for ~3 h after injection (Fig. 8*D*). In contrast, injection of the 5-HT_{2A} antagonist in 5-HT_{2A}^{-/-} mice did not modify the pattern of respiration after sighs (*n* = 6; data not shown). However, these mutants exhibited larger delayed bradypneas than 5-HT_{2A}^{+/+} mice (Fig. 8*E*), a phenotype that contrasted with the bradypnea-preventive effects of MDL 100907 observed in 5-HT_{2A}^{+/+} mice.

Discussion

This paper provides the first exhaustive characterization of the functional role of 5-HT₂ receptor subtypes in sleep–wakefulness and breathing pattern regulation during sleep in mice. Our results demonstrate an opposite implication of 5-HT_{2A} and 5-HT_{2B} receptors in NREMS regulation. The present study also shows compensatory mechanisms between these two receptor subtypes, because mice lacking 5-HT_{2A} receptors have an apparent decrease in the contribution of 5-HT_{2B} receptors to NREMS control.

5-HT_{2A} and 5-HT_{2B} receptors are involved in NREMS regulation

The NREMS is under the regulatory influence of several neurotransmitters among which 5-HT plays a key role, notably through the activation of 5-HT₂ receptors. In the present work, we found that the selective blockade of 5-HT_{2A} receptors by MDL 100907 induced a dose-dependent increase in NREMS in 5-HT_{2A}^{+/+} mice. Thus, this 5-HT_{2A} receptor antagonist mimicked the

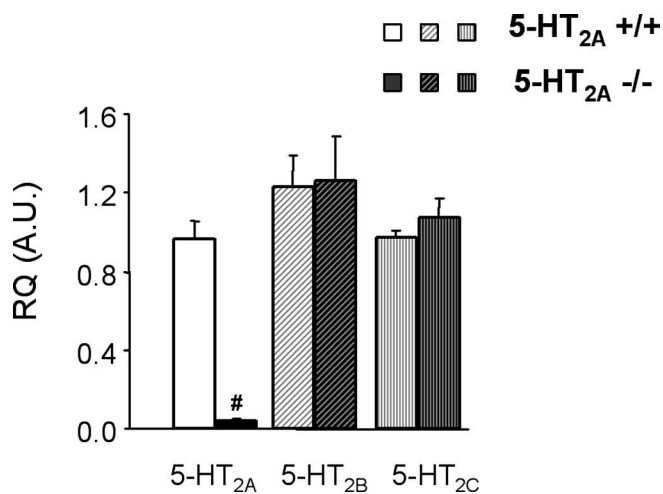


Figure 7. 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} mRNA expression in the hypothalamus of 5-HT_{2A}^{+/+} (white bars) and 5-HT_{2A}^{-/-} (black bars) mice. Results [relative quantity (RQ), mean \pm SEM of 4 animals in each group] are expressed in arbitrary units (A.U.) after normalization to an endogenous reference gene (HPRT). Normalization with GAPDH endogenous gene (see Materials and Methods) yielded similar results. #*p* < 0.05, significantly different from respective 5-HT_{2A}^{+/+} value; unpaired Student's *t* test. Error bars represent SEM.

NREMS-promoting action of wide-spectrum 5-HT₂ antagonists, as reported previously in rats and in humans (Dugovic et al., 1989; Idzikowski et al., 1991; Stutzmann et al., 1992).

In contrast, the blockade of 5-HT_{2B} receptors by SB 215505 produced a decrease in NREMS, an effect also observed in the rat (Kantor et al., 2004). Reciprocally, activation of 5-HT_{2B} receptors by BW 723C86 induced an increase in NREMS. These effects of antagonist and agonist aimed at 5-HT_{2B} receptors are opposite the respective effects of the wide-spectrum 5-HT₂ receptor antagonists, such as ritanserin or RP 62203, and agonists, such as DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] (Dugovic et al., 1989). This apparent discrepancy might be caused by the relatively low affinity of the latter compounds for 5-HT_{2B} versus 5-HT_{2A} and 5-HT_{2C} receptors (Leysen, 2004).

Acute blockade of 5-HT_{2C} receptors by SB 242084 induced no effect on NREMS in 5-HT_{2A}^{+/+} mice, which is surprising because mutant mice lacking 5-HT_{2C} receptors exhibited reduced NREMS amounts (Frank et al., 2002). Such a discrepancy might be attributable to receptor adaptations occurring in knock-outs (see below). Additional study is thus required to understand the involvement of 5-HT_{2C} receptors in NREMS control.

Altogether, our data indicate that 5-HT_{2A} and 5-HT_{2B} receptors are involved, in an opposite way, in NREMS regulation in mice. Opposite involvement of 5-HT₂ receptor subtypes has already been described in the control of various behaviors (Barendsen and Broekkamp, 1990; Kennett et al., 2000; Vickers et al., 2001). Finally, the hypnotic effect of 5-HT_{2B} receptor stimulation may be of interest in the treatment of insomnia.

Functional adaptation of 5-HT₂ receptor subtypes in 5-HT_{2A}^{-/-} mice

As expected, the acute blockade of 5-HT_{2A} receptors by MDL 100907 induced no effects on sleep in 5-HT_{2A}^{-/-} mice. This indicates that the effects obtained in 5-HT_{2A}^{+/+} mice with this compound were specifically caused by its 5-HT_{2A} receptor antagonistic properties and also that the residual 5-HT_{2A} mRNA in 5-HT_{2A}^{-/-} mutants was too low to maintain a physiological contribution to sleep regulation. However, although 5-HT_{2A} receptor blockade produced an increase in NREMS in 5-HT_{2A}^{+/+}

mice, 5-HT_{2A}^{-/-} animals expressed reduced NREMS, suggesting the existence of adaptive mechanisms in these constitutive mutants. Actually, a deficit in 5-HT_{2B} receptor function, consecutive to 5-HT_{2A} receptor gene knock-out, could be partly responsible for this paradoxical decrease in NREMS observed in 5-HT_{2A}^{-/-} mice. Indeed, in these mutants, the blockade of 5-HT_{2B} receptors by SB 215505 or SB 206553 induced no effect, and their activation by BW 723C86 caused a slight increase in NREMS, in sharp contrast with the marked NREMS changes caused by these ligands in 5-HT_{2A}^{+/+} animals. We found that, in both strains of mice, 5-HT_{2B} receptor gene is expressed in the brain (Duxon et al., 1997). However, no changes in 5-HT_{2B} mRNA levels in areas that are implicated in sleep regulation (hypothalamus, raphe) were found in 5-HT_{2A}^{-/-} compared with 5-HT_{2A}^{+/+} mice. Accordingly, 5-HT_{2B} receptor hyposensitivity in mutants is more likely attributable to desensitization rather than downregulation of these receptors.

The adaptive mechanisms leading to the reduced sensitivity of 5-HT_{2B} receptors might take place during development in 5-HT_{2A}^{-/-} mice. Indeed, 5-HT plays a critical role in the maturation of brain and behavior (Gaspar et al., 2003; Ansorge et al., 2004), notably through 5-HT_{2B} receptors (Lauder et al., 2000; Nebigil et al., 2000). Although 5-HT_{2A} and 5-HT_{2C} receptors are involved in a wide range of brain functions, the very low density of 5-HT_{2B} receptor in the adult brain suggests an involvement of these receptors in a more limited set of functions, such as NREMS.

5-HT₂ receptors and REMS

In 5-HT_{2A}^{+/+} mice, the amounts of REMS were decreased after treatment with all of the compounds used in this study. Similar results have been obtained in the rat after administration of various 5-HT₂ ligands, whether agonists or antagonists (Dugovic et al., 1989; Kantor et al., 2004) [but see Benington and Heller (1995), in rats, and Idzikowski et al. (1991), in human]. These data suggest that the effects of 5-HT₂ ligands on REMS are mediated by complex mechanisms. Nevertheless, 5-HT_{2A}^{-/-} mice were no longer responsive to the REMS inhibitory effects of 5-HT_{2A} and 5-HT_{2B} receptor blockade by MDL 100907 and SB 215505, respectively, supporting the idea that these effects might be normally mediated by these receptor subtypes, because the corresponding targets were either absent (5-HT_{2A}) or desensitized (5-HT_{2B}) in mutants. Furthermore, the REMS inhibitory effect of 5-HT_{2C} receptor blockade was more striking in 5-HT_{2A}^{-/-} than 5-HT_{2A}^{+/+} mice, as expected from adaptive changes in 5-HT_{2C} receptors also in the mutants. Our data are consistent with an involvement of 5-HT₂ receptors over the regulation of REMS, but it appears complex and remains to be elucidated.

5-HT_{2A} receptors in psychiatric disorders

The REMS latency was shorter, and the EEG theta rhythm, which parallels the phasic events of REMS (Karashima et al., 2004), was accelerated in 5-HT_{2A}^{-/-} mutants compared with 5-HT_{2A}^{+/+} mice. Such modifications were also found in various depression models in the rat (Adrien et al., 1991; Dugovic et al., 2000). Furthermore, in contrast to 5-HT_{2A}^{+/+} mice, 5-HT_{2A}^{-/-} mutants failed to exhibit an increase in the delta power (a marker of NREMS homeostasis) after sleep deprivation (Tobler et al., 1997; Borbely and Achermann, 1999; Franken et al., 2001; Lena et al., 2004). This sharply contrasts with the exaggerated response to sleep deprivation observed in 5-HT_{2C}^{-/-} mutants (Frank et al., 2002) and suggests differential implications of 5-HT₂ receptor

Table 1. Respiratory parameters during sleep in mice after invalidation of the gene coding for or pharmacological blockade of 5-HT_{2A} receptors

	Respiration rate during REMS	Respiration rate during NREMS	Sigh index per minute of NREMS	Spontaneous apnea index per minute of NREMS	Postsigh apnea index per minute of NREMS
Baseline conditions					
5-HT _{2A} ^{+/+} (10:00 A.M. to 4:00 P.M.) (n = 14)	3.5 ± 0.1 Hz	3.0 ± 0.1 Hz	0.56 ± 0.08	0.13 ± 0.05	0.41 ± 0.10
5-HT _{2A} ^{-/-} (10:00 A.M. to 4:00 P.M.) (n = 12)	3.5 ± 0.1 Hz	2.8 ± 0.1 Hz	0.54 ± 0.06	0.13 ± 0.06	0.62 ± 0.14
MDL 100907 (2 mg/kg, i.p.)					
5-HT _{2A} ^{+/+} (10:00 A.M. to 1:00 P.M.) (n = 7)	109 ± 16%	124 ± 10%	144 ± 18%	99 ± 53%	26 ± 9%*

Postsigh apneas were preceded by a sigh within the 10 previous seconds, whereas spontaneous apneas were not preceded by a sigh. 5-HT_{2A}^{+/+} and 5-HT_{2A}^{-/-} mice exhibited similar respiratory parameters under baseline conditions. In 5-HT_{2A}^{+/+} mice, acute treatment with the 5-HT_{2A} receptor antagonist MDL 100907 (2 mg/kg, i.p.) reduced the postsigh apnea index. The effects of the drug are shown as percentage of the value after vehicle injection. Data are expressed as mean ± SEM of the number of independent determinations indicated in parentheses. *p < 0.05, significantly different from vehicle; paired Student's *t* test.

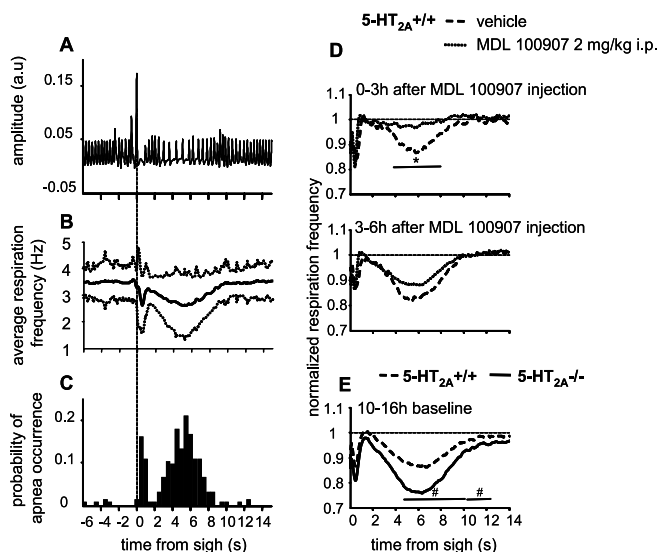


Figure 8. Implication of 5-HT_{2A} receptors in the dynamics of breathing after sighs. Sighs are followed by both an immediate and a delayed decrease in respiration frequency, and apnea occurs in these two phases of bradypnea. **A**, Example of plethysmograph recording of a sigh (0 on abscissa). a.u., Arbitrary units. **B**, Average rate of respiration after a sigh. Dotted lines correspond to ± SD. **C**, Distribution of apneas after a sigh; the counts of apneas have been normalized to the number of sighs (bin size, 0.5 s). **A–C** are from the same 5-HT_{2A}^{+/+} mouse recorded from 10:00 A.M. to 4:00 P.M. **D–F**, The delayed decrease in breathing frequency after a sigh is almost completely suppressed after 5-HT_{2A} receptor blockade by MDL 100907 in 5-HT_{2A}^{+/+} mice but is more pronounced in 5-HT_{2A}^{-/-} mutants. **D**, Average changes in respiration frequency after a sigh in 5-HT_{2A}^{+/+} during the first 3 h (between 10:00 A.M. and 1:00 P.M.) and during the next 3 h (between 1:00 P.M. and 4:00 P.M.) after MDL 100907 (2 mg/kg, i.p.) administration (n = 7). The rate of breathing is normalized to the rate before the sigh. **E**, Average changes in spontaneous respiration frequency after a sigh in 5-HT_{2A}^{+/+} (n = 14) and 5-HT_{2A}^{-/-} (n = 12) mice. Recordings were made between 10:00 A.M. and 4:00 P.M. #*p < 0.05, significantly different from vehicle or 5-HT_{2A}^{+/+} mice, respectively; Student's *t* test.

subtypes in NREMS homeostasis. Interestingly, a decrease in NREMS fragmentation was observed in mutants after sleep deprivation, which suggests that these mice still express NREMS homeostasis and which is consistent with a differential regulation of delta power and fragmentation in mice (Franken et al., 1999).

All of the impairments in 5-HT_{2A}^{-/-} mutants are evocative of those observed in depressed patients (Benca, 2000) who also exhibit 5-HT₂ receptor functional alterations (Bonnier et al., 2002). Additional behavioral investigations have to be performed to assess whether 5-HT_{2A}^{-/-} mice actually exhibit depression-like deficits in validated paradigms.

5-HT_{2A} receptors and respiration

Mice have been used recently as a model for studying the respiratory regulation in relation to pathologies such as sleep apnea and sudden infant death syndrome (Nakamura and Kuwaki,

2003). In mice, the large majority of bradypneas/apneas during NREMS were found to occur either immediately after the sighs (Nakamura and Kuwaki, 2003; Lena et al., 2004) or within the subsequent 10 s (this study) and are presumably central apneas (Nakamura et al., 2003) (supplemental Fig. A, available at www.jneurosci.org as supplemental material). Because 5-HT_{2A} receptor stimulation excites the respiratory motor system at the level of the pre-Bötzinger complex (Pena and Ramirez, 2002) or the hypoglossal nucleus (Jeleu et al., 2001; Fenik and Veasey, 2003), 5-HT_{2A} receptor blockade was expected to increase the occurrence of central apneas. Surprisingly, we found the opposite (with MDL 100907) in 5-HT_{2A}^{+/+} mice, but only apneas after sighs were decreased. Indeed, sighs are known to trigger the Hering-Breuer reflex, which produces a transient bradypnea via the afferent vagal system. The latter system conveys the inhibitory action of 5-HT on respiration through activation of 5-HT₂ receptors (Yoshioka et al., 1992). Furthermore, activation of 5-HT₂ receptors at the level of the nucleus tractus solitarius elicits a baroreceptor-like reflex response (Merahi et al., 1992) that typically comprises a decrease in respiration frequency. Thus, 5-HT_{2A} receptor blockade could reduce the serotonergic contribution to bradypnea/apnea generation (Hilaire et al., 1993) in the dorsal vagal complex.

5-HT_{2A}^{-/-} mice presented no modifications in respiratory parameters during NREMS, except for a stronger delayed (3–7 s) bradypnea after sighs, which contrasted markedly with the action of 5-HT_{2A} receptor blockade in 5-HT_{2A}^{+/+} mice. In this case also, adaptive mechanisms might explain the discrepancy between 5-HT_{2A} pharmacological blockade and 5-HT_{2A} gene knock-out, especially because 5-HT contributes to the maturation of the neuronal networks controlling breathing (Bou-Flores et al., 2000).

In conclusion, the combined use of gene knock-out and specific pharmacological treatments allowed us to assess the differential contribution of 5-HT₂ receptor subtypes to sleep and respiratory control in mice. Our results also showed that genetic invalidation triggered adaptations in these systems and produced a phenotype opposite to the effect of acute pharmacological blockade. Additional work is required to determine whether chronic treatments could trigger similar adaptations that should be taken into account when considering 5-HT₂ receptors as potential therapeutic targets.

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