Behavioral/Systems/Cognitive

# β2-Containing Nicotinic Receptors Contribute to the Organization of Sleep and Regulate Putative Micro-Arousals in Mice

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**The cholinergic system is involved in arousal and in rapid eye movement sleep (REMS). To evaluate the contribution of nicotinic acetylcholine receptors (nAChRs) to these functions, we studied with polygraphic recordings the regulation of sleep in mice lacking the β2 subunit gene of the nAChRs, a major component of high-affinity nicotine binding sites in the brain. Nicotine (1–2 mg/kg, i.p.) increased wakefulness in wild-type but not knock-out animals, indicating that β2-containing nAChRs mediate the arousing properties of nicotine. Under normal conditions, the β2−/− mice displayed the same amounts of waking, non-REM sleep (NREMS) and REMS as their wild-type counterparts. However, they exhibited longer REMS episodes and a reduced fragmentation of NREMS by events characterized notably by a transient drop in EEG power and frequently associated with EMG activation, tentatively referred to as micro-arousals. Respiration monitoring showed that these events were accompanied with, but not caused by, breathing irregularities. Sleep deprivation of β2−/− mice resulted in a normal increase in REMS episode duration and NREMS δ power but yielded a reduction of the number of micro-arousals in NREMS. In contrast, in β2−/− mice, a 1 hr immobilization stress failed to produce the normal rebound in REMS in the following 12 hr and, instead, was associated with increased NREMS fragmentation and sustained corticosterone levels. Our results show that the β2-containing nAChRs contribute to the organization of sleep by regulating the transient phasic activity in NREMS, the REMS onset and duration, and the REMS-promoting effect of stress.**

*Key words:* **nicotinic receptors; sleep; arousal; mice; sleep deprivation; stress**

## Introduction

The regulation of the sleep–wake cycle is a complex process involving several brain structures and neurotransmitters (Steriade and McCarley, 1990; Pace-Schott and Hobson, 2002). The cholinergic system is classically considered to be a positive effector of rapid eye movement sleep (REMS) at the pontine level (Sakai et al., 2001) and to contribute to cortical arousal through its ascending component (Sarter and Bruno, 2000; Jones, 2003). Indeed, acetylcholine (ACh) release in the forebrain is higher during REMS and wake (Jasper and Tessier, 1971; Marrosu et al., 1995). The contribution of nicotinic ACh receptors (nAChRs) to this dual function of the cholinergic system is still primarily unknown.

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Nicotine intake has long been known to cause cortical electroencephalography (EEG) desynchronization (Yamamoto and Domino, 1965). Nicotine increases arousal and attention (Lawrence et al., 2002; Levin, 2002), and the nicotinic antagonist mecamylamine causes drowsiness and decreases the performance in attention-demanding tasks (Pickworth et al., 1997). Although nicotine injections acutely increase waking, chronic nicotine treatment results in higher amounts of REMS (Salin-Pascual et al., 1999), indicating that nAChRs may intervene at different levels in the control of vigilance. Moreover, mutations in the α4 and β2 subunits of nAChRs have been linked to nocturnal frontal lobe epilepsies that occur during light slow-wave sleep (Sutor and Zolles, 2001). The importance of nAChRs in sleep regulation has also been suggested by their implication in the sudden infant death syndrome (SIDS) (Slotkin, 1998).

The nAChRs are pentameric ligand-gated ion channels with subunits that are encoded by 10 different genes in the nervous system (Le Novère et al., 2002). Mice lacking the β2 subunit gene (β2−/− mice) are devoid of high-affinity nicotine binding sites in the CNS (Picciotto et al., 1995; Zoli et al., 1998) and exhibit a loss or reduction of nicotine-elicited currents in neurons from various brain regions (Picciotto et al., 1995; Zoli et al., 1998; Marubio et al., 1999; Klink et al., 2001). Therefore, these mice offer the opportunity to investigate the contribution of nAChRs

to sleep and wakefulness. Indeed, recent work has demonstrated that, compared with wild-type (WT) mice, they exhibit an increased ventilatory response to a hypoxic challenge in sleep associated with a reduction of transient movements, which suggests that nAChRs contribute to awakening under these conditions (Cohen et al., 2002). The present study aims at testing the involvement of nAChRs in the regulation of vigilance stages and in breathing pattern during sleep. For this purpose, we performed polygraphic recordings in $\beta2-/-$ and WT mice under baseline conditions, as well as in response to nicotine injections and to behavioral manipulations (sleep deprivation and immobilization stress) known to alter sleep patterns (Boutrel et al., 2002).

## Materials and Methods

*Animals.* The animals used were adult male, age-matched, WT and homozygous $\beta2-/-$ mice deleted for the $\beta2$ nAChR subunit gene. Specifically, C57BL/6J mice (IFFA-Credo, Les Oncins, France) were compared with $\beta2-/-$ mutant siblings from parents backcrossed for 12 generations to C57BL/6J inbred mice. The animals were housed under a 12 hr light/dark (lights on 7:00 A.M. to 7:00 P.M.) cycle with *ad libitum* food and water. All experiments followed the European Economic Community (EEC) Directive (86/609/EEC), and every effort was made to minimize the number of animals used and any pain and discomfort.

*Surgical procedure.* Animals were implanted under xylazine/ketamine anesthesia (5 and 100 mg/kg, i.p., respectively). The electrodes were made of enameled nichrome wire (diameter, 100 $\mu$m). These electrodes were positioned in holes perforated in the skull (1 mm caudal and 1 mm lateral to bregma, and medial reference electrode 1 mm caudal to $\lambda$) for epidural EEG. A pair of nichrome wire electrodes (with the insulant removed over ~1 mm) was inserted in the neck muscles for electromyographic (EMG) recording (Boutrel et al., 1999). A fraction of the animals also received a pair of subcutaneous stainless steel electrodes located under the chest and at the level of the tail for electrocardiogram (ECG) recordings in the plethysmograph experiments. The animals were allowed to recover at least 10 d after the surgical procedure.

*Recordings.* The mice were connected to the recording system with a light-weight cable and a swivel allowing free movements in the cage. Animals were recorded by groups of four, usually two WT and two $\beta2-/-$ mice. The signals were fed into an Embla device (Medcare, Reykjavik, Iceland) for differential amplification, sampled at 100 Hz (EEG, pressure signal in plethysmographic experiments) or 200 Hz (EMG, ECG), and recorded with the software Somnologica (Medcare, Reykjavik, Iceland). Recordings started after a 48 hr habituation period and were collected during 48 consecutive hours for analysis of basal sleep–waking patterns.

Sleep deprivation was performed by moving the cage into a new room and by enriching the cage with new objects during 6 hr (7:00 A.M. to 1:00 P.M.). The animals were visually monitored and new stimuli were added, or the animals were gently handled when behavioral signs of drowsiness were observed. The immobilization stress was performed by wrapping the mice in a nylon-covered metal mesh during 1 hr (6:00–7:00 P.M.). Matched control recordings were obtained for each animal by disconnecting and reconnecting back the animals to the recording leads at the same time of the day as in the sleep deprivation and immobilization stress conditions. These controls were performed first, and in all cases, the two protocols were inter-spaced by at least 1 week. For pharmacological experiments, animals received the drug (nicotine bitartrate in saline injected intraperitoneally) at 10:00 A.M. Two successive injections of nicotine were separated by at least 2 d. Most animals underwent either the deprivation and stress protocols or the pharmacological treatments; otherwise, the drug injections were always performed last.

*Plethysmography.* Respiration was monitored with a two-chambers, 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 by a Capnomac Ultima (Datex, Helsinki, Finland) and maintained within physiological range. All of the signals were recorded with the same Embla device as above. The recordings were performed between 10:00 A.M. and 4:00 P.M. Cycle by cycle tidal volume and breathing period were evaluated with custom Matlab (MathWorks, Natick, MA) routines. "Sighs" were isolated as single respiratory cycles of amplitude higher than two times the average amplitude over a baseline taken over the 30 preceding cycles (~10 sec) and followed by a pause; this criterion corresponds approximately to events deviating more than five times the SD of the cycle amplitude. The respiratory pause that followed immediately after the sighs qualified as an apnea (see below) in only 5–15% of the cases. Apnea is defined as cessation of breathing during at least twice the average respiratory cycle duration in non-REM sleep (NREMS). Apneas were scored independently from desaturation events. The measurement of effort was not recorded; however, it is only required to determine the mechanism of the apneas (Martin et al., 1985). Because a large fraction of apnea occurred in the seconds following a sigh, we considered separately "spontaneous apnea," defined as apnea not preceded by sighs in the preceding 15 sec. The respiratory flow is the ratio of tidal volume over the respiratory cycle duration. The traces of each candidate sigh and apnea were visually inspected to avoid artifacts. We only considered sighs and apneas in NREMS. The heart rate was obtained from the R-R intervals in the ECG.

*Serum corticosterone assay.* Serum corticosterone levels were measured immediately and 4 hr after the immobilization stress (6:00–7:00 P.M.). For this purpose, animals were decapitated, and blood was collected and centrifuged at 4000 rpm (4°C) for 25 min. Separated serum was stored at −20°C until additional analysis. Corticosterone was quantified by radioimmunoassay (ICN Biochemicals, Orsay, France).

*Scoring and analysis.* The three vigilance states, wake, NREMS, and REMS, were scored manually by 15 sec segments using primarily the power spectrum of EEG as well as the EMG activity (Fig. 1). WT and $\beta2-/-$ animals had similar EEG power spectra across the different vigilance stages, consistent with the spectra published for C57BL/6J mice (Fig. 1*C*) (Franken et al., 1998; Huber et al., 2000). We also scored events in NREMS corresponding to 5–15 sec drop of at least 50% of the EEG power with a spectral content mostly in the $\delta$ band (1–5 Hz). These events were scored as micro-arousals (MA) (see Discussion). They are distinct from single episodes in NREMS, where the EEG spectral content increases in the $\theta$ band (6–9 Hz) with an EMG activation and that were thus scored as "short awakenings" and counted as Wake. Events corresponding to our short awakenings and micro-arousals are presumably pooled into "brief awakenings" in other studies in the rat (Franken et al., 1991) and in the mouse (Tobler et al., 1996; Franken et al., 1999). The reliability of the score was assessed by two different persons scoring independently 24 hr from one WT and one $\beta2-/-$ mouse and yielded a correspondence of 95% in the score (WT, 95.5%; $\beta2-/-$, 95.7%). The accuracy of the estimates of sleep state amount and duration was verified by rescoring with a resolution of 5 sec, 6 hr of recordings from seven WT and seven $\beta2-/-$; the average difference in amounts and episode durations (estimated over periods of 3 hr as in most of the present study) between the 5 sec score and the 15 sec score was in the interval of $\pm3\%$. The analysis was made using Matlab (MathWorks) routines and R (http://cran.r-project.org/mirrors.html), a free implementation of S-plus, for statistical analysis. Amounts and durations were evaluated over 3 hr intervals. Significance was tested by performing an ANOVA using a linear mixed effect model with repeated measures over time (3 hr segments) and treatment (sleep deprivation, stress), when appropriate, followed by unpaired or paired *t* tests as needed. The symbols used in the figures indicate the following: $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$ for paired *t* tests (control vs treatment); and $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for unpaired *t* tests (difference in genotypes). Symbols in parenthesis are used to indicate significant *t* test probabilities but nonsignificant ANOVA. Data are presented as mean ± SEM.

## Results

### Nicotine injections transiently increase wakefulness in WT but not in $\beta2-/-$ mice

In WT animals, nicotine at 1–2 mg/kg, intraperitoneally, caused a significant increase in the amounts of wakefulness in the first hour after the injection (Fig. 2). These doses of nicotine also

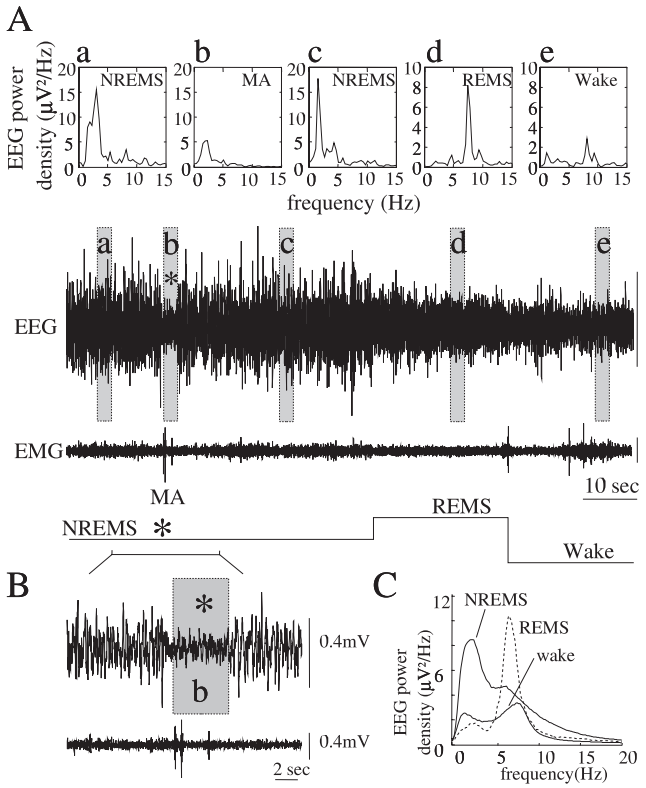


**Figure 1.** Example of recording and scoring of an MA. *A*, Polygraphic traces of EEG, EMG, and EEG spectrum in a WT mouse. Calibration bars on the right correspond to 0.4 mV. Scoring is performed with three levels: wake, REMS, and NREMS. The transient reduction in EEG power accompanied by a slight EMG activation during NREMS is scored as MA (*). The power spectrum of the EEG (estimated with 256-point fast Fourier transform and using a Hanning window) for 5 sec segments at the times indicated by the shaded area labeled a–e on the EEG trace are plotted on top in the graphs with the corresponding letters. The spectrum during the MA is characteristic of NREMS but has a low power. *B*, Detail of EEG and EMG for the seconds preceding and following the MA, with low-frequency oscillations of small amplitude during the MA. *C*, EEG spectral content from a knock-out $\beta2-/-$ mouse for the three states scored over 24 hr. These spectra are similar to the ones of WT mice (C57BL/6J) used in this study and previously (Franken et al., 1998; Huber et al., 2000). The relative amplitude of the peaks was found to vary from animal to animal, presumably depending on the implantation site.

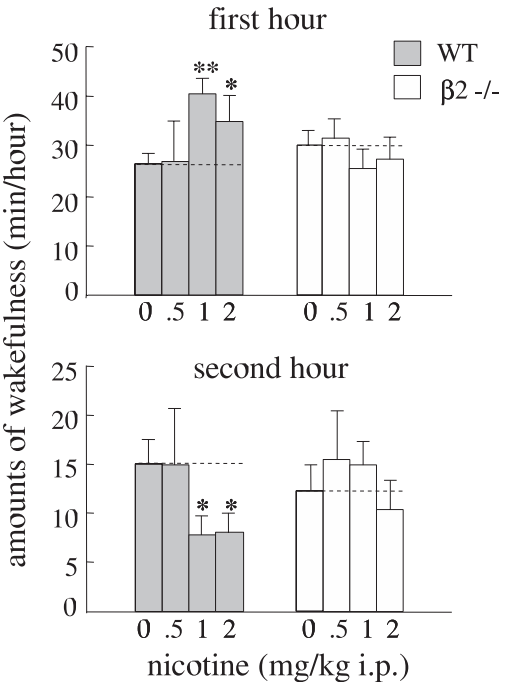


**Figure 2.** Nicotine injection (intraperitoneally) alters the amount of wake in WT but not in $\beta2-/-$ mice. The injections were performed at 10:00 A.M. Compared with saline injection (0 on abscissa), nicotine at 1 mg/kg (10 WT; 7 $\beta2-/-$) and 2 mg/kg (8 WT; 7 $\beta2-/-$) increases the amount of wake during the first hour. A rebound decrease is observed in the second hour in WT but not in $\beta2-/-$ mice. Nicotine (0.5 mg/kg) does not affect the amount of wake (5 WT; 6 $\beta2-/-$). The data are taken from 14 WT and 14 $\beta2-/-$ mice. *$p < 0.05$; **$p < 0.01$; significantly different from control; paired Student's *t* test.

caused a significant decrease in NREMS and REMS during the first hour after the injection (NREMS: vehicle, 31 ± 2 min; nicotine 1 mg/kg, 19 ± 3 min, $p < 0.01$; and nicotine 2 mg/kg, 24 ± 5 min, $p < 0.05$; REMS: vehicle, 2.6 ± 0.4 min; nicotine 1 mg/kg, 0.5 ± 0.3 min, $p < 0.001$; nicotine 2 mg/kg, 0.9 ± 0.5 min, $p < 0.001$). Thereafter, a "rebound" decrease in wake could be observed during the second hour (Fig. 2), and this effect was associated with an increase in NREMS amounts (vehicle, 39 ± 2 min; nicotine 1 mg/kg, 46 ± 1 min, $p < 0.001$; nicotine 2 mg/kg, 45 ± 1 min, $p < 0.05$). No remaining effect was observed in the third and fourth hours after the injection. In knock-out $\beta2-/-$ animals, nicotine failed to change the amounts of wake and sleep at any dose tested in the first 2 hr. However, a consistent decrease in wake was observed during the third hour for the highest dose of nicotine (vehicle, 17 ± 3 min; nicotine 2 mg/kg, 7 ± 1 min, $p < 0.001$). This effect was not further investigated. These results indicate that the awakening effects of nicotine are mediated by $\beta2$-containing nAChRs ($\beta2^*$ nAChRs).

### NREMS and REMS are more stable in $\beta2-/-$ mice

Under basal conditions, both groups of mice exhibited a diurnal sleep–wake rhythm characteristic of C57BL/6J mice (Franken et al., 1999; Huber et al., 2000). WT ($n = 14$) and $\beta2-/-$ ($n = 18$) mice expressed similar amounts of wake ($F_{(1,30)} = 0.020$; $p = 0.89$), NREMS ($F_{(1,30)} = 0.022$; $p = 0.88$), and REMS ($F_{(1,30)} = 0.0037$; $p = 0.95$).

Although the amounts of various vigilance states were similar, the organization of sleep differed significantly between the strains (Figs. 3, 4). REMS episodes were markedly longer in $\beta2-/-$ animals ($F_{(1,30)} = 11.5$; $p = 0.0019$) (Fig. 3), notably during the night. The fragmentation of NREMS by short awakenings (up to 15 sec of wake between NREMS episodes) was similar in both strains (Fig. 4*A*) ($F_{(1,30)} = 0.82$; $p = 0.37$). In contrast, the fragmentation of NREMS by MA (expressed as the number of MA per minute of NREMS) was significantly lower in $\beta2-/-$ animals ($F_{(1,30)} = 17.2$; $p = 0.0003$) (Fig. 4*A*). Interestingly, the duration of sustained NREMS segments, defined as segments of NREMS not interrupted by MA, was particularly longer in $\beta2-/-$ animals for segments of sleep preceding REMS (Fig. 4*B*). The sleep that immediately precedes REMS (pre-REMS) is characterized by shifts in the EEG power content (Benington and Heller, 1994; Franken et al., 1998; Franken, 2002) (Fig. 4*C*). WT and $\beta2-/-$ mice showed a similar time course of spectral changes in pre-REMS (Fig. 4*C*), indicating that the difference between the strains in the duration of sustained NREMS before REMS is more likely to correspond to a stabilization of NREMS rather than a change in the duration of the pre-REMS. Overall, these results indicate that REMS and NREMS are more stable in $\beta2-/-$ animals.

### Similarity of events fragmenting NREMS in WT and $\beta2-/-$ mice

The MA interrupting the NREMS were composite events associating a diminution in EEG power with several peripheral signs

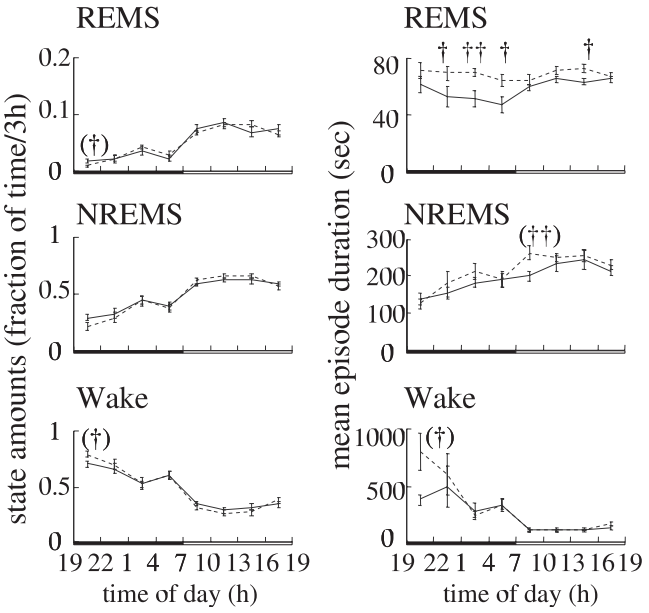


**Figure 3.** $\beta2-/-$ mice express normal amounts of Wake, NREMS, and REMS but longer episodes of REMS. Left column, WT and $\beta2-/-$ mice exhibit similar amounts (fraction of time) of Wake, NREMS, and REMS. The data are expressed for 3 hr time bins (but similar results are obtained for 1 hr time bins). When ANOVA failed to detect any difference (see Results), significance with the *t* test is indicated in parenthesis. Right column, The duration of REMS episodes is longer in $\beta2-/-$ mutants than in WT mice, whereas NREMS and Wake episodes have a similar duration in both strains. The data are taken from 14 WT and 18 $\beta2-/-$ mice. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$; significant difference between strains; unpaired Student's *t* test.

such as EMG activation, heart beat acceleration, and respiratory events (Fig. 5*A*). The relationship between EEG power changes and EMG activation was assessed in a subset of recordings with little or no artifacts on the EMG channel during MA. MA were associated with a neck EMG activation in ~85% of the cases (WT, 87.3 ± 5.5% ($n = 7$); $\beta2-/-$, 86.6 ± 2.4% ($n = 8$); $p = 0.90$). The onset of EMG activation coincided generally with the beginning of the reduction in EEG power (Fig. 5*B*, left). EMG activation in NREMS was associated with a similar reduction in EEG power in WT and $\beta2-/-$ animals [average EEG power over 2 sec centered on 3–15 sec long EMG activation was: WT, 37.7 ± 3.1% ($n = 7$); $\beta2-/-$, 40.2 ± 3.5% ($n = 8$); $p = 0.46$, of the baseline EEG power taken 20–30 sec before EMG activation] with no increase of power in the $\theta$ band visible in averaged spectrograms (data not shown). During NREMS, EMG activation was thus associated with a similar drop in EEG power in both strains.

The observation that hypopnea results in less behavioral activation in $\beta2-/-$ mutants than in WT mice (Cohen et al., 2002) raised the hypothesis that the decreased NREMS fragmentation observed in $\beta2-/-$ mice might result from a diminution of transient stage shifts in response to spontaneous hypopnea/apnea during NREMS. This led us to run a series of experiments combining respiration monitoring with polygraphic recordings.

Spontaneous apneas in NREMS rarely lasted more than 1 sec [average duration: WT, 0.68 ± 0.03 sec ($n = 7$); $\beta2-/-$, 0.75 ± 0.03 sec ($n = 5$); $p = 0.11$] and were associated with a mild reduction of EEG power (Fig. 5*B*, right) [average EEG power over 2 sec around the apnea was, respectively: WT, 81.5 ± 7.4% ($n = 7$); $\beta2-/-$, 69.9 ± 3.1% ($n = 5$); $p = 0.17$ of the EEG power 30–20 sec before the apnea]. In contrast, sighs in NREMS were generally associated with a strong reduction of EEG power of similar amplitude in both strains (Fig. 5*B*, middle) [average EEG power over 2 sec around the sigh was: WT, 43.0 ± 2.9% ($n = 7$); $\beta2-/-$, 42.7 ± 3.2% ($n = 5$); $p = 0.96$, of the EEG power 30–20 sec before the sigh] but tended to be followed by a stronger decrease in EEG power in $\beta2-/-$ mice (Fig. 5*B*). In both WT and $\beta2-/-$ mice, the sighs (and apneas) occurred in an average of 5–10 sec after the beginning of the decline in EEG power (Fig. 5*B*), and average spectrograms failed to evidence a change in EEG spectrum during these events. The MA were accompanied by sighs in half of the cases [WT, 48.3 ± 4.8% ($n = 7$); $\beta2-/-$, 55.7 ± 11.3% ($n = 5$); $p = 0.48$] and with spontaneous apnea in a smaller number of cases [WT, 15.9 ± 4.7% ($n = 7$); $\beta2-/-$, 21.0 ± 4.7% ($n = 5$); $p = 0.42$]. In summary, apneas had little effect by themselves on EEG. The main respiratory events associated with the MA were the sighs, and these events occurred after the decline in EEG power had started. An abnormal coupling between respiration and MA generation in $\beta2-/-$ mice is thus unlikely to be the cause of the lower NREMS fragmentation in these mutants compared with WT mice.

The increase in heart rate tended to occur in a late phase of the MA. Sigh-triggered average showed that the peak of heart rate was reached within the second that follows the sighs (data not shown), with a similar delay in WT and $\beta2-/-$ mice ($p = 0.16$). Interestingly, the amplitude of the maximal increase in beat per min (bpm) was relatively larger in $\beta2-/-$ mice [increase at peak relative to the baseline heart rate taken 30–20 sec before the sigh: WT, +13 ± 2% ($n = 5$); $\beta2-/-$, +29 ± 3% ($n = 5$); $p < 0.01$], but this could be caused in part by the lower baseline in $\beta2-/-$ animals [WT, 548 ± 17 bpm ($n = 5$); $\beta2-/-$, 459 ± 10 bpm ($n = 5$); $p < 0.01$]. In summary, the $\beta2-/-$ animals exhibited a normal or slightly enhanced sympathetic activation during the MA.

### Sleep deprivation is followed by a marked reduction in NREMS fragmentation in $\beta2-/-$ mice

Sleep deprivation increases the sleep pressure and challenges the homeostatic processes of sleep regulation. After a 6 hr sleep deprivation (by enriching the environment and by gentle handling), WT ($n = 8$) and $\beta2-/-$ ($n = 7$) animals showed a similar transient increase in the EEG power in the $\delta$ band, a marker of NREMS homeostasis (Borbely and Achermann, 1999), during the first hour of recovery (Fig. 6*A*) (WT, +43 ± 15%, $p < 0.05$; $\beta2-/-$, +55 ± 15%; $p < 0.01$; no significant difference between the strains: $F_{(1,13)} = 0.067$; $p = 0.80$). Sleep deprivation also influenced sleep episode duration; REMS episode duration (Huber et al., 2000) increased similarly in WT and $\beta2-/-$ mice during the first 3 hr of the recovery period (Fig. 6*B*) (WT, +51 ± 17%; $\beta2-/-$, +40 ± 19%; $p = 0.62$). Moreover, sleep deprivation caused a marked decrease in the frequency of short awakenings (Franken et al., 1999; Huber et al., 2000) during the first 3 hr period of recovery (WT, −39 ± 6%; $\beta2-/-$, −37 ± 2%; $p = 0.84$), these short awakenings occurring primarily between REMS and NREMS. In contrast, the fragmentation of NREMS by MA was strongly decreased in $\beta2-/-$ but not in WT animals (Fig. 6*C*) (first 3 hr: WT, −10 ± 12%; $\beta2-/-$, −47 ± 5.9%; $p < 0.05$). Overall, the $\beta2-/-$ animals exhibited normal homeostatic changes in response to sleep deprivation with the exception of a marked decrease in NREMS fragmentation by MA.

### Immobilization stress disrupts sleep in $\beta2-/-$ mutants

Depending on its intensity and duration, a stress may either promote or disrupt sleep (Marinesco et al., 1999). We examined whether the stabilization of sleep could also be promoted in $\beta2-/-$ animals after an immobilization stress (Fig. 7) as de-

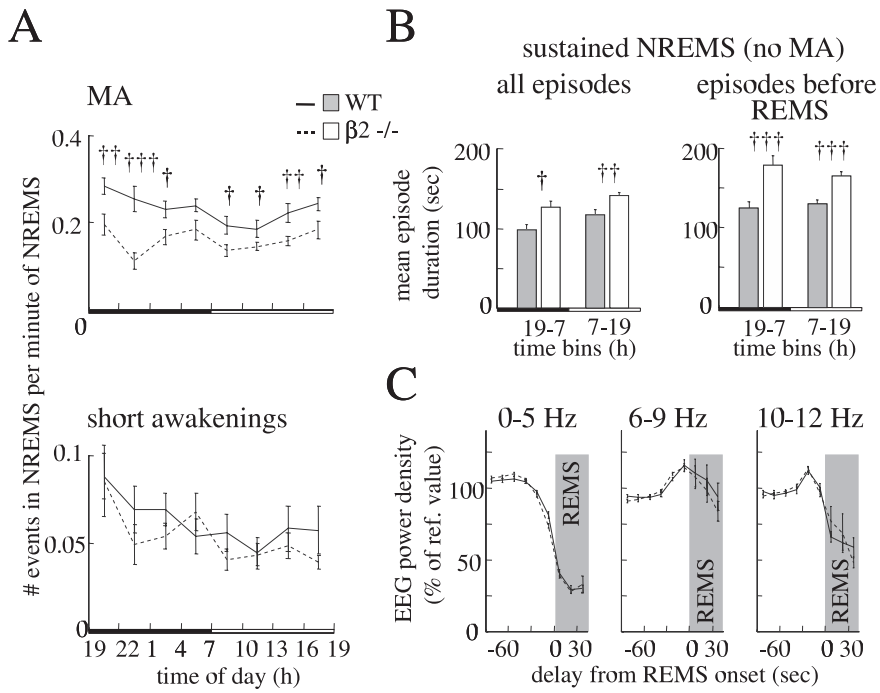


**Figure 4.** The NREMS in $\beta2-/-$ mice is less fragmented by MA, notably for the NREMS immediately preceding REMS. *A*, The fragmentation of NREMS by MA is significantly lower in $\beta2-/-$ compared with WT mice, whereas the fragmentation of NREMS by short awakenings is similar in both strains. *B*, The segments of sustained NREMS (i.e., devoid of MA) are significantly longer in $\beta2-/-$ notably for the NREMS that immediately precedes REMS. *C*, The power content of the EEG shows a similar time course in both strains during the 30 sec that immediately precedes REMS, indicating that the pre-REMS is not altered in $\beta2-/-$ mice. The data are taken from 14 WT and 18 $\beta2-/-$ mice. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$; significant difference between strains; unpaired Student's *t* test.

scribed previously for WT mice with the same genetic background (Meerlo et al., 2001). One hour immobilization of WT mice ($n = 9$) significantly increased the amount of REMS during the following dark period ($F_{(1,56)} = 7.76$; $p = 0.0073$) but affected neither the amount nor the fragmentation of NREMS. When subjected to the same stress, $\beta2-/-$ animals ($n = 8$) exhibited a different response during the following night, notably with no increase in the amounts of REMS ($F_{(1,49)} = 0.19$, $p = 0.66$; with a significant genotype-treatment interaction $F_{(1,105)} = 4.48$, $p = 0.037$) (Fig. 7*A*) and an increased fragmentation of NREMS by MA ($F_{(1,48)} = 14.0$, $p = 0.0005$; with a significant genotype-treatment interaction $F_{(1,103)} = 14.7$, $p = 0.0002$) (Fig. 7*B*). There was also a small increase in the frequency of short awakenings during the night, but this effect did not differ between the two strains (10:00 P.M. to 7:00 A.M.: WT, $+28.8 \pm 18.3\%$; $\beta2-/-$, $+14.1 \pm 14.8\%$; genotype-treatment interaction term: $F_{(1,103)} = 0.89$, $p = 0.35$). Mild effects of stress on sleep fragmentation could be observed during the following lights-on period (7:00 A.M. to 7:00 P.M.) (Fig. 7*B*), but they were similar in WT and $\beta2-/-$ mice. In summary, after a 1 hr immobilization stress, the $\beta2-/-$ animals displayed a reduction in the quality of NREMS instead of the increase in REMS that occurs in WT animals.

The immobilization stress is known to induce a large response of the stress hormonal system. The REMS rebound is thought to require a return of corticosterone to baseline levels (Boutrel et al., 2002). We then examined the time course of serum corticosterone levels after the immobilization. We measured the corticosterone in WT and $\beta2-/-$ mice ($n = 8$ for each condition) at the end and 4 hr after an immobilization stress. Both genotypes showed a similar immediate response of corticosterone to the stress (Fig. 7*C*). These levels were decreased in the WT and $\beta2-/-$ mice 4 hr after the immobilization session, but they remained higher than control values in the $\beta2-/-$ mice ($p < 0.05$) (Fig. 7*C*). Thus, the response of the hormonal system was not terminated in $\beta2-/-$ mice at the time where the hormonal response was terminated, and the REMs rebound occurred in WT mice.

## Discussion

Our results demonstrate that $\beta2^*$nAChRs are involved in processes that control NREMS and REMS stability (but not homeostasis): generating the MA that fragment NREMS, limiting REMS continuity, and facilitating the sleep-promoting effects of an acute stress.

### $\beta2^*$nAChRs promote waking states

As previously reported in the rat (Salin-Pascual et al., 1999), the injection of nicotine produced an increase in the time spent in the awake state in WT mice. In the latter species, it also caused a rebound increase in sleep during the second hour after the injection. This secondary change was not described in the rat and might correspond to either a recovery from the sleep deficit caused by nicotine in the first hour or a delayed pharmacological action of nicotine and its metabolites. These two effects of nicotine were not observed in $\beta2-/-$ mutants. In contrast with these pharmacological data, the amounts of spontaneous wake, NREMS, and REMS were similar in WT and $\beta2-/-$ mice, thereby suggesting that the physiological activation of $\beta2^*$nAChRs does not contribute critically to overall sleep regulation. Instead, our experiments indicate that $\beta2^*$nAChRs play a role in shaping sleep episodes. Notably, the frequency of interruptions of NREMS by MA, particularly before REMS, is decreased, whereas REMS duration is increased in $\beta2-/-$ animals.

### $\beta2^*$ nAChRs are involved in transient reduction of NREMS depth

NREMS is segmented by MA, transient events characterized by a decreased EEG power accompanied by polygraphic changes with notably EMG activation, sighs, and heart beat acceleration. Our results show that the occurrence of such events is reduced in $\beta2-/-$ animals, indicating an involvement of $\beta2^*$nAChRs in their generation. The combination of EEG changes, and peripheral polygraphic activity in these events is reminiscent of micro-arousals observed in humans (for review, see Halász, 1998). They occur typically at a frequency of ~0.2–0.3 events per minute (one every 3–5 min of NREMS), which matches the frequency of micro-arousals in human sleep (Halász, 1998; De Gennaro et al., 2001). Their onset is followed by breathing irregularities as observed in humans for NREMS arousals elicited by sounds (Khoo et al., 1996). Events that could be similar to our MA have been described previously in the rat as "arousal-like period" (Roldán et al., 1963), "low-amplitude sleep" (Bergmann et al., 1987), "brief awakenings" (Franken et al., 1991), or "sleep small-amplitude irregular activity" and "a state of increased alertness" described

by Jarosiewicz et al. (2002). We propose that the MA described herein correspond to micro-arousals in mice.

The lower frequency of MA during NREMS observed in β2−/− mice resembled the deficit in putative arousals (detected by artifacts in the respiratory pressure signal) that was reported in the same mutant mice after a hypoxic challenge (Cohen et al., 2002). However, our plethysmographic recordings showed that the sighs, which are the main respiratory events observed during the arousals, clearly occurred several seconds after the beginning of the decline in EEG power. Still, the protective awakenings evoked by a hypoxic challenge observed by Cohen et al. (2002) might recruit the same pathway as the MA described in our study.

Sleep deprivation enhances sleep pressure. It is followed by an enhancement of EEG synchronization illustrated by an increased power in the δ band (Borbely and Achermann, 1999). WT and β2−/− mice exhibited the same pattern of increased synchronization with similar amplitude and time course as published for the C57BL/6J mouse strain (Franken et al., 1999; Huber et al., 2000), indicating that β2*nAChRs are not involved in this phenomenon. In accordance with previous observations (Franken et al., 1999; Huber et al., 2000), a 6 hr sleep deprivation also reduced the number of short awakenings both in WT and β2−/− mice. In β2−/− mutants, sleep deprivation further reduced the frequency of MA by ~40% compared with WT. Therefore, instead of lowering the values of sleep fragmentation in WT mice down to those in β2−/− mutants, sleep deprivation tended, rather, to exaggerate the difference between WT and β2−/− mice.

Our results indicate that nAChRs are involved in the phasic expression of arousal-promoting mechanisms (Halász, 1998; Terzano and Parrino, 2000) observed in NREMS in normal conditions and after sleep deprivation. Interestingly, nAChRs have also been proposed to regulate (Vazquez et al., 1996) and to relay in the cat (Hu et al., 1988) the pontogeniculo-occipital waves, a phasic activity observed in REMS. Whether the transient activity in REMS and the transient arousals in NREMS share some common mechanisms remains to be investigated.

Our results might also be relevant to frontal lobe nocturnal epilepsies (FLNE) in humans that are caused by the hyperfunction of α4 or β2*nAChRs (Itier and Bertrand, 2002) and that tend to occur in a stage of light sleep with intense cyclic micro-arousals activity (cyclic alternating pattern) (Terzano and Parrino, 2000). Indeed, in some cases of FLNE, a direct temporal correlation was found between micro-arousals and epileptic manifestations (Zucconi and Ferini-Strambi, 2000). The understanding of the physiological involvement of β2*nAChRs in the phasic activity in NREMS might thus help to clarify the causes of nocturnal epilepsies caused by mutations in nAChRs subunits encoding genes.

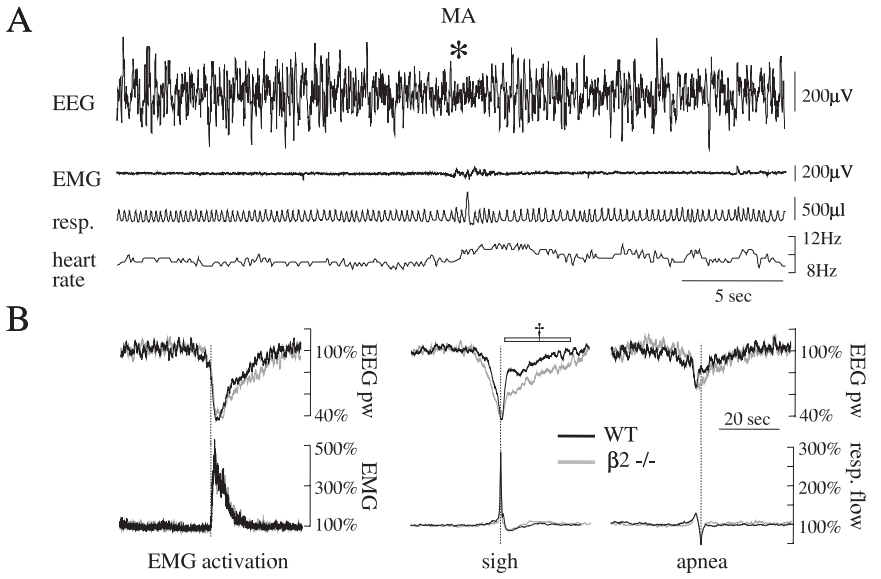


**Figure 5.** Similarity of the MA in relation to peripheral signs (notably breathing) in WT and β2−/− mice. *A*, Example of polygraphic recording from a WT mouse showing an MA combining a reduction in EEG power with EMG activation, changes in respiration (resp.), and an increase in heart rate. *B*, EEG in WT and β2−/− mice exhibits a similar reduction in power during EMG activation and sighs. Left, Events of EMG activation lasting 3–15 sec were used to compare the associated reduction in EEG power. EEG traces were aligned around the beginning of EMG activation (30–100 events per animal in the interval 7–13 hr) and averaged for each animal. These traces were then normalized to the average of 20–30 sec preceding the EMG activation, pooled together, and smoothed by run-average with a 1 sec square window (left, top). The same treatment (without the run-average) was applied in parallel to the EMG traces (left, bottom) for seven WT and eight β2−/− mice during NREMS. The same analysis was performed for EEG power and respiration flow (see Materials and Methods) around sighs and isolated apneas monitored in plethysmographic experiments (right). These data are taken from seven WT and five β2−/− mice. The reduction of EEG power begins shortly before the onset of the EMG activation, whereas sighs and apneas occur clearly after the decline in EEG power has started. The apneas are associated with a minor reduction in EEG power. The bar indicates a significant difference ($^{\dagger}p < 0.05$; unpaired *t* test) between the normalized traces smoothed by run-average with a 1 sec window.

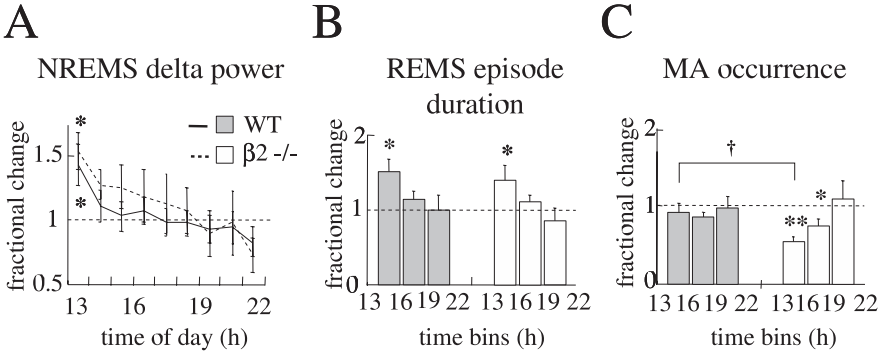


**Figure 6.** Effects of a 6 hr sleep deprivation (7:00 A.M. to 1:00 P.M.) in WT and β2−/− mice. *A*, *B*, Similarity in WT and β2−/− mice of the increase in δ power (a marker of NREMS pressure) and of REMS episode duration (an index of stabilization) during recovery from sleep deprivation. The increase in δ power during the 1:00–2:00 P.M. interval is significant, but equivalent, in both strains (see Results). The increase in duration of REMS episode, a normal consequence of sleep deprivation, is limited to the first 3 hr bin and is similar in both strains. *C*, Sleep deprivation is followed by a marked reduction in NREMS fragmentation by MA during the first 3 hr of recovery in β2−/− mice. Fractional change is calculated relative to controls. $^{*}p < 0.05$; $^{**}p < 0.01$; significantly different from control; paired Student's *t* test. $^{\dagger}p < 0.05$; significant difference between strains, unpaired Student's *t* test.

### β2*nAChRs participate in the stabilization of REMS episodes

REMS episodes in β2−/− mutants have a longer duration but are also preceded by a longer period of sustained NREMS (not

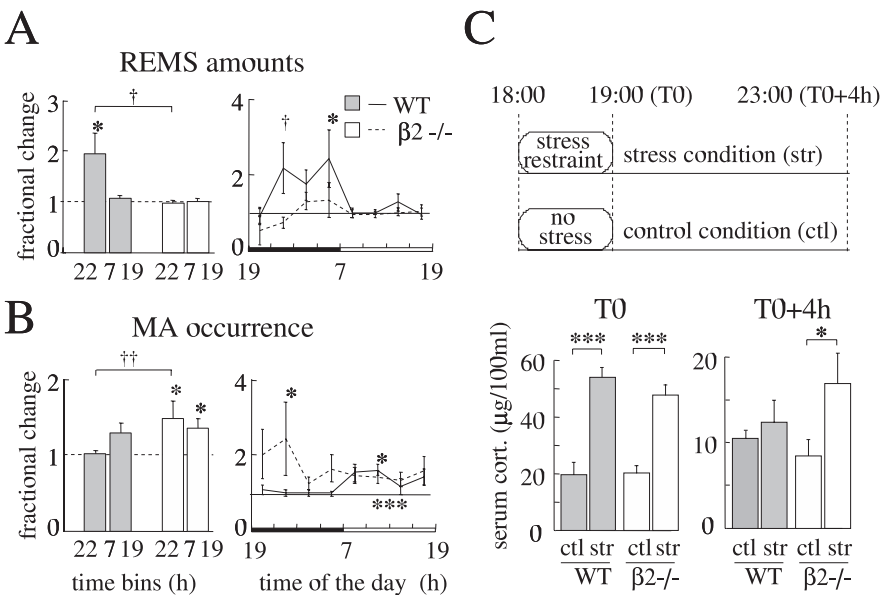


**Figure 7.** Differences in the impact of a 1 hr immobilization stress in WT and $\beta2-/-$ mice. *A*, The immobilization stress (6:00–7:00 P.M.) increases REMS in WT mice and disrupts NREMS in $\beta2-/-$ mice during the following night. *A*, *B*, The fractional changes in the amount of REMS (*A*) and in NREMS fragmentation by MA (*B*) are plotted for 9–12 hr time bins (left) and 3 hr time bins (right). Fractional change is calculated relative to controls. *C*, Time course of corticosterone after an immobilization stress. The serum corticosterone has been measured in WT and $\beta2-/-$ mice immediately (T0) or 4 hr after (T0 + 4 hr) the end of the stress (str) and is compared with the levels in control mice with the same genotype killed at the same hours (ctl). Corticosterone levels is increased similarly in $\beta2-/-$ and WT mice just after the stress, but the corticosterone levels remained higher than controls only in $\beta2-/-$ mice after 4 hr. Each data point is from eight animals. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; significantly different from control; paired Student's *t* test. $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$; significant difference between strains; unpaired Student's *t* test.

interrupted by MA) than in WT mice. REMS is a period of intense cholinergic activity in the brain, and REMS termination is classically thought to result from the disinhibition of REM-off neurons such as locus coeruleus noradrenergic neurons or raphe nuclei serotoninergic neurons (Steriade and McCarley, 1990). Indeed, both of these structures express high amounts of $\beta2^{*}$nAChRs (Léna et al., 1999; Marubio et al., 1999), which might contribute to the disinhibition of aminergic neurons and would thus trigger earlier REMS termination in WT mice compared with $\beta2-/-$ mutants.

Moreover, according to models of REMS homeostasis (Benington and Heller, 1994; Vivaldi et al., 1994; Franken, 2002), the longer REMS episodes observed in $\beta2-/-$ mutants would reset the REMS pressure and delay the onset of the next REMS episode, an effect that would be reflected in the longer sustained NREMS preceding REMS in the $\beta2-/-$ mutants. Thus, a deficit in REMS termination could result in alterations of the short-term homeostasis of the REMS-NREMS cycle consistent with our observations in $\beta2-/-$ mice.

### NREMS disruption by acute immobilization stress in $\beta2-/-$ animals

Nicotine is a positive effector of the hypothalamo-pituitary-adrenal axis (Matta et al., 1998), and it was thus expected that an acute stress would have less effect on sleep in $\beta2-/-$ mutants than in WT mice. A 1 hr immobilization stress in the rodent is normally followed by an increase in REMS amounts in the following hours (Rampin et al., 1991; Boutrel et al., 2002). Such an effect was not observed in $\beta2-/-$ mice, and an increased fragmentation of NREMS was observed instead. A similar pattern has been described in the rat after a prolonged restraint stress (4 hr) that triggered a delayed return of corticosterone levels to baseline, a suppression of the REMS rebound and a light reduction of NREMS episode duration (Marinesco et al., 1999). The REMS rebound is also suppressed by a chronic stress and is then accompanied by a decrease in sleep continuity (Kant et al., 1995). The pattern observed in $\beta2-/-$ mutants might thus correspond to a slower poststress decline of corticosterone levels (Boutrel et al., 2002) as it has been suggested already for these animals (Zoli et al., 1999). Indeed, a cholinergic deafferentation of the hippocampus results in a delay in the decline of corticosterone levels after a stress episode (Han et al., 2002). Consistently, our measures of corticosterone 4 hr after the termination of the stress evidenced a sustained increase in corticosterone plasma levels of $\beta2-/-$ mice that was not observed in WT mice. Therefore, the delayed decline of corticosterone levels after a stress episode might result in the absence of REMS rebound observed in our study. Alternatively, the REMS rebound and the maintenance of NREMS continuity might require the release of prolactin (Meerlo et al., 2001) triggered by the recruitment of $\beta2^{*}$nAChRs (Matta et al., 1998).

### Conclusion, perspectives

The main finding of this work is the existence of a contribution of nAChRs to the organization of sleep, notably to the regulation of transient events occurring during NREMS (which are putative micro-arousals), to the duration and onset of REMS episodes, and to the REMS-promoting effects of stress.

In humans, micro-arousals form a heterogeneous population of events of varying structure and intensity (Halász, 1998; Sforza et al., 2000). Additional characterization of the corresponding events in mice and of their neurobiological mechanisms should help to clarify the contribution of $\beta2^{*}$nAChRs to sleep regulation and potentially to SIDS and to the autosomal dominant FLNE caused by mutations in nAChRs subunits encoding genes.

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