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Magnesium Involvement in Sleep: Genetic and Nutritional Models

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Alterations of peripheral magnesium (Mg) concentration have been reported in association with several behavioral disorders and sleep organization. Blood Mg regulation is under a strong genetic control, whereas brain Mg regulation does not seem to be affected. We have studied peripheral and central levels of Mg and analyzed sleep in two lines of mice selected for low (MGL) and high (MGH) red blood cell (RBC) Mg levels. The same variables were also studied in C57BL/6J mice before and after 3 weeks of Mg deficiency. Whereas blood Mg was highly affected by the selection, brain Mg exhibited only small differences between the two lines. In contrast, Mg deficiency strongly decreased both central and peripheral Mg levels. Sleep analysis indicated that in both models the amount of paradoxical sleep was lower in mice with higher Mg levels. The amplitude of daily variation in sleep and slow-wave sleep delta power was markedly decreased in MGH line. Quantitative electroencephalogram (EEG) analysis also revealed a faster theta peak frequency in MGH mice, irrespective of behavioral states. Central Mg showed significant correlations with the amount of paradoxical sleep and sleep consolidation. However, because the direction of these correlations was not consistent, it is concluded that optimal, (physiological) rather than high or low, Mg levels are needed for normal sleep regulation.

KEY WORDS: MGL and MGH mice; brain; blood; paradoxical sleep; theta, EEG.

INTRODUCTION

Involved in neuronal processes from presynaptic to postsynaptic events, magnesium (Mg) is thought to play a major role in the excitability of both the peripheral and the central nervous system (CNS). Because of its action on many enzymes involved in the neurotransmitter synthesis, exocytosis and recycling (Boadle-Biber, 1978; Classen, 1986; Chutkow, 1990), on the modulation of ion channel conductivity (Kuner and Schoepfer, 1996; Li-Smerin and Johnson, 1996a, 1996b; Wollmuth *et al.*, 1998) and inward rectifier potassium channels (Matsuda *et al.*, 1987; Chuang *et al.*, 1997), and because of its involvement in the binding of most of neurotransmitters to their receptors (Nelson *et al.*, 1980; Usdin *et al.*, 1980; Okada and Kaneko, 1998), Mg has been proposed to take part in biochemical disregulation contributing to behavioral disorders. *In vivo* observations in animals and humans indicate that Mg deficiency is implicated in the pathophysiology of seizures (Belknap *et al.*, 1977; Nuytten *et al.*, 1991; Bac *et al.*, 1998), schizophrenia, anxiety, aggression, and stress (Kantak, 1988; Kirov and Tsachev, 1990; Henrotte *et al.*, 1995, 1997). Conversely, high levels of Mg have been shown to affect the control of locomotor activity in rats (Modak *et al.*, 1979), and correlate with low psychomotor activity in human depressed subjects (Widmer *et al.*, 1995, 1998). Thus, Mg is likely to play an important role in the regulation of central excitability and, therefore, in the modulation of behavioral

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states. Nevertheless, the role of this cation in the regulation of sleep and wakefulness has been poorly investigated. Some studies have suggested a relationship between blood Mg and the amount of sleep, with increased slow-wave sleep and decreased paradoxical sleep when serum Mg is high (Dralle and Bödeker, 1980; Popoviciu *et al.,* 1991). In rats, a Mg-restricted diet induced increased waking and decreased sleep time as well as pronounced sleep disorganization after prolonged Mg deficiency, whereas reintroduction of Mg in the diet restored sleep organization to its original pattern (Poenaru *et al.,* 1984; Depoorter *et al.,* 1993). Although *in vitro* electrophysiological studies clearly show the importance of Mg in neuronal excitability, most *in vivo* studies both in animals and humans has only related peripheral Mg levels to changes in behavior. In a recent study involving six inbred strains of mice, we have shown that brain Mg does not follow the strain specific distribution observed for blood Mg (Chollet *et al.,* 2000a). Therefore, the central-peripheral balance in Mg homeostasis and its effects on behavior remain unknown. Because peripheral Mg has been shown to be genetically regulated in humans and mice (Darlu *et al.,* 1982; Henrotte, 1993; Henrotte *et al.,* 1984, 1990), a mouse model has been generated to further investigate the mechanism and the biological significance of this genetic regulation (Henrotte *et al.,* 1997). This model consists of two lines of mice selected over 18 generations for high (MGH) or low (MGL) erythrocyte Mg concentration. MGL mice exhibited lower erythrocyte, plasma, kidney, and bone Mg levels, lower Mg exchangeable pool mass, and higher urinary Mg excretion than MGH mice (Henrotte *et al.,* 1997; Feillet-Coudray *et al.,* 2000). MGL mice have been shown to differ in their brain catecholamine levels, with increased adrenaline and noradrenaline, and in behavior, with increased aggressive behavior and acute stress reactivity as compared to MGH mice (Henrotte *et al.,* 1993; Aymard *et al.,* 1995; Henrotte *et al.,* 1995, 1997). The number of gastric ulcers induced by immobilization stress was greater in MGL mice and in Mg-deficient Swiss mice than in MGH mice and in control Swiss mice (Henrotte *et al.,* 1995). This model has also been tested for its sensitivity to audiogenic seizure (Motta and Louis, 1999), but compared with results from nutritional Mg restriction models (Belknap *et al.,* 1977; Bac *et al.,* 1998), the MGL line has been found to be less sensitive than the MGH line.

In the present study, we have investigated central levels of Mg in these two lines of mice in order to establish the effect of selection for peripheral Mg on brain Mg. We have also analyzed their sleep to determine the role of peripheral and central Mg in sleep regulation. These variables were also studied in C57BL/6J (B6) mice before and after 3 weeks under severe dietary-induced Mg deficiency.

MATERIAL AND METHODS

MGH and MGL Mice

Eight adult male mice from each line were used (age: 8 to 10 weeks at the beginning of the study; weight: 20 to 32 g, from the stock maintained at INRA, Clermont-Ferrand, France). Animals were kept under a 12:12 light-dark cycle (light on at 0800 h), an ambient temperature of 24 ± 1°C, and humidity of 45% to 65%. Food and water were available *ad libitum.* Animals were kept under these conditions for at least 18 days before sleep recordings.

B6 Mice

Eight C57BL/6J mice were purchased from Jackson Laboratory (Maine, USA) and were kept under the same conditions as MGH and MGL mice for 2 weeks. Animals were separated in two groups of four mice, one of which was given a control diet (Mg 40 mmol/kg) and the other a Mg-deficient diet (Mg 2 mmol/kg) for 3 weeks, at the end of which sleep was recorded. At the end of the recordings, dietary regimes were reversed for the two groups and 3 weeks later sleep was recorded a second time. Only distilled water was provided in order to better control mineral intake.

Surgery

Electroencephalogram (EEG) and electromyogram (EMG) electrodes were implanted under pentobarbital anesthesia (ip, 65 to 75 mg/kg, depending on strain). Two gold-plated screws served as EEG electrodes and were screwed through the skull over the right cerebral hemisphere. Two other screws were implanted over the left hemisphere and were used as anchor screws. Two semi-rigid gold wires served as EMG electrodes and were inserted between the two neck muscles. The electrodes were soldered to a miniature connector and cemented to the skull. The recording leads were connected and attached to a swivel-contact 1 week after surgery and animals were then allowed another week for recovery and habituation before the experiment.

Sleep Recording and Data Acquisition

EEG and EMG recordings started at lights-on (0800 h) and lasted for 2 days. The first day (24 h) served as baseline. On the second day starting at lights-on, mice were sleep deprived for 6 hours by gentle handling and the remaining 18 hours of this day were considered as recovery. The analog EEG and EMG signals were amplified and filtered and analog-to-digital converted. The EEG signal was subjected to a Fast-Fourier Transform (FFT) analysis, yielding power spectra between 0.125 and 25.125 Hz, with a 0.25 Hz frequency and a 4-second time resolution. Digitized EEG and EMG signals and the FFT results performed on-line were stored on magneto-optical disk.

Magnesium Determination

Because the aim of this study was to investigate the involvement of basal Mg in sleep regulation, all animals were sacrificed 3 days after the end of the sleep recordings to counteract any possible changes in brain and peripheral Mg due to the sleep deprivation. Animals from different strains were sacrificed between 0800 h and 1230 h under deep anesthesia with pentobarbital at 75 mg/kg (no major circadian variation of plasma Mg, the fraction which is most sensitive to changes, has been reported [Touitou *et al.*, 1989]). Five hundred to 800 μL of blood were collected by cardiopuncture in heparinized tubes and brains were quickly removed, frozen, and stored at −80°C.

Blood samples were centrifuged for 10 min. at 3500 g and 4°C and the RBC pellets were washed three times in a KCl isotonic solution. RBC pellets were diluted in KCl isotonic solution (2:3 vol/vol) from which hematocrit was measured. One hundred μL of this solution were mixed with 9900 μL of distilled water. Plasma samples were centrifuged for 10 min. at 3500 g and room temperature to remove residual blood cells. Plasma and RBC Mg concentrations were then measured in triplicate by the usual atomic absorption spectrometry (AAS; Perkin Elmer [Archer *et al.*, 1972]), with minor modifications. Results are reported as mmol/L of plasma or RBC. It should be pointed out that Mg determination in the present study concerns total Mg (bound + free Mg^{2+}), whereas only free Mg^{2+} constitutes the active form. However, although the determination of free Mg^{2+}, especially *in vivo*, may be more relevant to its function, it has been well-established that total intracellular Mg is highly correlated with free Mg^{2+} concentration (Resnick *et al.*, 1984; Fujise *et al.*, 1991).

Brains were microdissected at −20°C into nine blocks, containing the following main structures: amygdala (AM); frontal cortex (FC); basal forebrain (BF); cerebellum (CB); motor cortex (MC); somatosensory cortex (SC); thalamus (TH); mesencephalon (ME); and brainstem (BS). Tissue blocks are identified by the name of their main structure, although they may contain parts of other structures (e.g., SC contained the hippocampus). Dissections were performed according to the rapid method described by Heffner *et al.* (1980), with adaptations for mice based on the stereotaxic atlas by Franklin and Paxinos (1997). The frozen blocks were sonicated in distilled water in preweighted glass tubes, lyophilized for 16 hours, and weighted to obtain dry weight. A second lyophilization did not indicate any change in weight. Samples were then mineralized for 12 hours in 1.5 mL acid solution (3:1 solution of HNO_3 65% and $HClO_4$ 70%), and dissolved in 4.5 mL of distilled water by sonication. After centrifugation for 10 min. at 3500 g, supernatants were used for Mg determination in triplicate by AAS. Total brain Mg concentration in each mouse was calculated as the sum of the products of Mg concentration (in μmol/g of dry weight) and dry weight per structure divided by the total weight of all structures. In the same way, Mg concentration was calculated for cortical (FC + MC + SC + AM) and subcortical (BF + TH + ME) structures.

Determination of Behavioral States and Sleep-Data Analysis

Off-line the animals' behavior for each of the 43,200 consecutive 4-second epochs was classified as paradoxical sleep, slow-wave sleep, or wakefulness by visual inspection of the EEG and EMG signals as previously described (Franken *et al.*, 1998). The amount and distribution of behavioral states were analyzed by expressing them as a percentage of recording time in the time interval of interest. As an amplitude measure of changes in sleep and wakefulness across the 24-hour day, the difference between the mean amount of sleep in the light and dark periods was taken. Behavioral state quality was assessed by analyzing its fragmentation and for slow-wave sleep the level of EEG delta power. Fragmentation was estimated by calculating frequency histograms for episode duration (Franken *et al.*, 1999). According to their length, episodes were allotted to one of nine bins of logarithmically increasing size (4, 8–12, 16–28, 32–60, 64–124, 128–252, 256–508, 512–1020, and >1024 sec). The frequency in each bin was corrected for the total amount of each state by expressing it per

hour of behavioral state. Effects of sleep deprivation on slow-wave sleep fragmentation were also assessed by counting the number of brief arousals during slow-wave sleep resulting in short slow-wave sleep episodes (<60 sec) or long slow-wave sleep episodes (>60 sec), as defined in Franken *et al.* (1999). For the evaluation of the sleep deprivation effect on sleep fragmentation, the data from the first 6 h of recovery (last 6 h of light) were compared with the last 6 h of baseline (light), in order to compare data from the same circadian period. Delta power was calculated by averaging power density in the frequency bins from 1 to 4 Hz. Only delta power values from 4-second epochs, which themselves and their two adjacent epochs were scored as artifact-free slow-wave sleep, were included in the analysis. The time course of delta power in slow-wave sleep was calculated by averaging over 1 hour (light periods) or 1.5-hour (dark periods). Theta peak frequency was determined as outlined in Franken *et al.* (1998).

Statistics

All main effects of factors "line" (MGH and MGL) or "diet" (control and Mg deficiency), "structure" (nine brain structures, RBC and plasma) for Mg content, and "day" (recovery vs. baseline), "light/dark" (light vs. dark), and "bin" (bins 1 to 9 of episode-length distribution) for sleep only were analyzed by analysis of variance (ANOVA) for repeated measures. Whenever main effects reached the significant level ($p < 0.05$), post-hoc pairwise comparisons were performed using the student *t*-test to evaluate differences between groups ("line" or "diet") or using the Tukey's studentized range test for multiple comparisons analyses. The relationship between brain and blood Mg concentrations, but also within the brain and with sleep parameters, was analyzed by the Pearson's correlation test.

RESULTS

MGH and MGL Mice

Peripheral Mg was, as expected, significantly higher in MGH (2.97 ± 0.07 mmol/L vs. 2.12 ± 0.04 mmol/L, $p < 0.0001$, for RBC Mg, and 1.17 ± 0.02 mmol/L vs. 0.98 ± 0.04 mmol/L, $p < 0.0007$ for plasma in MGH and MGL mice, respectively). As previously observed (Henrotte *et al.*, 1993, 1997), body weight did not differ between MGH and MGL, whereas total brain weight was 12% higher in the MGL line (88.2 ± 1.2 mg vs. 100.9 ± 1.5 mg in MGH and MGL, respectively, $p < 0.0001$).

Compared with blood Mg, brain Mg did not differ between the two lines, except in the somatosensory cortex, where values were significantly higher in the MGH line (Fig. 1A). As we have recently reported in six inbred mouse strains (Chollet *et al.*, 2000a), brain Mg showed a structure dependent distribution that was not affected by genotype (two-way ANOVA with repeated measures for factor "structure": factor "line", $F(1,14) = 0.7$, $p = 0.41$; factor "structure", $F(8,112) = 31.8$, $p < 0.0001$; interaction, $F(8,112) = 1.1$, $p = 0.36$; Fig. 1A).

Some significant correlations were found between peripheral and central Mg concentrations. In MGH mice, plasma Mg was negatively correlated with the brainstem, cortical, and total brain Mg ($r = -0.80$, $p < 0.004$; $r = -0.69$, $p < 0.03$ and $r = -0.66$, $p < 0.04$, respectively). In MGL mice, RBC Mg showed a significant positive correlation with the frontal cortex Mg ($r = 0.85$, $p < 0.005$).

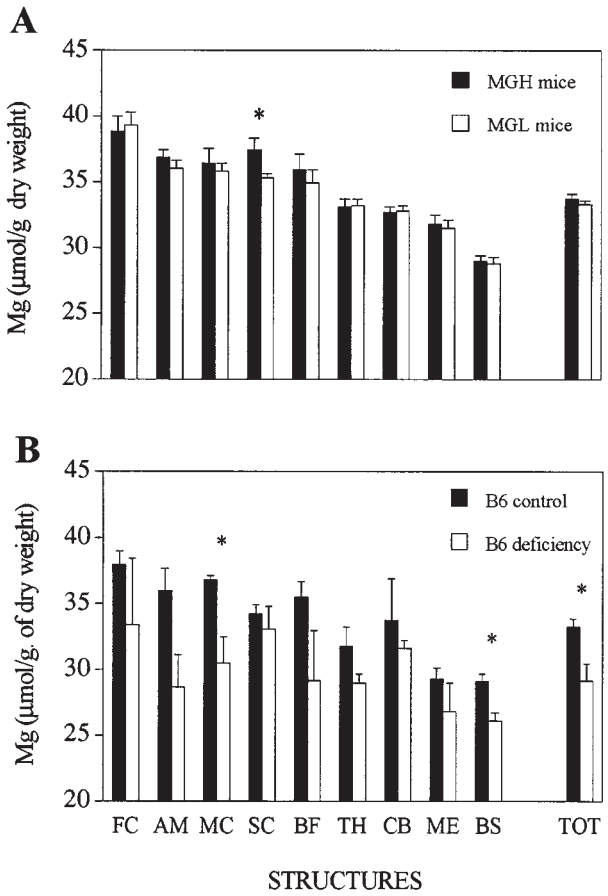

Fig. 1. Brain magnesium distribution in MGH (solid) and MGL (open bars in A) and in B6 under control (solid) and Mg-deficient (open bars in B) diet. FC, frontal cortex; AM, amygdala; MC, motor cortex; SC, somatosensory cortex; BF, basal forebrain; TH, thalamus; CB, cerebellum; ME, mesencephalon; BS, brainstem; TOT, total brain. *$p < 0.05$; Tukey's range test.

Behavioral State Distribution and Effects of Sleep Deprivation

Baseline: The daily amount of sleep and wakefulness did not differ between the lines (Table I), even though MGH mice tended to have less paradoxical sleep than did MGL mice. However, during the light period, MGH mice showed significantly more waking and less paradoxical sleep than MGL mice (Table I, Fig. 2A). The altered distribution in baseline resulted in significantly lower light-dark amplitudes for all behavioral states in MGH mice (Fig. 3). Paradoxical sleep and slow-wave sleep were affected in the same way, leaving the paradoxical sleep/total sleep ratio not significantly changed (Fig. 3A). Finally, sleep quality assessed by the analysis of its fragmentation did not show any difference between the two lines (data not shown).

Delta oscillations are characteristic of the EEG during slow-wave sleep. Power in the delta frequency range varies according to the distribution of sleep and wakefulness, i.e., decreasing in the presence of slow-wave sleep and increasing in its absence (Franken *et al.*, 2001). Parallel to the lower daily amplitude in sleep time, MGH mice showed lower amplitude in delta power within the baseline (Fig. 2A).

Theta oscillations (5 to 10 Hz), which originate from the hippocampus, characterize the EEG during paradoxical sleep and exploratory behavior and are associated with learning and memory consolidation (Vinogradova, 1995; Vertes and Kocsis, 1997). The comparison of EEG spectral profiles between the two lines showed that the prevailing theta frequency was significantly higher for MGH in paradoxical sleep (6.52 ± 0.11 Hz vs. 7.02 ± 0.10 Hz in MGL and MGH, respectively, $p < 0.005$) and in waking (5.74 ± 0.14 Hz vs. 6.47 ± 0.10 Hz in MGL and MGH, respectively, $p < 0.002$). A similar tendency was also evident in slow-wave sleep (5.60 ± 0.14 Hz vs. 5.92 ± 0.10 Hz in MGL and MGH, respectively, $p < 0.08$).

Recovery: The occurrence of brief slow-wave sleep episodes during sleep deprivation cannot be avoided (Franken *et al.*, 1999, 2001), especially toward the end, but our subjective impression that MGL

TABLE I. Behavioral States Distribution in MGH and MGL Mice

Baseline	Strain	W	SWS	PS	PS/tot
24 h	**MGH**	58.5 (1.1)	36.6 (1.0)	4.9 (0.3)	11.8 (0.6)
	MGL	58.3 (1.5)	36.1 (1.7)	5.6 (0.2)	13.7 (1.0)
	p	0.92	0.79	0.05	0.13
12-h L	**MGH**	50.2 (0.8)	43.8 (1.0)	6.0 (0.4)	12.1 (0.9)
	MGL	45.3 (1.9)	46.9 (2.1)	7.7 (0.4)	14.4 (1.1)
	p	**<0.03**	0.19	**<0.008**	0.13
12-h D	**MGH**	66.8 (2.2)	29.4 (1.9)	3.8 (0.3)	11.5 (0.5)
	MGL	71.3 (2.2)	25.2 (2.0)	3.5 (0.3)	12.4 (1.0)
	p	0.16	0.15	0.48	0.40
last 6-h L	**MGH**	56.3 (2.2)	38.2 (2.0)	5.5 (0.5)	12.6 (0.9)
	MGL	52.3 (2.3)	40.5 (2.3)	7.2 (0.5)	15.5 (1.5)
	p	0.23	0.47	**<0.04**	0.14
Recovery	Strain	W	SWS	PS	PS/tot
18 h	**MGH**	57.7 (1.7)	36.7 (1.6)	5.6 (0.3)	13.3 (0.6)
	MGL	56.7 (1.0)	37.1 (1.2)	6.2 (0.4)	14.3 (1.1)
	p	0.63	0.84	0.23	0.38
6-h L	**MGH**	45.6 (1.2)	47.1 (1.1)	7.3 (0.5)	13.4 (0.8)
	MGL	40.3 (1.2)	50.8 (1.3)	8.9 (0.8)	14.9 (1.3)
	p	**<0.007**	**<0.05**	0.09	0.35
12-h D	**MGH**	63.9 (2.3)	31.4 (2.1)	4.7 (0.4)	13.0 (0.8)
	MGL	65.1 (1.6)	30.2 (1.5)	4.8 (0.4)	13.7 (1.1)
	p	0.68	0.62	0.89	0.60

Mean ± SEM percentage of recording time for wakefulness (W), slow-wave sleep (SWS), paradoxical sleep (PS), and PS/total sleep ($n = 8$ per line) for the 24-h, light (L), and dark (D) periods of baseline and recovery. *p* indicates the probability of the *t*-tests comparing the lines.

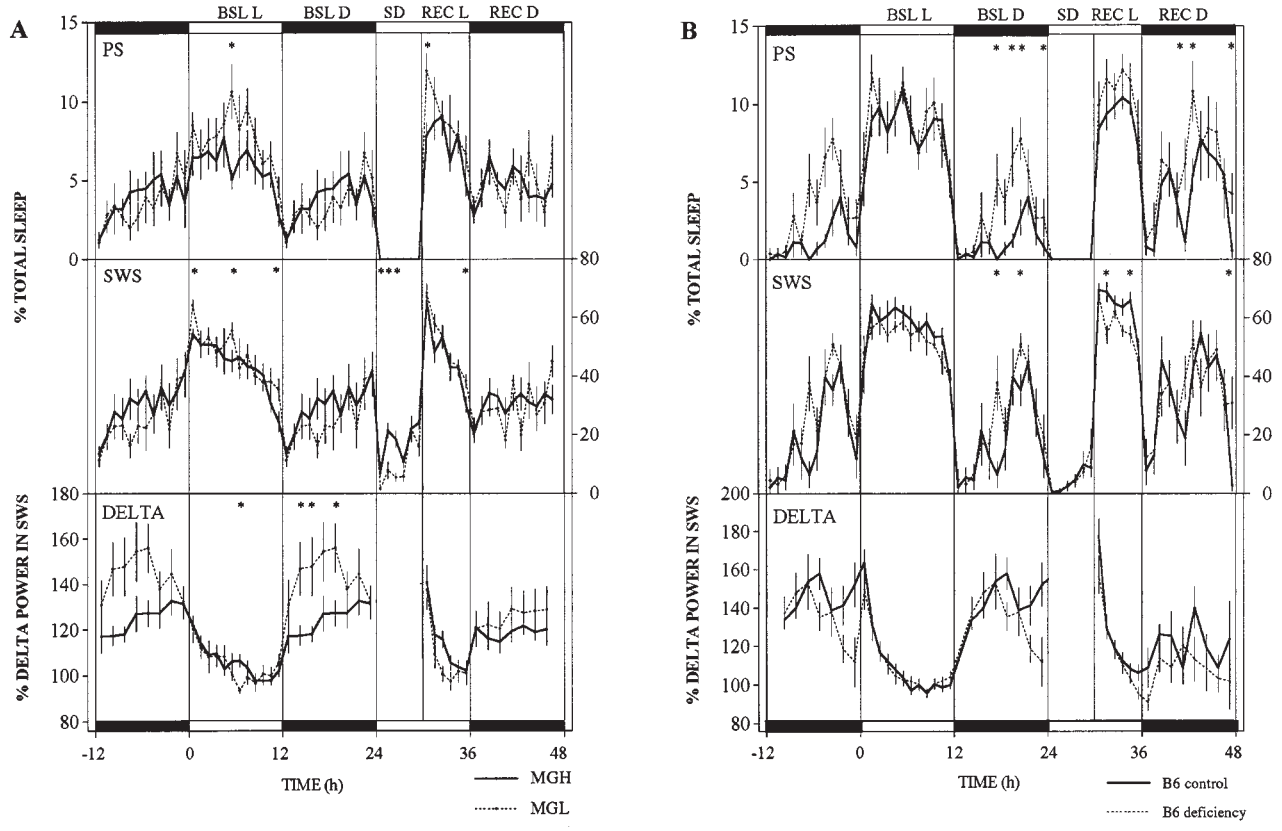

Fig. 2. Time course of paradoxical sleep (PS), slow-wave sleep (SWS), and SWS delta power (DELTA) in MGH (solid) and MGL (dotted lines in A) and in B6 under control (solid) and Mg deficient (dotted lines in B) diet. Values are mean ± SEM hourly (or 1.5 h for DELTA in the dark periods) values in the course of baseline light and dark (BSL L, BSL D) periods, sleep deprivation (SD), and recovery light and dark (REC L, REC D) periods. The values for the baseline dark period are depicted twice to illustrate the changes at the transition from the dark to the light period. Vertical lines and horizontal black bars in the top and bottom mark the dark periods. *$p < 0.05$ indicates intervals with significant genotype or diet differences; *t*-test.

mice, and even more so MGH mice, were more difficult to sleep deprive compared with other mouse strains was confirmed by a large amount of slow-wave sleep observed during the 6-hour sleep deprivation (Fig. 2A). When calculated at 1-hour intervals over the course of sleep deprivation, the amount and distribution of slow-wave sleep was significantly different between the two lines (two-way ANOVA with repeated measures for factor "interval": factor "line", $F(1,14) = 8.8$, $p < 0.01$; factor "interval", $F(5,70) = 25.8$ $p < 0.0001$; interaction, $F(5,70) = 3.7$, $p < 0.01$). Note that MGH mice have significantly more sleep already during the first 3 h of sleep deprivation. The number of forced arousals to prevent sleep showed the same trend (two-way ANOVA with repeated measures for factor "interval": factor "line", $F(1,14) = 4.7$, $p < 0.05$; factor "interval", $F(5,70) = 22.3$, $p < 0.0001$; interaction, $F(5,70) = 5.8$, $p < 0.0002$).

During the light period of recovery, MGH mice showed significantly more waking and less slow-wave sleep, and tended to have less paradoxical sleep than MGL. During the dark period of recovery, compared with baseline, MGL mice showed a significant decrease in waking, with a concomitant increase in slow-wave sleep and paradoxical sleep (*t*-test; $p < 0.004$, $p < 0.01$, and $p < 0.002$ for waking, slow-wave sleep, and paradoxical sleep, respectively). A larger amount of paradoxical sleep was also observed for MGH during the dark period of recovery ($p < 0.02$), but none of the sleep deprivation-induced differences were affected by genotype (two-way ANOVA with repeated measures for factor "day": factor "line", waking: $F(1,14) = 1.1$, $p = 0.31$, slow-wave sleep: $F(1,14) = 1.28$, $p = 0.28$, paradoxical sleep: $F(1,14) = 0.1$, $p = 0.80$; factor "day", waking: $F(1,14) = 14.2$, $p < 0.005$, slow-wave sleep: $F(1,14) = 9.8$, $p < 0.01$, paradoxical sleep: $F(1,14) = 33.3$, $p <$

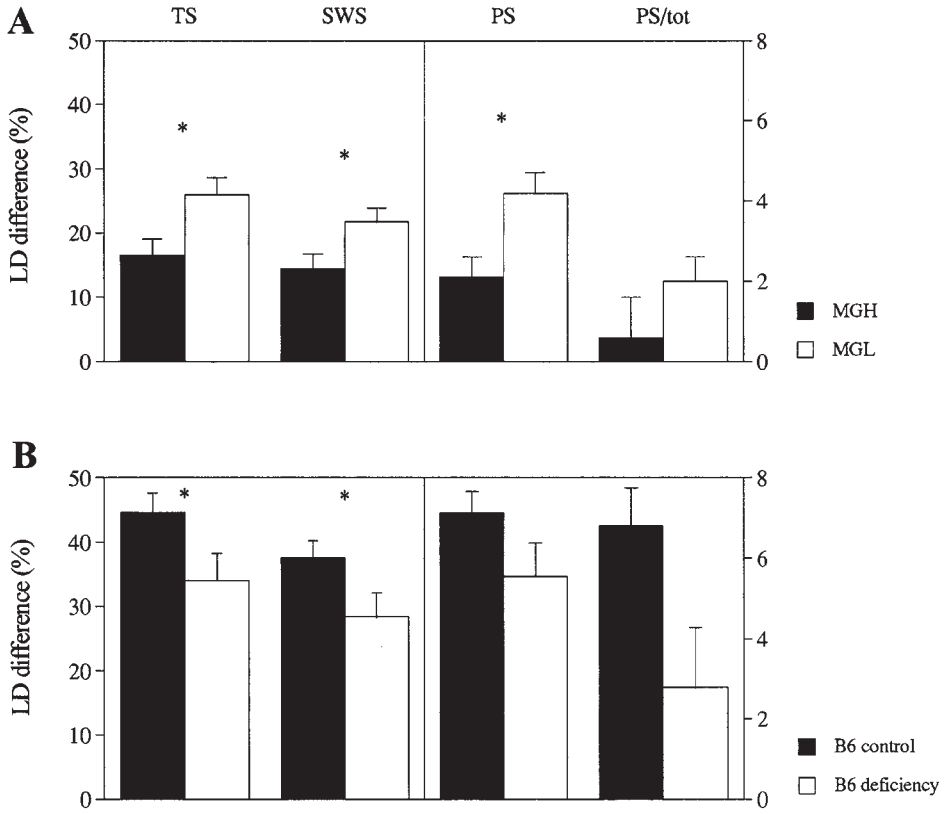

Fig. 3. Daily amplitude in total sleep (TS), slow-wave sleep (SWS), paradoxical sleep (PS), and PS/total sleep in MGH (solid) and MGL (open bars in A) and in B6 under control (solid) and Mg deficient (open bars in B) diet. Bars indicate the mean ± SEM difference ($n = 8$ in each group) in percentage of the recording time between the values in the light and dark (LD) periods in baseline. *$p < 0.05$ indicates significant genotype or diet differences; *t*-test.

0.0001, interaction: waking: $F(1,14) = 1.9$, $p = 0.19$, slow-wave sleep: $F(1,14) = 1.8$, $p = 0.21$, paradoxical sleep: $F(1,14) = 1.2$, $p = 0.30$).

The typical delta power rebound after sleep deprivation was not evidenced, possibly due to the unavoidable amount of slow-wave sleep accumulated during sleep deprivation (Fig. 2A, Franken *et al.*, 2001). Note that in contrast to delta power the amount of sleep showed a significant increase during recovery.

Magnesium and Sleep

In order to establish a link between peripheral and central Mg concentrations and sleep, correlation analysis was performed between Mg levels and sleep parameters. During the baseline dark period, high Mg levels in the brainstem were associated with less sleep in MGH mice ($r = -0.86$, $p < 0.006$, $n = 8$) and with less paradoxical sleep in MGL mice ($r = -0.91$, $p < 0.002$, $n = 8$). Furthermore, in MGL mice, high Mg levels in the amygdala were associated with less sleep during the 24-hour baseline ($r = -0.87$, $p < 0.005$, $n = 8$) and less slow-wave sleep during recovery ($r = -0.94$, $p < 0.0007$ for the entire 18 h and $r = -0.88$, $p < 0.005$ for the dark period of recovery, $n = 8$).

Although sleep fragmentation did not show significant differences between the two lines, correlations with brain Mg showed discrepancies. Total brain Mg was negatively correlated with the number of long episodes of slow-wave sleep during the recovery period in MGL mice ($r = -0.89$, $p < 0.007$, $n = 8$), whereas it tended to be positively correlated in MGH mice ($r = 0.84$, $p < 0.04$, $n = 8$). In MGH mice, high brainstem Mg levels were correlated with low occurrence of short episodes of sleep ($r = -0.94$, $p < 0.0006$, $n = 8$), but with increased occurrence of long slow-wave sleep ($r = 0.88$, $p < 0.006$, $n = 8$) during the recovery period.

B6 Mice

Mg deficient mice showed extremely low values for RBC and plasma Mg (RBC Mg: 2.35 ± 0.05 mmol/L and 1.05 ± 0.08 mmol/L for control and deficient mice, respectively; *t*-test, $p < 0.0001$; Plasma Mg: 1.28 ± 0.06 mmol/L and 0.21 ± 0.02 mmol/L for control and deficient mice, respectively; *t*-test, $p < 0.005$; $n = 4$), indicating a severe Mg deficiency. RBC Mg was highly significantly correlated with plasma Mg in the two groups ($r = 0.98$, $p < 0.0001$; $n = 8$). Mg deficiency slightly decreased both body and brain weights but the changes were not significant.

In three Mg-deficient animals, spontaneous seizure activity was observed in the EEG recordings and one of them died just after a generalized seizure (this animal was replaced so that $n = 4$).

The concentration of brain Mg was influenced by the diet but not its regional distribution (two-way ANOVA with repeated measures for factor "structure": factor "diet", $F(1,6) = 6.2$, $p < 0.05$; factor "structure", $F(8,48) = 4.1$, $p < 0.001$; interaction, $F(8,48) = 0.7$, $p = 0.69$). Significant decreases under Mg deficiency were found for total brain, motor cortex, and brainstem Mg (Fig. 1B).

Brain Mg showed positive correlations with blood Mg (e.g., for RBC vs. total brain Mg, $r = 0.75$, $p < 0.002$; vs. amygdala Mg, $r = 0.71$, $p < 0.006$; vs. motor cortex Mg, $r = 0.71$, $p < 0.005$; vs. brainstem Mg, $r = 0.69$, $p < 0.008$, $n = 8$). Note that these brain structures are the same (except for the amygdala) as those showing a significant decrease in Mg content under Mg deficiency.

Behavioral State Distribution and Effects of Sleep Deprivation

Baseline: The daily amount of paradoxical sleep was significantly higher in Mg-deficient mice (130%), mainly due to a large difference during the dark period (300%). The daily amount of slow-wave sleep was unchanged, but the deficient group tended to have less slow-wave sleep within the light period and more in the dark period. The inverse relation was observed for waking (Table II, Fig. 2B). As a result of the differences in behavioral state distribution, the daily amplitude (light-dark difference) of waking and slow-wave sleep was significantly lower in the deficient mice (Fig. 3B).

Significant differences were observed in sleep fragmentation between the control and the Mg-deficient group. Thus, animals under the Mg-deficient diet showed a significantly higher number of short waking episodes (102 ± 10 vs. 90 ± 9 for deficient and control mice, respectively; *t*-test, $p < 0.02$) and short slow-wave sleep episodes (109 ± 13 vs. 88 ± 11 for deficient and control mice, respectively; *t*-test, $p < 0.02$).

Recovery: As in baseline, during the 18 h of recovery after sleep deprivation, Mg-deficient mice had significantly more paradoxical sleep than control mice, again mainly due to high values in the dark period (Table II, Fig. 2B). Now the tendency for a lower amount of slow-wave sleep in the Mg deficient mice observed in the baseline light period became significant (Table II).

Similar sleep deprivation induced changes were observed in both groups (two-way ANOVA with repeated measures for both factors: factor "diet", waking: $F(1,7) = 14.6$, $p < 0.01$; slow-wave sleep: $F(1,7) = 86.4$, $p < 0.0001$; paradoxical sleep: $F(1,7) = 2.2$, $p = 0.18$; factor "day", waking: $F(1,7) = 72.0$, $p < 0.0001$; slow-wave sleep: $F(1,7) = 44.7$, $p < 0.0005$; paradoxical sleep: $F(1,7) = 23.0$, $p < 0.005$; interaction, waking: $F(1,7) = 2.0$, $p = 0.20$; slow-wave sleep: $F(1,7) = 4.9$, $p = 0.07$; paradoxical sleep: $F(1,7) = 3.6$, $p = 0.10$).

Finally, spectral analysis failed to reveal any significant difference in delta power or theta peak frequency between the two groups either in baseline or recovery (data not shown).

Magnesium and Sleep

Although in each group only four animals were available for Mg measures at the end of the experiments, in Mg-deficient mice a significant positive correlation was found between the brainstem Mg and the baseline dark period amount of paradoxical sleep ($r = 0.99$, $p < 0.008$).

Analysis of sleep fragmentation before and after sleep deprivation indicated that although not different between the two groups, B6 control mice increased more efficiently the duration of long slow-wave sleep episodes during the 18-hour recovery period after sleep deprivation (from 1.6 ± 0.1 min. to 2.3 ± 0.1 min. in control vs. from 1.4 ± 0.1 to 1.8 ± 0.1 in Mg-deficient mice, $p < 0.05$). Among brain structures, the mesencephalon showed a positive correlation with the number of long slow-wave sleep episodes in baseline for Mg-deficient mice ($r = 0.96$, $p < 0.04$).

TABLE II. Behavioral States Distribution in B6 Mice

Baseline	Diet	W	SWS	PS	PS/tot
24 h	**Control**	58.0 (1.0)	37.3 (1.2)	4.7 (0.2)	11.3 (0.8)
	Deficiency	55.6 (1.1)	38.3 (0.9)	6.1 (0.4)	13.6 (0.7)
	p	0.13	0.51	**<0.01**	**<0.05**
12-h L	**Control**	35.7 (1.0)	56.0 (1.2)	8.3 (0.5)	12.9 (0.8)
	Deficiency	38.6 (1.2)	52.5 (1.2)	8.8 (0.6)	14.4 (0.9)
	p	0.09	0.053	0.48	0.24
12-h D	**Control**	80.3 (2.4)	18.5 (2.2)	1.1 (0.2)	6.1 (1.0)
	Deficiency	72.7 (3.1)	24.1 (2.6)	3.3 (0.5)	11.6 (1.1)
	p	0.07	0.13	**<0.003**	**<0.003**
last 6-h L	**Control**	38.8 (0.8)	53.3 (1.3)	7.9 (0.5)	13.0 (1.0)
	Deficiency	42.3 (2.0)	49.7 (1.7)	8.2 (0.7)	14.2 (1.1)
	p	0.07	**<0.03**	0.36	0.13
Recovery	Diet	W	SWS	PS	PS/tot
18 h	**Control**	52.3 (2.2)	42.0 (2.1)	5.8 (0.3)	12.2 (0.6)
	Deficiency	51.1 (2.3)	41.4 (2.0)	7.4 (0.5)	15.3 (0.8)
	p	0.73	0.85	**<0.009**	**<0.006**
6-h L	**Control**	28.0 (1.0)	63.0 (1.1)	9.1 (0.5)	12.6 (0.8)
	Deficiency	33.3 (1.2)	56.1 (1.4)	10.7 (0.6)	16.0 (0.9)
	p	**<0.005**	**<0.002**	0.06	**<0.02**
12-h D	**Control**	64.6 (3.5)	31.3 (3.1)	4.1 (0.4)	11.6 (0.5)
	Deficiency	60.3 (3.2)	33.9 (2.8)	5.8 (0.5)	14.8 (0.8)
	p	0.37	0.53	**<0.02**	**<0.004**

Mean ± SEM percentage of recording time for wakefulness (W), slow-wave sleep (SWS), paradoxical sleep (PS), and PS/total sleep ($n = 8$ per diet) for the 24-h, light (L), and dark (D) periods of baseline and recovery. *p* indicates the probability of the *t*-tests comparing the diets.

DISCUSSION

Central and Peripheral Mg

We have recently confirmed that blood Mg is under genetic control, whereas brain Mg is not (Chollet *et al.,* 2000a). Therefore, we postulated that the selection for peripheral Mg in MGH and MGL mouse lines might not affect or select for brain Mg. This was previously shown in the course of the lines' selection because at the 12th generation where, in addition to the RBC and plasma, the kidney and bone Mg levels have shown highly significant differences, the total brain Mg was virtually identical in the two lines (Henrotte *et al.*, 1997). This is also partly verified in the present study because overall brain Mg did not differ between the two lines, although subtle differences cannot be ruled out, e.g., in one structure, the somatosensory cortex, Mg content was significantly higher in the MGH line. Although these findings strongly suggest that mechanisms through which central Mg is regulated differ from those regulating peripheral Mg, the two compartments are not independent. Diet-induced Mg deficiency affects both central and peripheral Mg, as evidenced in this study and in others (Chutkow, 1972; Altura *et al.*, 1997). Therefore, one possibility is that brain Mg is regulated as, for instance, brain glucose: Under physiological conditions, changes in peripheral levels have little effect on the central concentration, whereas dramatic changes at periphery ultimately affects the brain. Also, both in MGH-MGL and in B6 mice, some correlations were found between blood and brain Mg levels and in B6 mice the brain structures that were affected the most by Mg deficiency had the highest correlations with blood Mg. However, correlations were negative in MGH and positive in B6 mice, again suggesting different mechanisms under physiological and experimental conditions. We had also noticed a negative correlation between RBC and brain Mg in inbred mice under normal conditions (Chollet *et al.,* 2000a). The brain is therefore protected against variations of Mg concentration by a rigorous, still unknown, homeostatic control mechanism.

Mg and Sleep

The most conspicuous effect of Mg on behavioral states is the low amount of paradoxical sleep when Mg concentration is high. This effect was found both in MGH and in B6 control mice and was most evident during the dark period. Jouvet (1962) demonstrated that a section rostral to the brainstem induces a complete disappearance of paradoxical sleep while after a transection caudal to the brainstem all paradoxical sleep-related activities are still present (Jouvet, 1962). Further lesion and electrophysiological experiments clearly indicated that the pons (brainstem and medulla) is necessary and sufficient for paradoxical sleep expression (Sakai, 1988; Siegel, 1989). In the present study, brainstem Mg was negatively correlated with the amount of paradoxical sleep during the dark period in MGH, MGL, and B6 mice. In B6 mice before and after Mg deficiency, the brainstem Mg was negatively correlated with the amount of paradoxical sleep during 24-hour baseline, 18-hour recovery, and 12-hour dark period of recovery. In these mice, RBC Mg was also negatively correlated with the amount of paradoxical sleep. Note that plasma Mg in MGH-MGL lines and RBC Mg in B6 mice were positively correlated with the brainstem Mg. Therefore, we believe that Mg affects the expression of paradoxical sleep by acting at the level of the brainstem, where the executive mechanisms of paradoxical sleep are contained. Additionally, paradoxical sleep expression is under the reciprocal interaction of pontine noradrenergic (locus coeruleus), serotonergic (raphe), and cholinergic systems. Mg is involved in the synthesis of catecholamines (low Mg concentration is associated with high catecholamine concentration) and increased noradrenaline and dopamine levels were reported in MGL mice (Henrotte *et al.,* 1993; Aymard *et al.,* 1995) and in rats under Mg deficient diet (Poenaru *et al.,* 1984; Kantak, 1988). Increased catecholaminergic transmission is associated with increased waking, decreased paradoxical sleep (Crochet and Sakai, 1999), and changes in behavior (increased motor activity and aggressive behavior, [Kantak, 1988]). All these changes were also observed in the present study with lower Mg concentration, except for the amount of paradoxical sleep. Thus, whether or not the brainstem Mg affects paradoxical sleep amount through its action on catecholaminergic systems requires further investigation.

As in our previous study (Chollet *et al.,* 2000a), higher brain and blood Mg levels were associated with a more consolidated or less fragmented sleep in both models, confirming that Mg plays a role in the modulation of sleep quality. However, because in MGH-MGL and B6 mice, some correlations tended to be in the opposite direction, optimal (physiological) levels of Mg more than high or low values seem to be involved.

Mg and Theta Peak Frequency

The theta peak frequency was significantly higher both during paradoxical sleep and waking in MGH than in MGL mice. We have shown, in inbred and segregating populations of mice, that theta peak frequency is one the most heritable EEG features in the mouse (heritability estimates between 80% and 94%, [Franken *et al.,* 1998; Tafti *et al.,* 1998]). The septum seems critical for the expression of hippocampal theta (Vertes and Kocsis, 1997; Vinogradova, 1995), and with increasing excitatory input from the brainstem to the septum, hippocampal theta frequency increases (McNaughton and Sedgewick, 1978; Vertes, 1981). Therefore, the brainstem, septum, and hippocampus are critically involved in the regulation of theta activity and its frequency. The concentration of Mg was found to differ between the MGH and MGL lines in the somatosensory cortex only. This tissue block included the hippocampus. Hallak *et al.* (1992) showed that within the rat brain the highest concentration of Mg occurs in the hippocampus, where it is also supposed to have an inhibitory action on seizure activity (Hallak *et al.,* 2000). Therefore, faster theta peak frequency in MGH mice may be related to increased Mg concentration in the hippocampus. As previously mentioned, Mg also has an essential role in monoaminergic systems, for instance, as the cofactor of the rate-limiting enzyme tryptophan hydroxylase in the synthesis of serotonin, which has been also implicated in the regulation of paradoxical sleep (Boutrel *et al.,* 1999). Thus, changes in serotonergic output from the raphe nuclei can change the frequency of hippocampal theta (Vinogradova, 1995). Other mechanisms such as hippocampal size and anatomy may have contributed to the faster theta peak frequency observed in MGH mice. Finally, note that Mg deficiency did not induce significant changes in either the somatosensory cortex Mg or the theta peak frequency.

Differences Between MGL, B6 Mg Deficiency, and MGH Models

MGL mice have a blood Mg concentration within the range of normal variations in inbred mice (Chollet *et al.,* 2000a), whereas under our experimental conditions, Mg deficiency induced a severe reduction in both blood and brain Mg. Also, the diet-induced deficiency

is usually accompanied by changes in other ions such as calcium (Chutkow, 1972), which is not observed in MGL mice (Henrotte *et al.,* 1997). Additionally, the effects of acute and prolonged reduction in Mg concentration are not documented. Also, several aspects of sleep such as the daily amplitude of sleep time (Fig. 3), the ability to resist to sleep deprivation (Fig. 1), or the theta peak frequency clearly deviated in the two models. As did Belknap *et al.* (1977) and Hallak *et al.* (1992, 2000), we have also noticed that Mg deficiency induced seizure activity, whereas there was no sign of seizure activity in MGL mice. In agreement with others (Henrotte *et al.,* 1995; Motta and Louis 1999; Feillet-Coudray *et al.,* 2000), we conclude that the two models, MGL and Mg deficient mice, are not comparable.

On the other hand, MGL mice were previously shown to have high Mg and catecholamine urinary excretion and greater sensitivity and/or reactivity to stress, as demonstrated by stress-induced aggressive behavior and gastric ulcers (Henrotte *et al.,* 1995, 1997; Feillet-Coudray *et al.,* 2000). In contrast, MGH mice appeared to exhibit characteristics similar to those of control animals. The present results show clearly that, as far as sleep is concerned (behavioral and electrophysiological correlates), for most parameters MGH mice deviate from other inbred mice more than MGL mice. Similar conclusions were reached by Motta and Louis (1999) studying audiogenic seizures in these lines. Consequently, MGH and MGL lines constitute two distinct models, each of them diverging or not diverging from control values according to the biological characteristics involved. Whatever the mechanisms underlying these findings, MGH mice may represent a unique model for hypermagnesemia as observed in human depression (Widmer *et al.,* 1995, 1998). As in depressed patients, for whom we have reported higher psychomotor retardation being strongly related to higher RBC Mg, MGH mice showed lower daily amplitude in sleep-wake distribution, mainly due to a lower "activity" (wake) level during the dark (active) period. We have also studied behavioral differences between MGH and MGL in the open field and found that MGH mice were significantly less active than MGL or B6 mice (Chollet *et al.,* 2000b). Finally, the reduced daily amplitude of vigilance states was accompanied by a dramatic decrease in the delta activity during the dark period. We have recently shown a very similar pattern of changes in delta activity in mice lacking the albumin D-binding protein gene (DBP), which we could attribute to a reduced circadian amplitude in the distribution of the vigilance states possibly in combination with a marked reduction of locomotor activity in the dark period (Lopez-Molina *et al.,* 1997, Franken *et al.,* 2000). We believe that as in DBP knock-out mice, the reduction in delta activity found in MGH can be interpreted as a secondary effect of the reduced daily amplitude of slow-wave sleep (Fig. 2) and the general locomotor activity (Chollet *et al.,* 2000b).

Do Mg and Sleep Regulation Share the Same Genetic Factors?

MGH and MGL lines were obtained by a bidirectional selective breeding based on high and low RBC Mg levels, respectively. This selective breeding was carried on from a heterogeneous population derived from F2 hybrids between four founding inbred strains (C57BL/6, AKR, DBA, and C3H). Through this process, the relevant alleles of genes regulating Mg levels were fixed in each line, whereas the genetic background in the two lines was a random distribution of the gene pool of the four founding strains. In this way, consistent differences appearing in MGH and MGL lines are most likely due either to genes involved in Mg regulation or to unrelated genes in tight linkage with them. Nevertheless, random fixation of unrelated genes cannot be excluded. The molecular bases of Mg as those of sleep regulation are poorly understood in mammals. Therefore, it seems premature to draw any conclusion. One possibility may be that sleep differences present in the four founding strains (Franken *et al.,* 1999) have been differentially fixed in each line early during the selection process. An alternative explanation may be that genes regulating Mg have nothing in common with those regulating sleep but that Mg, by being involved in so many key steps in the biology of the nervous system, can easily affect multiple unrelated systems involved in many behaviors, including sleep. Therefore, although the present findings strongly indicate that Mg affects the regulation of several aspects of sleep, whether this effect is mediated through common genes underlying the regulation of Mg and sleep remains to be elucidated.

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