Vitamin B_{12} Promotes Sleep and Modulates the Circadian Rhythm of Sleep and Brain Temperature in Rats

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Vitamin B_{12} improves circadian rhythm sleep disorders in humans. Since little is known for its underlying mechanism, we investigated sleep and brain temperature (Tbr)-modulatory activity of four different analogs of vitamin B_{12} by infusing into the third ventricle of freely behaving male rats for a 10-h nocturnal period. Three doses (1, 10 and 100 nmol) of methylcobalamin (methyl-B_{12}) dose-dependently enhanced both rapid-eye-movement (REM) sleep and non-REM sleep during the infusion period and further induced fluctuations of circadian sleep-waking rhythm for two post-infusion days. The time-course pattern of non-REM sleep and REM sleep was interrelated before and during the drug infusion period but dissociated thereafter. The administration of 100 nmol methyl-B_{12} resulted in a significant reduction of Tbr during the dark period in phase with the enhancement of sleep. Subsequently, significant fluctuations of circadian rhythm of Tbr occurred over two consecutive days independently from sleep-waking rhythm: a nocturnal elevation on the first post-infusion day followed by a diurnal reduction and a nocturnal elevation on the second post-infusion day. Lower doses of methyl-B_{12} exerted little effect on Tbr. Two analogs, 5'-deoxyadenosylcobalamin and hydroxocobalamin, at 100 nmol induced similar but less effects on sleep, whereas another analog, cyanocobalamin, at 100 nmol was ineffective. These results suggest that vitamin B_{12} acutely induced an enhancement of sleep by a direct action on the sleep-regulatory mechanisms, being accompanied by a reduction of Tbr, and that the drug further mediated a modulation of circadian rhythm of sleep-wakefulness and Tbr via its nonspecific action on the biological clock. (Sleep and Hypnosis 1999;1:98-104)

Key words: brain temperature, circadian rhythm, intracerebroventricular infusion, methylcobalamin, non-REM sleep, rat, REM sleep, vitamin B_{12}

INTRODUCTION

In human patients with circadian rhythm sleep disorders, chronic administration of a vitamin B_{12} analog, a-(5,6-dimethylbenzimidazolyl)-Co-methyl-cobamide (methylcobalamin, methyl-B_{12}), may improve their desynchronized sleep-wake cycles to entrain a 24-h nycthemeral cycle (1-4). However, the central mechanism involved in this phenomenon remains unknown. In an attempt to investigate the mechanism, we undertook a series of animal experiments to analyze effects of vitamin B_{12} on the circadian clock of free-running rats (5-10). We also undertook experiments to analyze direct effects of vitamin B_{12} on sleep in freely behaving rats by means of our long-term intracerebroventricular (icv) infusion technique, which we originally developed to quantify physiological sleep-promoting activity of sleep substances (11).

Since four different analogs of vitamin B_{12} such as cyanocobalamin (cyano-B_{12}), 5,6-dimethylbenzimidazolyl cobamide coenzyme (5'-deoxyadenosylcobalamin, DBCC), hydroxocobalamin (hydroxo-B_{12}) and methyl-B_{12} are registered, we conducted an experimental analysis of sleep-

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modulatory activity of all these analogs in rats. Further, on the basis of our preliminary observations (12) that methyl-B_{12} enhanced sleep for a prolonged period in freely behaving rats, we examined a possible modulatory effect of this substance on the circadian clock. In this connection, we concurrently analyzed the time-course changes in brain temperature (Tbr), since the circadian rhythm of Tbr is closely correlated with that of sleep. In the present paper, we deal with dose-dependent modulatory effects of methyl-B_{12} on sleep and Tbr as well as on their circadian rhythms. We further discuss the presumable involvement of transmethylation process in the action of the vitamin B_{12} analog.

METHOD

Male rats of the Sprague-Dawley strain raised in our closed colony were used. They were kept on a 12-h light and 12-h dark schedule (lights on: 08.00 to 20.00 h) in a constant air-conditioned environment of 25 – 1 ßC and 60 – 6 % relative humidity with free access to rat chow and water. At the age of 60-70 days, rats weighing 300-350 g were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally), fixed on a stereotaxic apparatus and implanted with a stainless-steel cannula (outer diameter: 0.35 mm) in the third ventricle for continuous icv infusion, three cortical gold-plated screw electrodes for recording electroencephalogram (EEG), two nuchal stainless-steel electrodes for recording electromyogram (EMG), and a thalamic thermistor (outer diameter: 1.1 mm) for measuring Tbr. The electrodes, thermistor and cannula were chronically fixed over the skull by a dental acrylic resin. The surgical techniques are described elsewhere (13). After the operation each animal received a total 40,000 U of penicillin G potassium subcutaneously and locally. Then they were individually housed in a special experimental cage which continuously enabled icv. infusion and monitoring of EEG, EMG and Tbr.

The experimental cages were placed in a soundproof, electromagnetically shielded room with the same environmental conditions as described above. Lead wires of the electrodes and thermistor were connected with a polygraph (Nihon-Kohden EEG-4317). The external end of the cannula was connected via a thin polyethylene tubing (inner diameter: PE10, 0.28 mm) to an infusion pump (Central Kagaku Boh-eki, CKI-100), which continuously flowed out a sterile physiological saline at a rate of 10 l/h. A cannular feed-through slip ring, inserted in between the lead wires along with the infusion tubing, and fixed above the experimental cage, guaranteed free movements to the animals. Polygraphic recordings of EEG, EMG and Tbr were done under continuous icv infusion of saline. Tbr data were fed into a computer and printed out at 3-min intervals.

After one week of recovery from surgery and acclimatization to icv infusion, a 4-day sleep assay was conducted under continuous recordings of EEG, EMG and Tbr along with continuous icv infusion: day 1 as baseline starting at the onset of the light period, day 2 as experiment when the saline infusion was replaced by an infusion of vitamin B_{12} solutions (1, 10 and 100 nmol of methyl-B_{12} in 100 l) for a 10-h period starting 1 h before the dark onset, and days 3 and 4 as recovery when saline was icv infused as before. Cyano-B_{12}, DBCC and hydroxo-B_{12} were also tested in the same manner at a single dose of 100 nmol. The vitamin samples were kindly gifted by Eisai Co., Ltd.

On the basis of polygraphic records of EEG and EMG, the vigilance state was classified into rapid-eye-movement (REM) sleep, non-REM sleep and wakefulness according to our routine criteria (14) and further analyzed statistically.

RESULTS

Effects of Methyl-B_{12} on Sleep

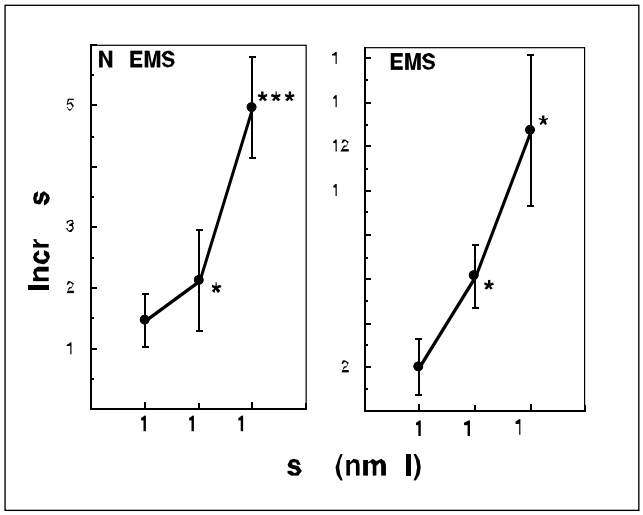
Similar to our previous observation (15), the rats kept under continuous icv infusion of saline exhibited a circadian rhythm of sleep and wakefulness that clearly manifested a night-active pattern of the rat. A 10-h icv infusion of methyl-B_{12} dose-dependently induced an enhancement of sleep at the expense of wakefulness during the 12-h dark period. The higher two doses of 10 and 100

Table 1. Effects of nocturnal 10-h icv infusion of 100 nmol methyl-B_{12} on sleep parameters in male rats (mean – S.E.M., n=8)

Parameters	Day 1 (Baseline)		Day 2 (Experiment)		Day 3 (Recovery 1)		Day 4 (Recovery 2)	
	NREMS	REMS	NREMS	REMS	NREMS	REMS	NREMS	REMS
Light period								
Total time (min	420.7 – 8.4	81.8 – 2.9	420.4 – 13.1	79.4 – 2.7	449.5 – 11.3*	64.3 – 2.8***	427.4 – 12.4	73.6 – 5.1
Episode number	83.3 – 6.7	34.3 – 1.5	88.6 – 8.0	34.5 – 1.9	65.8 – 4.3*	28.9 – 1.9*	57.4 – 3.1	28.1 – 1.6
Episode duration (min)	5.3 – 0.4	2.4 – 0.1	5.0 – 0.4	2.3 – 0.1	7.1 – 0.5*	2.3 – 0.1	7.6 – 0.4	2.6 – 0.2
Dark period								
Total time (min)	241.3 – 14.2[a]	33.9 – 6.7[b]	356.1 – 18.5[a],***	58.2 – 5.7[b],*	241.1 – 14.7[a]	28.5 – 5.3[b]	199.1 – 16.6[a]	24.3 – 4.9[b]
Episode number	65.0 – 7.5	18.4 – 3.0	67.8 – 7.7	32.0 – 3.1**	48.6 – 3.1	15.4 – 2.0	42.5 – 5.3	12.5 – 2.5
Episode duration (min)	3.9 – 0.3	1.7 – 0.1	5.6 – 0.6*	1.8 – 0.1	5.0 – 0.2**	1.8 – 0.2	5.0 – 0.4	1.9 – 0.1

NREMS: non-rapid-eye-movement sleep; REMS: rapid-eye-movement sleep.
[a] $P < 0.005$ (one-way ANOVA, $F_{3,28} = 17.585$), [b] $P < 0.005$ (one-way ANOVA, $F_{3,28} = 7.214$).
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Significantly different from the corresponding baseline values (Student's t-test).

Figure 1. Dose-response relation of icv infused methyl-B_{12} on the amount of non-rapid-eye-movement sleep (NREM) and rapid-eye-movement sleep (REM) during the 12-h dark period in rats (n = 8 for each group). Each value was expressed as percent change from the corresponding baseline (100 %). Vertical lines on each column stand for S.E.M.



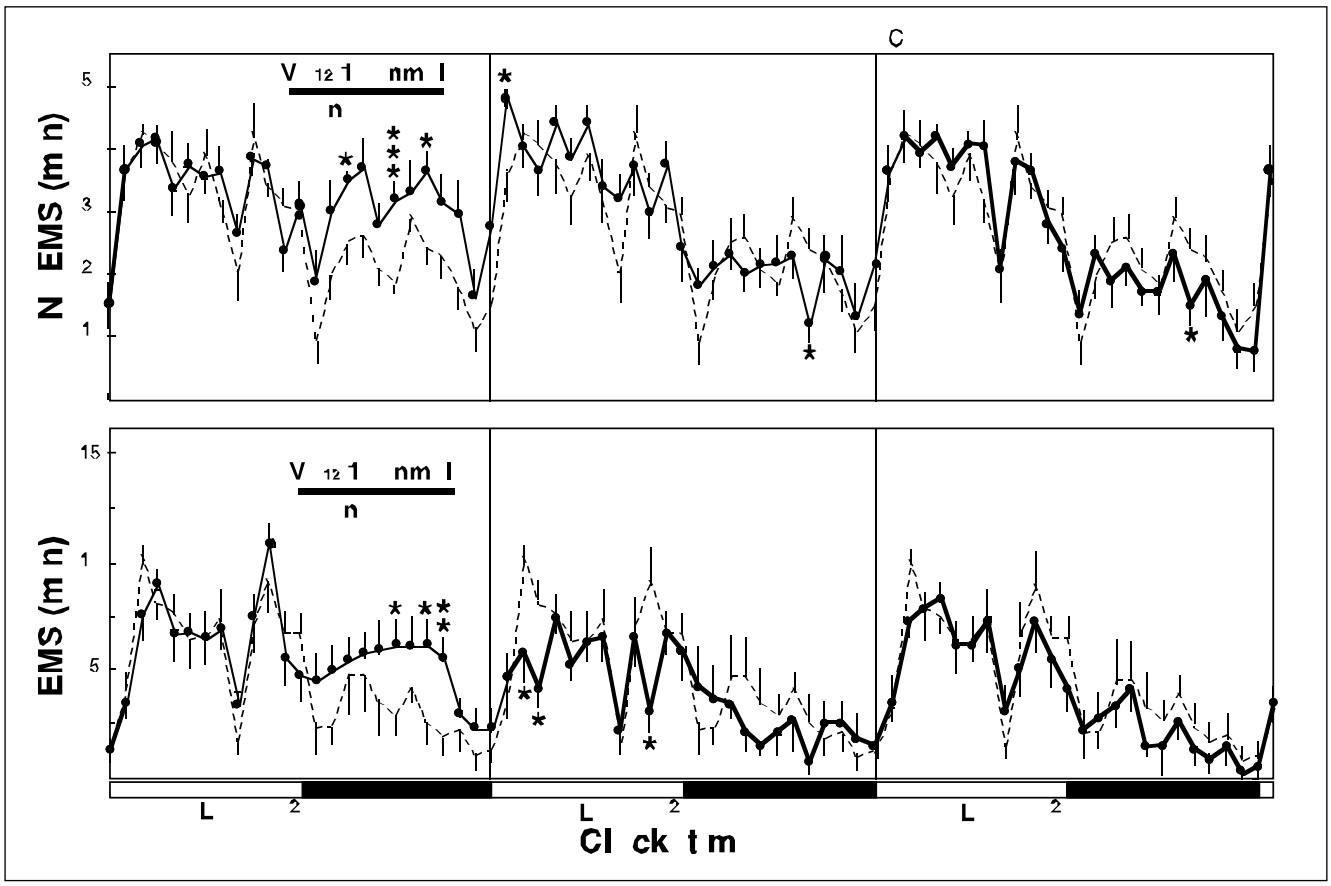
* P < 0.05, ** P < 0.01 (Student's t-test)

nmol significantly increased both non-REM sleep and REM sleep, whereas the lowest dose of 1 nmol exhibited a slight but insignificant somnogenic activity (Figure 1).

The effects of 100 nmol of methyl-B_{12} on sleep parameters were summarized in Table 1. The drug administration brought about a significant increase in the amount of non-REM sleep by 114.8 min (49.7 % above the baseline value, P < 0.001, Student s t-test) and that of REM sleep by 24.3 min (127.5 %, P < 0.05) during the 12-h dark period of day 2. The increment of non-REM sleep and REM sleep was mainly due to a prolongation of their duration and an increase in their number, respectively. The prolonged duration of non-REM sleep episodes was statistically not different from the duration observable in the light period, indicating that nocturnally administered methyl-B_{12} brought about almost the same non-REM sleep as occurring in daytime, i.e. the resting phase of the rat. During the subsequent light period (day 3), non-REM sleep was still significantly enhanced and elicited an increment by 28.8 min (7.0%, P<0.05) due to a significant prolongation of episode duration, while REM sleep was inversely suppressed by 17.5 min (20.8 below the baseline value, P<0.001). During the dark period of day 3, the episode duration of non-REM sleep were still significantly prolonged although the total amount of non-REM sleep returned to the baseline level. On the second day of recovery (day 4), all sleep parameters were no more different from those of the baseline values. Interestingly, however, nocturnal amounts of non-REM sleep and REM sleep were considerably lower than those of the corresponding baseline values and the episode duration of non-REM sleep was longer (Table 1).

The time-course changes in hourly values of non-REM

Figure 2. Effects of 10-h icv infusion (indicated by a solid bar) of 100 nmol of methyl-B_{12} on hourly amounts of non-rapid-eye-movement sleep (top, NREMS) and rapid-eye-movement sleep (bottom, REMS) for three consecutive days in rats, as compared with the corresponding baseline values (broken lines). A: day for infusion; B and C: days for recovery. Vertical lines on each hourly value stand for S.E.M.



* P < 0.05, ** P < 0.01, *** P < 0.001 (Student's t-test)

Table 2. Effects of nocturnal 10-h icv infusion of 100 nmol methyl-B_{12} on brain temperature (ßC) in male rats (mean – S.E.M., n=6)

	Day 1 (Baseline)	Day 2 (Experiment)	Day 3 (Recovery 1)	Day 4 (Recovery 2)
Light period	36.93 – 0.07	36.93 – 0.05	37.02 – 0.08	36.72 – 0.10**
Dark period	37.60 – 0.04	37.27 – 0.04***	37.92 – 0.04***	37.75 – 0.04*

* P < 0.02, ** P < 0.01, *** P < 0.001, Significantly different from the corresponding baseline values (Student's t-test).

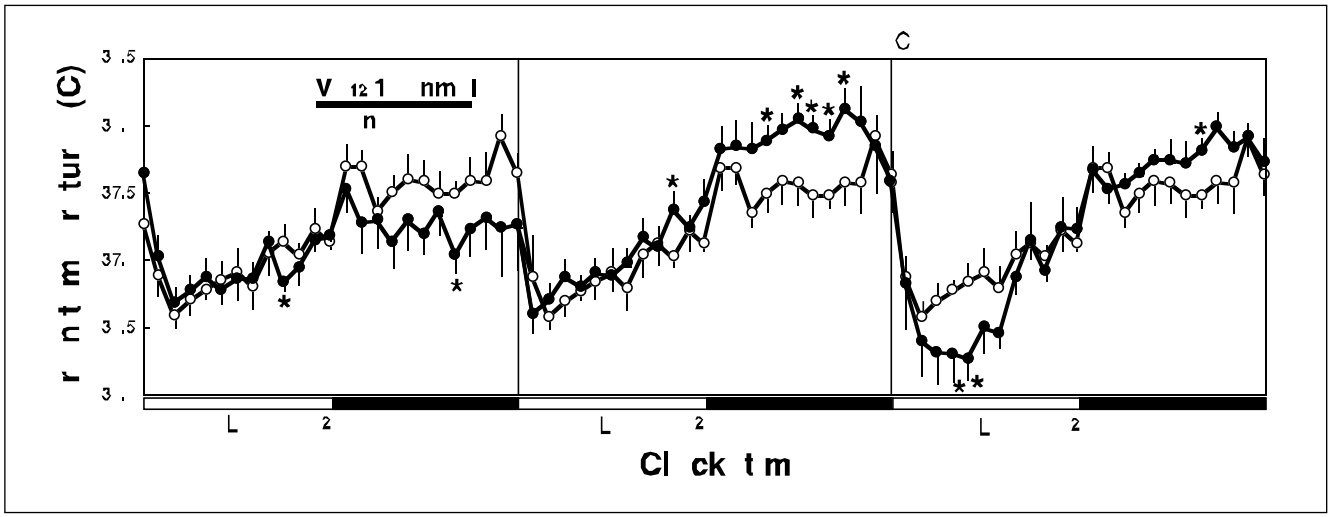
sleep and REM sleep caused by 100 nmol methyl-B_{12} are illustrated in Figure 2. During the experimental night, the temporal pattern of the enhancement of non-REM sleep was largely similar to that of REM sleep regardless of differences in magnitude (Figure 2A). Sleep-modulatory effects were still observable even after the termination of the drug infusion. However, the temporal pattern of the occurrence of non-REM sleep and REM sleep was quite differential, i.e. an enhancement of non-REM sleep and a suppression of REM sleep in the light period and a continued suppression of REM sleep in the dark period of day 3 (Figure 2B). The amount of non-REM sleep and REM sleep tended to return toward the baseline level thereafter, although fluctuations were still observed, especially a decrease in non-REM sleep and REM sleep during the night of day 4 (Figure 2C).

Effects of Methyl-B_{12} on Tbr

Similar to our previous observation (16), the rats kept under continuous icv infusion of saline exhibited a circadian rhythm of Tbr characterized with a night-active pattern of the rat: low in the light period and high in the dark period (Table 2). A 10-h icv infusion of 100 nmol of methyl-B_{12} induced significant changes in Tbr over three consecutive days. The time course of hourly changes in Tbr caused by this dose of methyl-B_{12} are illustrated in Figure 3 and average values during 12-h light or dark periods are shown in Table 2. As compared with the baseline, Tbr exhibited a remarkable reduction (-0.33 ßC) during the dark period of the day of administration, then a rebound (+0.09 ßC) in the subsequent light period through the dark period (+0.32 ßC), and again a reduction in the next diurnal period (-0.21 ßC) prior to an elevation in the nocturnal period (+0.17 ßC). All these changes except for the light period of the first post-infusion day were statistically significant as indicated in Table 2. Doses lower than 100 nmol exerted little Tbr-modulatory activity (data not shown).

The administration of 100 nmol methyl-B_{12} induced a long-lasting fluctuations in Tbr. The time-course of methyl-B_{12}-induced changes of Tbr was closely interrelated to that of sleep at the initial dark period but it became differential thereafter. Namely, during the experimental night, the temporal pattern of Tbr reduction was in phase with that of sleep promotion (Figures 2 and 3). However, shortly after the termination of drug infusion, i.e. from the beginning of the subsequent light period on, the temporal pattern of Tbr became largely dissociated from that of sleep.

Figure 3. Effects of 10-h icv infusion (indicated by a solid bar) of 100 nmol of methyl-B_{12} on hourly values of brain temperature for three consecutive days in rats, as compared with the corresponding baseline values (open circles). A: day for infusion; B and C: days for recovery. Vertical lines on each hourly value stand for S.E.M.

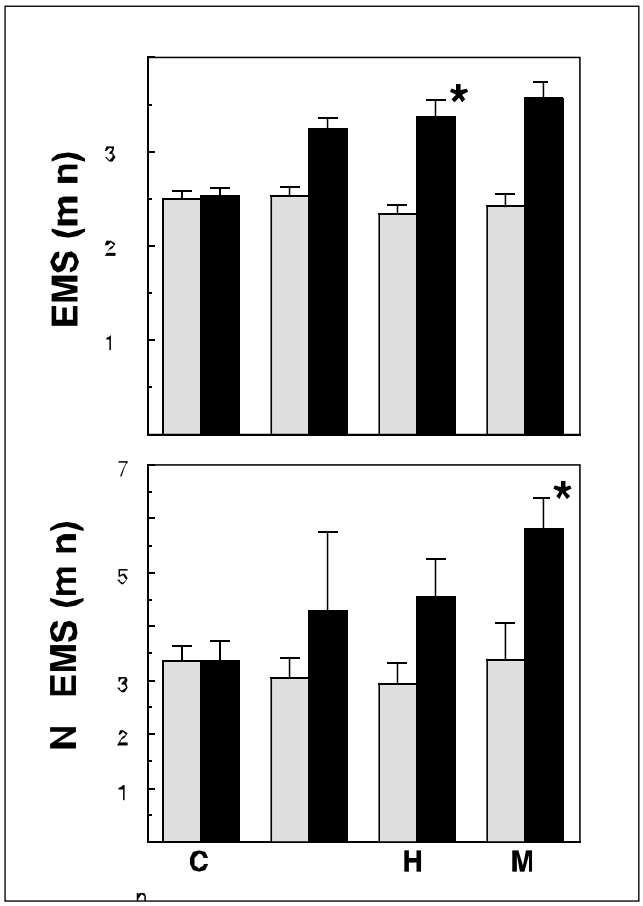


* P < 0.05, ** P < 0.01 (Student's t-test)

Effects of the Other VB_{12} Analogs on Sleep

A 10-h icv infusion of 100 nmol of DBCC and hydroxo-B_{12} also induced an enhancement of sleep during the 12-h dark period, while 100 nmol of cyano-B_{12} was entirely non-effective (Figure 4). A significant increase in the total amount of non-REM sleep caused by DBCC and hydroxo-B_{12} was mainly due to a considerable prolongation of their duration (for DBCC: from 3.8 – 0.2 to 4.5 – 0.3 min, P<0.2;

Figure 4. Effects of icv infused cyanocobalamin (C), 5,6-dimethyl-benzimidazolyl cobamide coenzyme (D), hydroxocobalamin (H) and methylcobalamin (M) on the total amount (black columns) of non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) during the 12-h dark period in rats, as compared with the corresponding baseline (gray columns). Vertical lines on each column stand for S.E.M.



* P < 0.05, # P < 0.001 (Student's t-test)

for hydroxo-B12: from 4.3 – 0.4 to 5.6 – 0.4 min, P<0.05, Student s t-test) but not due to an increase in the number of corresponding episodes. An increase in the total amount of REM sleep was also observable after administration of DBCC and hydroxo-B_{12} but the changes were statistically insignificant.

DISCUSSION

The most intriguing finding in the present study is that vitamin B_{12}, especially methyl-B_{12}, dose-dependently elicited sleep- and Tbr-modulatory activities in normal rats. Furthermore, the modulation of sleep and Tbr was long-lasting over two post-infusion days. The time-course changes in sleep and Tbr caused by methyl-B_{12} administration at the dose 100 nmol were closely associated with each other at the early phase, i.e. the initial 12-h dark period, but dissociated later. Interestingly, the methyl-B_{12}-induced enhancement of non-REM sleep and REM sleep at the early phase seems to be induced by differential mechanisms; the episode of nocturnal non-REM sleep prolonged its duration like that of diurnal one without changing its number, whereas the episode of nocturnal REM sleep increased its number without changing its duration. Since it is known that sleep, especially non-REM sleep, reduces Tbr (17), the methyl-B_{12}-induced excessive sleep at the early phase appears to be responsible for the reduction of Tbr, exerting a masking effect. However, it is likely that the long-lasting fluctuations in the circadian rhythm of Tbr was attributable to a mechanism different from that of the regulation of sleep. Dissociation between Tbr and sleep was also demonstrated by the fact that a lower dose (10 nmol) of methyl-B_{12} was ineffective on Tbr modulation but effective on sleep promotion.

The dissociation of the time-course changes in non-REM sleep, REM sleep and Tbr at the later period lead to two possible assumptions (18): (1) the initial modulation of sleep and Tbr at the early period was largely dependent on the direct and short-term effect of methyl-B_{12} on the neural mechanisms involved in the regulation of sleep and Tbr, respectively, and (2) the subsequent changes were predominantly mediated by the nonspecific long-term effect of methyl-B_{12} on the circadian biological clock.

The first assumption is substantiated by the fact that methyl-B_{12} counteracts cytotoxic action of glutamic acid in rat cortical neurons in vitro (19), since a blockade of neurotransmission mediated by glutamic acid, causes an enhancement of sleep in cats (20) and rats (21,22). This assumption is further substantiated by our recent findings that methyl-B_{12} acutely and directly affects firing activity of hypothalamic neurons including the preoptic area and the suprachiasmatic nucleus in vitro (23). The second assumption is substantiated by the fact that chronic repetitive administration of methyl-B_{12} is essentially required for an improvement of circadian rhythm sleep disorders in humans (1-3) and for a modification of free-running period of locomotor and drinking activity rhythms in rats (24,25). In this respect, we recently observed that, similar to rats in normal environment, rats kept under constant dim light, can exhibit differential changes such as an initial short-term sleep promotion and a subsequent long-term prolongation of free-running period of Tbr in response to methyl-B_{12} administration (6). In addition, Uchiyama et al. (26) reported that methyl-B_{12} amplifies circadian rectal temparature rhythm in healthy humans under the constant routine.

Okawa et al. (2) pointed out three possible mechanisms involved in the methyl-B_{12}-induced modulation of circadian rhythm in human patients: (1) alterations in the period of sleep-waking rhythm, (2) an improvement of entraining capacity of the endogenous sleep-waking rhythm to the environmental 24-h rhythm, and (3) an enhancement of sleep. For the first possibility, it is reported that free-running period of locomotor and drinking activity (24,25) and Tbr (6) rhythms are modified after methyl-B_{12} administration in rats. For the second possibility, it is reported that methyl-B_{12} mediates an improvement of sensitivity to light via a suppression of melatonin secretion (27,28), and that methyl-B_{12} accelerates the re-entrainment of body temperature and locomotor activity rhythms to shifted environmental rhythms (29). For the third

possibility, the present study as well as previous reports (6,10,30) clearly demonstrate that it is really the case. Thus, all these possibilities may be more or less responsible for the modulation of the circadian rhythmicity. Taking together, it may be plausible that methyl-B_{12} may nonspecifically and time-consumingly modulate the circadian clock system via an alteration of brain functions. Further analytical studies are required to unveil the brain mechanisms involved in the biological activities of methyl-B_{12}.

Among four different analogs of vitamin B_{12}, methyl-B_{12} exerted the most prominent sleep-modulatory activity. We recently found that cyano-B_{12} also failed to modulate sleep and Tbr in freerunning rats (6) and nalcoleptic dogs (31). This indicates that the methyl moiety of cobamide structure and thus a transmethylation reaction may play an important role in manifesting the biological activity in the brain. In this regard, it is well known that a transmethylation product S-adenosylmethione (SAM) elicits a wide variety of biological activities in the central nervous system (32). Although these analogs of vitamin B_{12} are mutually transferable via biochemical processes, the failure of sleep modulation by cyano-B_{12} is attributable to the fact that this compound is the excretory type having little tissue affinity (33).

In conclusion, it seems likely that methyl-B_{12}-mediated alterations of both sleep and Tbr in concert but differentially contribute to the modulation of the circadian clock system. Further studies are required to explain the brain mechanisms involved in these and other methyl-B_{12}-induced responses.

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