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0014-4754/92/080701-17$1.50 + 0.20/0

Research Articles

Effects of vitamin B12 on plasma melatonin rhythm in humans: increased light sensitivity phase-advances the circadian clock?

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Received 4 November 1991; accepted 14 April 1992

Abstract. Vitamin B12 (methylcobalamine) was administered orally (3 mg/day) to 9 healthy subjects for 4 weeks. Nocturnal melatonin levels after exposure to bright light (ca. 2500 lx) were determined, as well as the levels of plasma melatonin over 24 h. The timing of sleep was also recorded. Vitamin B12 was given blind to the subjects and crossed over with placebo. We found that the 24-h melatonin rhythm was significantly phase-advanced (1.1 h) in the vitamin B12 trial as compared with that in the placebo trial. In addition, the 24-h mean of plasma melatonin level was much lower in the vitamin B12 trial than with the placebo. Furthermore, the nocturnal melatonin levels during bright light exposure were significantly lower in the vitamin B12 trial than with the placebo. On the other hand, vitamin B12 did not affect the timing of sleep. These findings raise the possibility that vitamin B12 phase-advances the human circadian rhythm by increasing the light sensitivity of the circadian clock.
Key words. Circadian rhythm; melatonin; bright light; vitamin B12; entrainment.

Vitamin B12 has been reported to normalize the entrainment of circadian rhythms in delayed sleep phase insomnia (DSPI) and in non-24-h sleep-wake cycle [1–3], where disturbance of the entrainment of the circadian clock is assumed to be involved. DSPI is a sleep disorder in which sleep occurs regularly but is extremely delayed, and is thought to be a state where the circadian clock entrains at the border of the entrainment range [4]. Non-24-h sleep-wake cycle is a sleep disorder in which the timing of sleep is increasingly delayed, and is thought to be a state where the circadian clock is free-running in the presence of zeitgebers [5].

The effect of vitamin B12 can be explained theoretically by three mechanisms. First, vitamin B12 changes the free-running period of the circadian clock and thereby facilitates the entrainment to zeitgebers. Second, vitamin B12 increases the sensitivity of the circadian clock to photic or social zeitgebers. Third, vitamin B12 changes the quality of sleep or wakefulness, improving the internal organization of the circadian system. In the present study, we wanted to know whether vitamin B12 affects human circadian rhythms in normal subjects, and whether vitamin B12 increases the light sensitivity of plasma melatonin, in order to gain an insight into the mechanism of vitamin B12 action.

Materials and methods

Subjects were 10 male students (20–28 years old) who had been living in Sapporo City for at least 3 years. Before the start of the experiment, medical examinations

(including ECG, biochemical constituents in blood and urine, history of sleep disturbance, etc.) were performed to exclude unsuitable volunteers. The subjects gave written informed consent. Vitamin B12 (methylcobalamine; Ezai) was administered orally 3 × 1 mg/day for 4 weeks (B12 trial), which was crossed over to placebo (placebo trial) after an intermission period of 4 weeks. The placebo trial continued for 4 weeks. The order of administration and the types of drug (B12 or placebo) were not known to the subjects. Neither part-time jobs in the early morning or at night, nor travels across time zones, were permitted during the period of experiment.

A sleep diary was recorded for the entire period of the experiment (12 weeks). In the 4th week in each trial, blood sampling was performed in an experimental living facility. The facility consisted of a living room and bedrooms where temperature and humidity were controlled. The bedrooms had no windows and were sound-proof. The living room had a large window facing south through which natural daylight entered. The light intensity during daytime rose to 5000 lx on a sunny day. The subjects stayed there in pairs from 09.00 h until the next morning. A meal was supplied at 13.00, and 08.00 h. TV watching was allowed only during daytime. Blood sampling through an indwelling catheter was performed at 1-h intervals starting at 10.00 h until 10.00 h on the following day. At night, the living room was illuminated by a fluorescent light of 150–300 lx. Sleep was not permitted until 04.00 h. From 02.00 to 04.00 h, the subjects sat down in front of a source of bright light but without turning it on. During this period, the blood sampling interval was shortened to 30 min. The light intensity at the position of the subjects was about 250 lx. They gazed at the apparatus for 5 s every one min. The timing for this test period was controlled by chimes. Two days later, the subjects came to the laboratory at around 18.00 h and blood sampling was performed starting at 24.00 h and ending at 06.00 h. The sampling procedures and lighting conditions were the same as those in the previous experiment except that the bright light (ca. 2500 lx) was turned on from 02.00 h to 04.00 h. During the light exposure, blood was sampled at 30-min intervals.

Plasma melatonin was assayed by RIA using an Organon kit [6]. The sensitivity of the assay was 5 pg/tube. The inter- and intra-assay variances (CV) were 5.9% and 8.0%, respectively. Plasma vitamin B12 and folic acid were measured by RIA (SRL). The acrophase and the level of plasma melatonin rhythm were determined by a best-fitted cosine method [7]. Statistical significance was evaluated by non-parametric Wilcoxon signed rank test.

Results

One subject dropped out at the very beginning of this experiment because of his hospitalization due to an accident.

The mean plasma levels of vitamin B12 and folic acid determined 28 days after the start of each trial were 511 ± 48 (S E) pg/ml and 2.66 ± 0.12 ng/ml in the placebo trial, and 1047 ± 123 pg/ml and 2.97 ± 0.22 ng/ml in the B12 trial, respectively. Plasma vitamin B12 increased significantly after vitamin B12 administration, while plasma folic acid was not changed.

The timing of sleep was analyzed in 8 subjects (part of the result was lost in subject T Y) for 14 days, from 7 days after the start of each trial until the beginning of the week of the chronobiological examination. The group means were calculated from individual mean. Table 1 gives the times of go-to-bed, wake-up, and TIB (total time in bed) in individual subjects. Although the group means were not significantly different between the two trials, the timings of go-to-bed or wake-up of three subjects (subjects YM, MT, and ST) were phase-advanced in the B12 trial. TIB of subject MT was shortened in the B12 trial. On the other hand, no individual showed a phase-delay shift in the timing of sleep in the B12 trial.

Table 2 gives the levels of oscillation, amplitudes, and acrophases of individual melatonin rhythms determined by a best-fitted cosine method. Subject MT (medical student) had a problem with his social schedules during the examination week of the B12 trial. He had to stay up late at night because of obstetric duties, and the timing of his sleep was suddenly delayed. The acrophase of the plasma melatonin rhythm in this subject was regarded as extreme by Smirnoff test ($p < 0.01$), and so was omitted from this analysis. The acrophase of the plasma melatonin rhythm was located significantly earlier in the B12 trial than with the placebo ($n = 8$, $p < 0.01$). The level of rhythm was also significantly lower in the B12 trial than in the place-

Table 1. Timings of sleep (go-to-bed, wake-up, TIB) in placebo and B12 sessions. Individual values (h) were presented by the mean (n = 14) and the standard error of the mean (in parentheses).

Subject	Placebo Go-to-bed	Wake-up	TIB	Vitamin B12 Go-to-bed	Wake-up	TIB
S.O.	1.33 (0.20)	7.53 (0.24)	6.19 (0.27)	1.36 (0.46)	8.18 (0.41)	6.82 (0.41)
H.K.	1.68 (0.35)	8.49 (0.44)	6.82 (0.46)	1.56 (0.39)	7.62 (0.26)	6.06 (0.30)
S.T.	2.34 (0.19)	8.60 (0.30)	6.26 (0.40)	1.21 (0.15)	7.38 (0.18)	6.16 (0.21)
Y.M.	1.90 (0.32)	9.59 (0.32)	7.69 (0.47)	0.96 (0.24)	8.88 (0.36)	7.92 (0.36)
T.S.	1.08 (0.28)	8.27 (0.18)	7.19 (0.27)	1.62 (0.19)	8.59 (0.33)	6.97 (0.47)
T.O.	0.38 (0.21)	7.06 (0.18)	6.69 (0.31)	0.18 (0.13)	7.19 (0.24)	7.01 (0.18)
Z.M.	1.18 (0.35)	8.64 (0.29)	7.47 (0.28)	0.98 (0.40)	8.78 (0.14)	7.81 (0.44)
M.T.	1.74 (0.08)	10.47 (0.24)	8.73 (0.27)	1.82 (0.32)	9.17 (0.41)	7.35 (0.32)
Mean	1.45 (0.23)	8.58 (0.41)	7.13 (0.32)	1.21 (0.19)	8.22 (0.28)	7.01 (0.26)

Table 2. The level (pg/ml), amplitude (pg/ml) and acrophase (h) of plasma melatonin rhythm in placebo and B12 sessions. Asterisks indicate statistically significant differences from zero.! indicates an extreme value estimated by Smirnoff test ($p < 0.01$).

Subject	Placebo Level	Amplitude	Acrophase	Vitamin B12 Level	Amplitude	Acrophase	Difference Level	Amplitude	Acrophase
S.O.	16.0	16.6	3.3	10.1	8.6	1.4	6.0	6.0	1.9
H.K.	20.4	7.3	3.2	7.3	3.2	2.8	−13.2	4.2	0.4
T.Y.	12.6	8.1	3.3	11.8	5.6	2.4	0.8	2.5	0.9
S.T.	15.6	15.1	4.6	12.3	10.9	3.2	3.3	4.2	1.4
Y.M.	10.8	9.2	3.7	9.6	7.4	3.8	1.2	1.8	−0.1
T.S.	11.8	9.6	3.4	10.2	8.5	2.9	1.6	1.2	0.5
T.O.	7.1	4.9	4.3	6.2	3.2	2.4	0.9	1.7	1.9
Z.M.	29.3	17.8	5.8	29.2	22.6	3.9	0.1	−4.8	1.9
M.T.	8.2	4.4	2.9	12.7	4.2	6.8!	−4.5	−2.8	−3.9!
Mean	14.7	10.1	3.8	12.1	8.6	2.9	2.5*	1.5	1.1**
SE	2.3	1.6	0.3	2.3	1.9	0.3	1.6	1.1	0.3

bo ($p < 0.05$). On the other hand, the amplitude did not differ between the two trials.

Figure 1 illustrates the 24-h group rhythms of plasma melatonin in both trials. Because of a large interindividual difference in plasma melatonin level, melatonin levels in individual subjects were expressed as a percentage of the individual 24-h mean which was obtained by pooling the hormone values in 1-h bins. In this analysis, the results of subject MT were not included ($n = 8$). A significantly higher plasma melatonin level was observed in the B12 trial at 23.00, 24.00 and 01.00 h, when the nocturnal rise of plasma melatonin started ($p < 0.05$). The high nocturnal melatonin level also seems to start declining earlier in the B12 than in the placebo trial, and a statistically significant difference was detected in the melatonin level at 07.00 h.

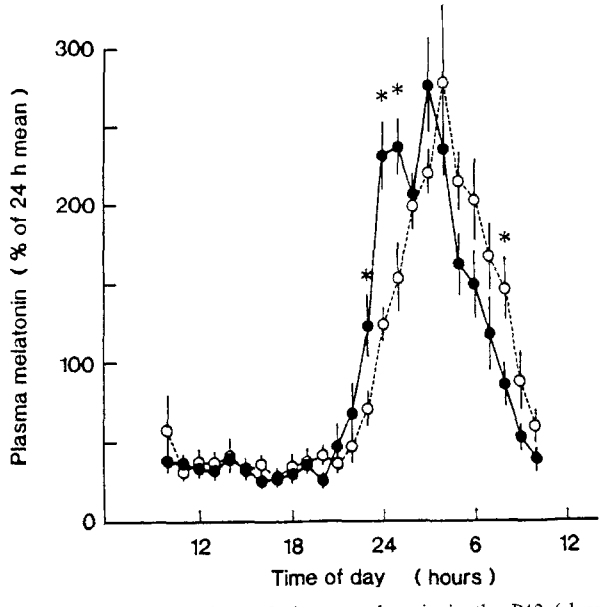

Figure 1. The 24-h rhythms of plasma melatonin in the B12 (closed circles) and placebo (open circles) trials. The melatonin levels were expressed as a percentage of the 24-h mean and presented by the mean ($n = 8$) and the standard error of the mean. Asterisk indicates a statistically significant difference between the two trials ($p < 0.05$).

Figure 2 illustrates the effects of vitamin B12 on the light suppression of nocturnal melatonin levels. The melatonin levels in individuals were expressed as a percentage of the melatonin level at 02.00 h in each measurement. In both trials, plasma melatonin levels were not suppressed by dim light (250 lx) when compared with the melatonin level at 02.00 h. There was no significant difference in plasma melatonin levels between the two trials, except for that at 05.00 h which was significantly lower in the B12 trial. On the other hand, the plasma melatonin level was suppressed by bright light (2500 lx) in both trials (at

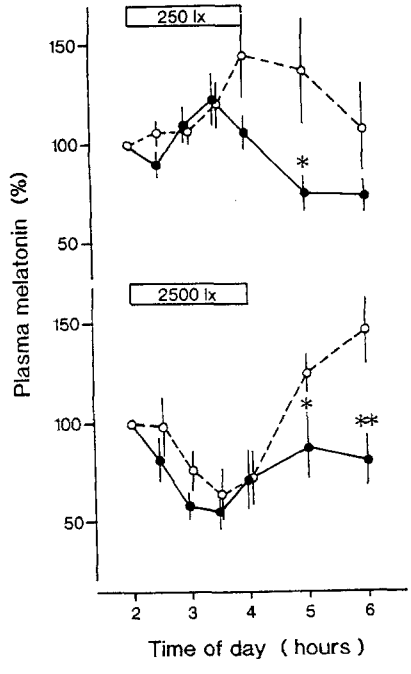

Figure 2. Light suppression of nocturnal melatonin levels in the B12 (closed circle) and placebo (open circle) trials. Effects of dim light exposure (250 lx) on plasma melatonin levels were illustrated in the upper panel and of bright light exposure (2500 lx) in the lower panel, respectively. The melatonin level is expressed as a percentage of the level at 02.00 h in each experiment and presented by the mean and the standard error of the mean. Asterisks indicate statistically significant difference between the two trials; *, $p < 0.05$: **, $p < 0.01$.

Table 3. Effects of bright light on nocturnal melatonin levels in the B12 and placebo trials. Values are expressed as a percentage of the melatonin level under exposure to dim light at each time, and presented by the mean and standard error of the mean (in parentheses). Asterisk indicates a statistically significant difference between the B12 and placebo trials ($p < 0.05$).

Trial	Time 24.00 h	01.00 h	02.00 h	02.30 h	03.00 h	03.30 h	04.00 h	05.00 h	06.00 h
Placebo	75.3	108.7	110.4	92.8	69.2	61.4	70.2	121.1	152.4
	(14.9)	(19.2)	(9.0)	(13.1)	(9.3)	(13.5)	(13.3)	(11.3)	(17.2)
B12	109.5	110.0	109.5	81.8	53.8*	42.1	56.1	100.9	165.6
	(15.0)	(15.3)	(13.7)	(8.8)	(7.3)	(5.7)	(12.1)	(17.7)	(25.0)

03.00 and 03.00 h: $p < 0.05$) by about 55–65% at maximum. Between the two trials, there was no significant difference in plasma melatonin at any time point during the light exposure, but the accumulated melatonin level from 02.30 h to 04.00 h was significantly lower in the B12 than in the placebo trial ($p < 0.05$). Significant decreases in plasma melatonin level were also detected in the B12 trial at 05.00 h and 06.00 h.

Table 3 demonstrates the effect of bright light on plasma melatonin level, using the same data as those in figure 2 but with a different analysis. In this analysis, the effect of bright light was assayed by comparing the melatonin levels not with value at 02.00 h but with the values under the dimlight exposure at different times. The melatonin level at 03.00 h was significantly lower in the B12 than in the placebo trial.

No systematic effect of the order of drug administration was detected.

Discussion

Vitamin B12, known as an anti-pernicious anemia factor, has been reported to normalize the sleep-wakefulness rhythm of DSPI and non-24-h sleep-wake cycle. The sleep-wakefulness rhythm was phase-advanced [3] in DSPI or stopped to free-run in non-24-h sleep-wake cycle [1, 2] within a few weeks of the start of vitamin B12 administration. In the present study, vitamin B12 was shown to exert a phase-advancing effect on the circadian rhythm in healthy subjects. The onset of the nocturnal rise in plasma melatonin as well as the acrophase of plasma melatonin rhythm were phase-advanced significantly in the B12 trial (table 2 and fig. 1).

The above conclusion was based on the plasma melatonin rhythms determined where the subjects were exposed to dim light of 250 lx from 02.00 to 04.00 h and were not permitted to sleep until 04.00 h. An acrophase calculated by a best-fitted cosine method is to some extent a function of the shape of rhythmicity [8]. A change in the shape of rhythmicity will change the acrophase, which may lead to a misinterpretation of the data. However, sleep deprivation or exposure to light less than 400 lx does not seem to modify the shape of the plasma melatonin rhythm significantly [9], and the shape of the melatonin rhythm was essentially the same in both trials (fig. 2). Moreover, the onset of the noctural rise in plasma melatonin which occurred before light exposure was also phase-advanced in the B12 trial.

A phase advance of an entrained circadian rhythm is caused by an increased phase response of the circadian clock to entraining time cues. For the human circadian rhythm, bright light above 2500 lx is proposed to be a potent time cue, and a single bright light in the subjective morning was demonstrated to induce a phase-advance of free-running human circadian rhythms [10]. Bright light was also demonstrated to phase-shift the plasma melatonin rhythm [11]. Therefore, one possible mechanism of vitamin B12 action is an enhancement of the light sensitivity of the circadian clock, which increases the phase responsiveness for light. In the present study, the light suppression of nocturnal plasma melatonin was used as an index of the light sensitivity. In mammals it is well established that the pathway responsible for the light suppression starts from the retina, goes through the retinohypothalamic tract, a pathway for the light entrainment of the circadian pacemaker located in the suprachiasmatic nucleus, and finally ends in the pineal at the sympathetic postganglionic neurons [12]. The phase shift of the circadian rhythm by light and the light suppression of pineal melatonin were demonstrated to occur in parallel [13], and to have similar characteristics [14, 15].

As illustrated in figure 2 and table 3, bright light of 2500 lx suppressed plasma melatonin levels more extensively in the B12 trial than in the trial with placebo. The 24-h mean of plasma melatonin level was also significantly lower in the B12 trial. These findings suggest that the sensitivity of pineal melatonin to bright light is increased by vitamin B12. The significantly lower melatonin levels at 05.00 h and 06.00 h in the B12 trial, which were detected by one analysis (fig. 2), are probably due to a phase-advance of plasma melatonin rhythm by B12. Such differences were not observed when plasma melatonin levels at each timepoint were compared with those under the dim light exposure (table 3).

By contrast, the sleep-wakefulness rhythm was not affected by B12 administration, although the timing of sleep was phase-advanced in some individuals. In humans, the sleep-wakefulness rhythm is thought to be regulated by a different mechanism from that regulating the circadian rhythms of rectal temperature or plasma melatonin [16, 17]. The entrainment of the sleep-wakefulness rhythm can be dissociated from that of the rectal temperature rhythm; the former is entrained by social schedules, the latter by bright light [18]. Taking this into consideration, vitamin

B12 does not seem to affect the entrainment of the sleep-wakefulness rhythm directly.

To our knowledge, the light sensitivity of the circadian clock or plasma melatonin has not been reported in patients with DSPI or non-24-h sleep-wake cycle. Recently we examined the circadian rhythms and the light sensitivity of one sighted subject with a non-24-h sleep-wake cycle under temporal isolation (in preparation). His free-running period (25.9 h) was within a normal range. But it is of special interest that his circadian clock did not respond at all to bright light of 5000 lx applied in the subjective morning. Moreover, the light suppression of nocturnal melatonin level was very weak. These findings suggested a reduced light sensitivity of the circadian clock as the primary cause of free-running in non-24-h sleep-wake cycle.

In animal experiments, a number of chemical substances are known to affect the circadian pacemaker[13]. Among them, carbachol (acetylcholine agonist) was reported to mimic the effects of light on the circadian pacemaker and pineal melatonin, and acetylcholine was proposed to be a neurotransmitter of the retinohypothalamic tract in rodents[19,20]. Methylcobalamine, used in the present study as vitamin B12, may act as a methyl donor in the brain. Choline, a precursor of acetylcholine, is synthesized from ethanolamine by methylation. It is possible to speculate that methylcobalamine increases acetylcholine synthesis by providing methyl groups in this step, thereby activating a cholinergic input to the system involved in the entrainment. This possibility, however, has not yet been examined.

It is concluded that vitamin B12 phase-advances the circadian rhythm of plasma melatonin, and increases the sensitivity of plasma melatonin to bright light. The phase-advance shift of plasma melatonin rhythm by vitamin B12 may be mediated by an increased sensitivity of the circadian clock to light.

Acknowledgments. The present study was supported in part by grants from the Hokkaido Prefectural Government and Ezai Corp.

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