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Vitamin B_{12} amplifies circadian phase shifts induced by a light pulse in rats

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Abstract. Vitamin B_{12} (VB_{12}) is a putative modulator of the human circadian clock, improving entrainability to the 24 h light-dark cycle. The present study was intended to elucidate the mechanism of VB_{12} action in an animal model. In male rats free-running under constant dim illumination, a single light pulse of 50–1000 lux for 20 min given at circadian time (CT) 20 induced a 0.28 to 1.08 h phase advance and at CT 14 induced a 0.54 to 2.10 h phase delay. A 3 h intracerebroventricular (icv) infusion of 30 nmol VB_{12} starting 2 h prior to a 20 min 200 lux light pulse significantly amplified phase shifts in comparison with saline-treated or untreated controls. The mean phase advance (1.13 h) was 1.8-fold greater than that of saline-infused controls, whereas the mean phase delay (2.28 h) was 2.9-fold greater. These values were comparable to the maximal phase shifts caused by 1000 lux light pulses in untreated rats. Since the same VB_{12} treatment alone had failed to induce a phase shift in a previous experiment, these results indicate that VB_{12} strongly enhanced light pulse-induced phase shifts and thus augmented the entrainability of the circadian clock to light.
Key words. Circadian rhythm; intracerebroventricular infusion; light pulse; phase shift; rats; vitamin B_{12}.

Vitamin B_{12} (VB_{12}), α-(5,6-dimethylbenzimidazolyl)-co-methyl-cobamide (mecobalamin), is a putative modulator of the mammalian circadian clock. Patients with circadian rhythm sleep disorders could be entrained to social 24 h cycles by chronic administration of VB_{12}[1–4]. Intracerebroventricular (icv) infusion of VB_{12} enhanced sleep and modulated the circadian rhythm of brain temperature (Tbr) in rats entrained to 24 h light-dark (LD) cycles[5–7] or free-running under constant dim illumination (dim LL)[7,8]. These findings suggest that VB_{12} may affect the clock system directly to facilitate its circadian entrainability. However, it remains still unknown how VB_{12} affects the circadian clock.

A light pulse given at an appropriate time under constant lighting conditions can shift the phase of free-running rhythms earlier or later (the phase response curve)[9]. The magnitude of phase shift may reflect the degree of the underlying entrainability of the circadian clock to light. Hence, in the present study, we investigated the effect of VB_{12} on a light pulse-induced phase shift of free-running drinking rhythms in rats kept under dim LL. Firstly, we determined illuminance dependency of light pulse-induced phase shifts for rats kept in constant darkness (DD)[10]. Then a light pulse of the appropriate illuminance was given to free-running rats in combination with VB_{12} administration. We report here that VB_{12} could actually modify the magnitude of light pulse-induced phase shifts.

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Materials and methods

Adult male rats of the Sprague-Dawley strain (310–450 g, 60–70 days old) reared under a 12:12 LD regime (L: 0800–2000 local time, 10–100 lux at the animal level) in a constant air-conditioned environment (25 °C and 60 ± 6% humidity) were used. Food and water were available *ad libitum*. Animals were anesthetized with intraperitoneal injections of pentobarbital sodium (50 mg/kg), placed on a stereotaxic apparatus, and a stainless steel cannula was implanted in the third ventricle for continuous icv infusion. The external tip of the cannula was connected to a polyethylene lead tube through an acrylic cap on the skull. Cranial support screws, the skull cap and the cannula were fixed with dental acrylic resin. One week was allowed for recovery from surgery. Then each animal was individually housed in a special cage equipped with an electro-cannular slip ring, which enabled continuous icv infusion. The animals were initially kept under the 12:12 LD cycles for about 3 days as before, then switched to dim LL (0.5–1.0 lux at the animal level). The intensity of illumination was adjusted by filtering ceiling 10 W fluorescent lamps through a dark brown acrylic plate, so that transillumination between 360 and 660 nm was attenuated to about 25% of the original intensity. The consumption of drinking water was measured by an electronic drop counter (Drinkometer LA-1), which was inserted in the aqueduct. The number of droplets was continuously counted and stored in a computer at 3 min intervals for further statistical analyses. After observing a stable free-running rhythm of drinking behavior for at

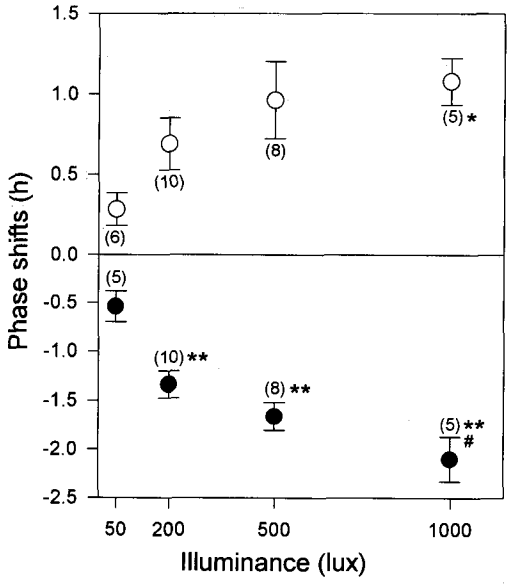

Figure 1. Mean magnitude of phase shifts ± SE of drinking rhythms caused by a 50, 200, 500, and 1000 lux light pulse in rats free-running under dim LL. Trial number is indicated in parentheses. Both phase advances caused by a light pulse given at CT 20 (open circles) and phase delays caused by a light pulse given at CT 14 (closed circles) are significantly different among the 4 illumination groups.
*$p < 0.05$, **$p < 0.01$ between phase shifts at 50 lux and #$p < 0.05$ between the shifts at 200 lux by Duncan's MRT following one way ANOVA.

least 2 weeks, a light pulse of 50, 200, 500 and 1000 lux was applied by 3 W fluorescent lamps (Hitachi L-2310) over the cage. The light pulse was applied for 20 min at CT 14 (2 circadian hours after activity onset) or at CT 20 (8 circadian hours after activity onset), because light pulses at these particular CTs are known to induce either a large phase delay or an advance[11]. During the light pulse exposure the cage was completely covered by a blackout curtain so as to avoid influencing the other animals.

After at least 2 weeks under dim LL, the animals were icv-infused with physiological saline (10 µl/h) for 3 h followed by a 3 h infusion of either VB_{12} solution (30 nmol VB_{12} dissolved in 30 µl physiological saline) or saline (30 µl), starting 2 h prior to a 200 lux light pulse exposure. This means that the VB_{12} infusion was initiated at CT 18 in the CT 20-light pulse group ($n = 6$) and at CT 12 in the CT 14-light pulse group ($n = 6$). The dosage and concentration of VB_{12} were the same as in our previous experiment[8]. In post-experimental confirmation by a chemilluminescent immunoassay[12], the whole brain concentration of total cobalamins was about 425 ng/g wet-tissue ($\cong$ 8-fold that of saline-treated control) in rats sacrificed 5 h after the VB_{12} infusion. Each animal was subjected to two light pulses at about a 20 day interval, although both light pulses were given at the same CT. In half of the members of each group, the first light pulse was combined with VB_{12} infusion and the second light pulse with saline infusion. In the other half, the sequence of infusions was reversed.

Event records for drinking behavior were double-plotted (48 h time scale with each day repeated once). The distribution of CTs of free-running drinking rhythms and the magnitude of phase shift were visually inspected by three experienced investigators who did not know the experimental schedules. CT 12 was determined by fitting a line to the onset time of drinking activity, and then free-running period (i.e., a cycle of the CTs) were inspected from the slope of the line. The time difference at CT 12 after the light pulse exposure was evaluated as the value of phase delay whereas that at the activity offset-point was determined as the value of phase advance, because manifest shifts were obtained at each point. The Duncan's new multiple range test (Duncan's MRT) following the one way analysis of variance (ANOVA) was used to compare the mean phase shift at 4 different levels of illumination and to evaluate the efficacy of VB_{12} on the phase responsiveness.

Results

Intact rats kept under dim LL showed a stable free-running rhythm of drinking behavior, whose period was 24.32 ± 0.12 h ($n = 27$, mean ± SE). The free-running rhythm was phase-shifted by a single light pulse given either at CT 14 or at CT 20. As shown in figure 1, the greater intensity of light pulse induced the greater phase shift. Significant illumination dependency for light pulse-induced phase shifts was observed both in phase delays (one way ANOVA, $F_{3,24} = 12.12$, $p < 0.01$) and in advances (one way ANOVA, $F_{3,24} = 3.23$, $p < 0.05$). For phase delays, significant differences were seen between the 50 lux-irradiated group and all the other groups (Duncan's MRT, $p < 0.01$) as well as between the 200 lux and the 1000 lux groups (Duncan's MRT, $p < 0.05$). For phase advances, a significant difference was only detected between the 50 lux and the 1000 lux groups (Duncan's MRT, $p < 0.05$). The maximal magnitude of phase delay (2.10 ± 0.23 h at 1000 lux) was significantly larger (Student's t-test, $p < 0.01$) than that of phase advance (1.10 ± 0.14 h at 1000 lux). Due to large individual variations in the magnitude of phase shifts, it was difficult to determine the critical illumination to induce a saturant response, especially for phase advances analysed by the Duncan's MRT. Thus we defined the 200 lux light pulse as a reference light pulse and used it to investigate the effects of VB_{12} in the subsequent experiment.

Significant enhancements of light pulse-induced phase shifts were observed after administration of VB_{12}. A typical example of amplification of phase shifts is shown in figure 2A for an advance and figure 2B for a delay.

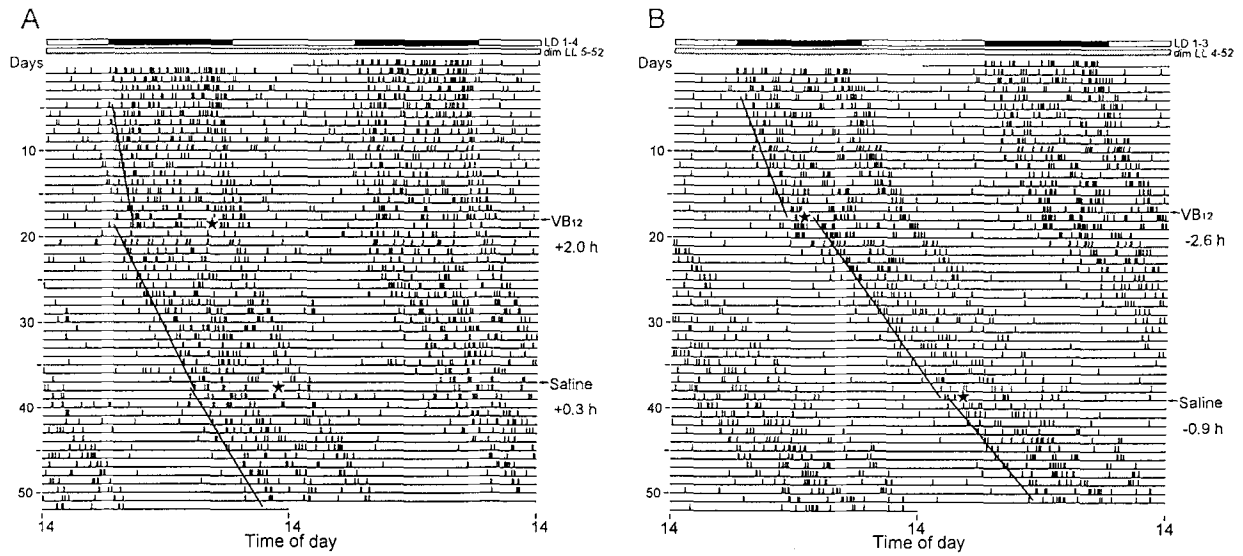

Figure 2. (*A*) An example of double-plotted actigram of drinking behavior in a rat free-running under dim LL, showing that the light pulse-induced phase advance was larger after 3 h icv infusion of VB_{12} than after that of saline alone. A 20 min light pulse of 200 lux (asterisk) was given at CT 20 in days 19 and 38 in combination with the infusion of VB_{12} and saline, respectively. (*B*) An actigram showing that a light pulse-induced phase delay was larger after a 3 h icv infusion of VB_{12} than after that of saline alone. A 20 min light pulse of 200 lux (asterisk) was given at CT 14 in days 18 and 39 in combination with the infusion of VB_{12} and saline, respectively.

The mean phase advance caused by the 200 lux light pulse at CT 20 was 1.7 and 2.9 times greater in the VB_{12}-infused group than in the untreated and saline-treated groups, respectively (fig. 3 upper, one way ANOVA, $F_{2,19} = 3.96$, $p < 0.05$, Duncan's MRT, $p < 0.05$). Similarly, the mean phase delay by the light pulse at CT 14 was also 1.7 and 1.8 times greater in the VB_{12}-treated group than in the untreated and saline-treated groups, respectively (fig. 3 lower, one way ANOVA, $F_{2,19} = 10.95$, $p < 0.01$, Duncan's MRT, $p < 0.01$). The amplified magnitudes of the phase advance (1.13 ± 0.20 h) and delay (2.28 ± 0.20 h) by VB_{12} were almost equivalent to the phase shifts by the 1000 lux light pulses in untreated rats (e.g. fig. 1). On the other hand, there were no significant differences between untreated and saline-treated groups either in advance and delay shifts (Duncan's MRT, $p > 0.05$).

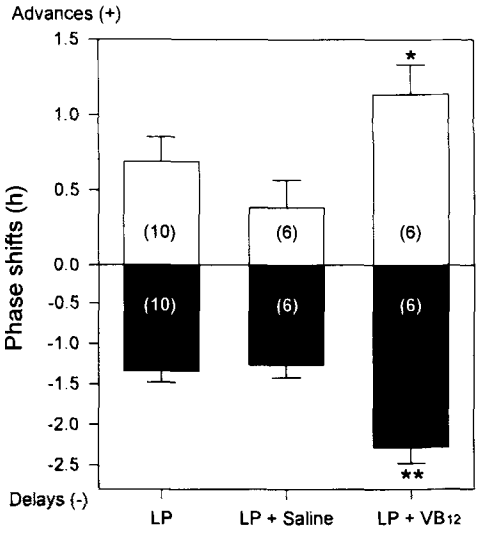

Figure 3. Comparison of mean phase shifts ± SE of drinking rhythms in rats exposed to a 200 lux light pulse in 3 groups treated with light pulse alone (LP), light pulse plus 3 h icv infusion of saline (LP + saline), and light pulse plus 3 h icv infusion of 30 nmol VB_{12} (LP + VB_{12}). The mean magnitude of phase advances caused by a light pulse at CT 20 (upper) and that of delays by a light pulse at CT 14 (lower) were both significantly larger in the VB_{12}-treated group than in the other two groups. Duncan's MRT following one way ANOVA, $^{**}p < 0.01$, $^{*}p < 0.05$.

With respect to the difference between two light pulses given at about a 20 day interval, the pooled mean magnitude of phase advances at the first trial (0.97 ± 0.29 h) was larger than that at the second one (0.55 ± 0.16 h), while that of delays at the first trial (1.60 ± 0.28 h) was smaller than that at the second one (1.95 ± 0.68 h). However, the difference between the first and the second trials was not significant either for phase advance or phase delay groups ($n = 6$ for each group, Student's t-test, $p > 0.05$).

Discussion

This study brought about two major findings. Firstly, illumination dependency of light pulse-induced phase shifts was clearly demonstrated in rats free-running under dim LL. Secondly, VB_{12} exerted an amplifying effect on the light pulse-induced phase shifts in these rats.

It was reported that the magnitude of light pulse-induced phase shifts largely depends on the illumination

of light pulses administered to rats free-running under DD[10]. However no literature has yet been available as to whether or not the same rule can apply to rats free-running under dim LL. The present study reveals for the first time that this is the case. Since light pulses of 170 lux for 5 min and 1.7–17 lux for 5 min were sufficient to induce maximal phase delays and advances under DD[10], saturant illumination estimated in this study was apparently higher than that under DD. This difference may be due to the presence or absence of background illumination. In spite of large variations in the magnitude of the phase shift, the maximal phase shifts at the respective CTs were well in accordance with those in the literature dealing with DD[10,11,13]. Since the magnitude of phase shifts may express the grade of circadian entrainability, our finding indicates that the entrainability to light pulse could depend on the applied illumination also in rats free-running under dim LL.

It is known that phasic modulation of motor activity results in a phase shift of circadian rhythms[14–16]. Recently, the vitamin B_1-related substance, sulbutiamine, was reported to amplify triazolam-induced phase shifts of motor activity[17]. In this connection we previously reported somnogenic effects of VB_{12} in LD-entrained and dim LL free-running rats[5–8]. If sleep could be regarded as equivalent to immobilization, sleep promotion caused by VB_{12} (see above) might be responsible for the phase delay. However, light pulse induced-phase advances as well as phase delays were both amplified by VB_{12} in this study. In addition, the VB_{12} infusions alone did not induce an abrupt phase shift of Tbr rhythms under dim LL[8]. Thus it seems likely that VB_{12} affected the endogenous clock system via modulation of photic inputs rather than via such an activity-related feedback function. Hence, our results suggest that VB_{12} promoted the sensitivity of the circadian clock to light, which eventually resulted in the enhancement of circadian entrainability.

Melatonin, a pineal hormone, is considered to be closely involved in photic signal transduction in the mammalian circadian system[18]. Recently it was reported that VB_{12} phase-advanced the 24 h melatonin rhythm and enhanced sensitivity to bright light in healthy humans[19]. Furthermore, it has been suggested that transmethylation by VB_{12} may involve signal transduction in the pineal via its β-adrenergic receptors[20]. Thus it is plausible that the amplification of light pulse induced-phase shifts by VB_{12} in the present study is accounted for a potentiation of photic signal transduction by the melatoninergic modulation.

In addition, more direct mechanisms may be involved in the circadian pacemaking system with respect to some effects of VB_{12}. The action of VB_{12} as a cofactor of methyltransferase is especially important, due to the need for production of methionine and adenosylmethionine, since methylation by these substances is required for the synthesis of proteins and neurotransmitters in the central nervous system[21]. Indeed, we recently found that VB_{12} modulated the concentration of amino acids (M. Ikeda, unpublished data) and spontaneous firing activity[7,22] in the hypothalamic suprachiasmatic nucleus (SCN), the circadian clock. Hence we assume that the sensitivity of circadian pacemakers like the SCN and/or conductivity of the visual input pathways like the retino-hypothalamic tract may be potentiated by VB_{12}. It is also conceivable that such a direct action of VB_{12} may play a role in modulating the circadian entrainability to photic inputs.

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