Enhanced Heat Loss and Age-Related Hypersensitivity to Diazepam

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Abstract: Whether elderly people suffer from age-related changes in pharmacokinetics and/or pharmacodynamics with administration of benzodiazepines is still a matter of controversy. We investigated the course of brain function and thermoregulation after oral administration of a standard benzodiazepine, diazepam (DZP), in 8 healthy young men (mean age, 19.8 years; range, 18 to 23 years) and 8 healthy middle-aged and older men (mean age, 60.9 years; range, 53 to 71 years). Placebo or DZP was administered in a single-blind crossover manner to the young men (placebo, 5-mg, 10-mg DZP) and to the older men (placebo, 5-mg DZP), and plasma DZP concentration, choice reaction time, proximal body temperature, and distal body temperature were monitored with high time resolution under a modified constant routine condition to exclude masking effects. Whereas there was no evidence of age-related alterations in pharmacokinetics between the 2 groups, the older subjects, in comparison to the young subjects, showed a more delayed choice reaction time in response to the same plasma DZP level, suggesting that hypersensitivity is related to increased age. DZP at 5 mg in the older subjects induced acute and transient hypothermia to the same degree as that induced by DZP at 10 mg in the young subjects. The distal-proximal body temperature gradient (difference between distal body temperature and proximal body temperature), an indicator of blood flow in distal skin regions, showed strong positive correlation with the delay in choice reaction time in both groups. These findings suggest that hypersensitivity to benzodiazepine in older persons may be due, at least in part, to age-related changes in thermoregulation, especially in the heat loss process.

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Benzodiazepine (BZP) is one of the most frequently prescribed drugs for elderly persons with anxiety or sleep disorders. Unfortunately, BZPs are often accompanied by undesirable side effects in the elderly, such as daytime sleepiness, cognitive decline, amnesia, rebound symptoms, ataxia, vertigo, and leading to falls and hip fracture.[1–5] An age-dependent increase in vulnerability of the elderly to these adverse effects has been discussed from the standpoint of pharmacokinetics (PK) and pharmacodynamics, that is, as the result of greater plasma concentrations of BZPs, increased sensitivity to BZPs, or a combination of the 2. Although numerous investigators have grappled with this issue, a definite conclusion has not been reached.[6–8]

Some studies showed age-related changes in the PK of BZPs such that elderly persons showed a higher plasma BZP concentration than that of young people.[9–13] Greenblatt et al[14] studied the PK and pharmacodynamics of orally administered triazolam in young and elderly subjects and concluded that the greater degree of sedation and impaired psychomotor performance observed in the elderly subjects was caused mainly by the reduced clearance and increased plasma concentration of triazolam rather than by increased intrinsic sensitivity to the drug. Another series of studies, however, showed that elderly persons might have greater sensitivity to BZP.[15–18] Platten et al[18] reported that midazolam induced more pronounced sedative effects in the brain, determined by choice reaction time (CRT) and visual analog scales, of elderly subjects compared with effects in young control subjects despite a lack of significant differences in PK parameters between the groups. This suggests altered pharmacodynamics, that is, increased sensitivity to BZPs with age. These conflicting study results may be due in part to differences in the drugs, to experimental protocols (including low time resolution), or to the limited number of available physiologic markers for exogenously administered BZP.

In the present study, we focused on the thermoregulatory response to the standard BZP, diazepam (DZP), as a reliable physiologic marker of sensitivity to BZP as well as a possible physiologic pathway by which brain function is impaired by DZP in the elderly. Many studies have consistently shown an intimate relation between thermoregulation and brain function, expressed in terms of alertness and psychomotor performance, not only under physiologic

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conditions[19–22] but also under various manipulations affecting sleep regulation: administration of melatonin, exposure to light, sleep deprivation, and passive body heating.[23–31] Recent studies have revealed that BZPs can also produce acute and transient changes in thermoregulation, that is, the heat loss process, in humans.[24,32–36] We aimed to investigate age-related changes in the sensitivity to DZP using thermoregulatory response as reliable physiologic marker. We performed CRT testing (responding to rare or frequent stimulation) with high time resolution and continuous monitoring of core body temperature (core BT) under a modified constant routine.[37] Under this routine, various masking effects on psychomotor performance and thermoregulation, produced by emotional stimulation, physical movement, posture, calorie intake, clothing, ambient temperature, and environmental light intensity are strictly controlled.

MATERIALS AND METHODS

Subjects

Healthy volunteers either younger than 25 years or between 50 and 75 years were each rigorously screened by 3 physicians via physical and psychologic evaluations. Volunteers were surveyed to exclude the following: irregularity in the sleep-wake pattern according to a self-registering 2-week sleep diary, history of physical disease that could affect sleep states, history of psychiatric disease identified by the Mini-International Neuropsychiatric Interview[38] or a structured diagnostic psychiatric interview for *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* disorders (including depression and anxiety disorders), use of medications (including BZP) that might modify sleep states during the previous 3 months, any airline travel during the previous 6 months that could cause jet lag, and any abnormal hematology or urinalysis findings. All older volunteers underwent Mini-Mental State Examinations (mean score, 29.3; range, 27 to 30) to rule out the presence of dementia. Finally, 8 young men (mean age, 19.75 years; range, 18 to 23 years) and 8 middle-aged and older men (mean age, 60.88 years; range, 53 to 71 years) gave written informed consent and participated in the study. All of the study subjects were paid volunteers. BMI in the young subjects was 20.8 ± 0.7 (SEM) (range, 17.6 to 23.5) and in the older subjects was 22.8 ± 1.0 (range, 17.3 to 26.8).

General Setting

All subjects abstained from tobacco, alcohol, heavy exercise, and medications for at least 7 days before the beginning of the study. During this prestudy period, all subjects were asked to maintain their daily home routines but to keep light intensity under 10 lux during the time in bed. Sleep quality and regularity were assessed by means of an actigraph (AMI Inc., Ardsley, NY) fitted to each subject's nondominant wrist; the data were analyzed for computer-calculated sleep-wake determinations by using Cole et al's algorithm.[39] For each subject, sleep onset times during the prestudy period were averaged; average onset time was defined as 0000 hours. Young subjects participated in 3 experiments (2 drugs, 1 placebo), and older subjects participated in 2 experiments (1 drug, 1 placebo). Experiments were conducted at 2-week intervals.

On the day before each experiment, the subject entered the sleep laboratory 8 hours before 0000 hours (−0800 hours). Before −0600 hours, the subject donned a cotton gown provided by the laboratory to control for the influence of clothing on thermoregulation. At −0600 hours, a 750-kcal meal and as much water as desired were given to each subject. From the time after the meal until the end of the study at 1630 hours the next day, the subject rested in the supine position on a reclining seat during the awake period. Standing and walking were prohibited, and movement of the limbs was discouraged. An adjoining room was fitted with a portable toilet, movement to the toilet was assisted, and sitting was permitted while the toilet was being used. Subjects were allowed to recline and sleep on a bed in the laboratory only from 0000 to 0800 hours. During the sleep time, each subject underwent polysomnography and was confirmed to have no sleep disorder as defined by the International Classification of Sleep Disorders.[40] Sleeping was forbidden outside the sleep period. Participants were subjected to continuous electroencephalography monitoring, and at least 2 laboratory workers were always present to observe the subjects and provide assistance. Laboratory lighting was maintained at less than 10 lux during the sleep period and at 100 lux near the subject's eyes at other times to avoid the alerting and alleviating effects of bright light on sleepiness and psychologic performance.[41] Room temperature was maintained at 23°C ± 1°C throughout the study period.

Drug Administration

On the day of the experiment, each subject ingested a 100-kcal meal and 100 mL of orange juice at 0830 hours to ensure alimentary canal absorption of DZP and to suppress the influence of calorie intake on temperature and psychomotor performance. We tested responses to placebo (lactose), 5-mg DZP, and 10-mg DZP for young subjects and to placebo and 5-mg DZP for older subjects. All substances were packed in indistinguishable gelatin capsules and were administered with 100 mL water at 1200 hours. The study was conducted in a randomized, single-blind, crossover fashion.

Evaluation of Plasma Concentrations of DZP and Desmethyldiazepam

Blood samples were used to monitor plasma DZP and its active metabolite desmethyldiazepam (dmDZP). Samples were drawn every 20 minutes from 1200 to 1500 hours and

again at 1600 hours via intravenous catheter placed in a forearm vein at 0900 hours. Blood samples were immediately centrifuged at 3000 rpm for 5 minutes, and the plasma was collected and frozen at −20°C for later assay. Plasma concentrations of DZP and dmDZP were determined by a high-performance liquid chromatography (HPLC) method previously described[42,43] but with some modifications. The apparatus used for HPLC was the EP-300 chromatography pump (EICOM, Kyoto, Japan) equipped with an EICOM NOD-10 NOx detector. The wavelength was set at 230 nm. Test samples were introduced with a 234 autoinjector (Gilson Inc., WI) at an effective volume of 75 μL. The HPLC column used was a Grand pack ODS-5 NK stationary phase (5 μm, Masis Inc., Owani, Japan). The column temperature was maintained at 25.0°C in an EICOM ATC-300 column oven. The mobile phase consisted of 0.5% KH_2PO_4 (pH 4.5)-acetonitrile (60:40, vol/vol), which was degassed in an EICOM DG-300 degasser before use. The flow rate was 1.0 mL/min. All solvents used were of HPLC grade (Wako Pure Chemical Industries, Osaka, Japan). After flunitrazepam (320 ng) in methanol (10 μL) was added to plasma samples (1 mL) as an internal standard, the plasma samples were diluted with 5 mL water, and the solution was mixed briefly. The mixture was applied to a Sep-Pak CN cartridge (Waters, Bedford, MA) that had been activated previously with 10 mL 100% acetonitrile and water. The cartridge was then washed with 10 mL water and 5 mL 20% acetonitrile in water. The desired fraction was eluted with 5 mL 70% acetonitrile in water. The eluate was evaporated in a vacuum at 40°C by a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan). The residue was dissolved in 50 μL methanol, and 100 mL mobile phase was added. The samples were injected into the HPLC apparatus. The ratios of drug to internal standard were calculated from the recorded peaks. The results obtained from spiked plasma samples containing known amounts of drug were calculated on the basis of linear regression analysis.

Evaluation of Psychomotor Performance

Psychomotor performance was monitored every 20 minutes from 1000 to 1600 hours. During the first 5 minutes of each 20-minute epoch, CRT testing was applied as described previously.[44] Two different brief tones (2000 and 1000 Hz), each with a duration of 100 milliseconds, were generated by an automatic stimulator (Nihon Kohden SS-1449, Nihon Kohden, Tokyo, Japan) and presented at 60 dB (normal hearing level). The 1000-Hz tone was presented 80% of the time, and the target 2000-Hz tone was presented 20% of the time. Order of presentation was randomized, and length of the interstimulus interval was also randomized within a range of 1.0 to 3.0 seconds (mean, 2.0 seconds). Subjects were instructed to respond only to target stimuli (2000-Hz tone) by pressing with the thumb a button attached to the palm of the right hand ('oddball' paradigm). The button-press response was converted to an electrical signal recorded on a personal computer. Older subjects had been screened at time of recruitment for their ability to accomplish CRT testing to avoid as much as possible the influence of hearing disability due to aging; they responded to 165 2000-Hz tones and 660 1000-Hz tones through 5 test trials. CRT testing at 1200 hours was performed immediately before drug administration. Thirty-three reaction time latencies from target stimulus to button press were averaged as the CRT data at each point of measure. All other CRT data were expressed relative to the data obtained at 1200 hours. For further analysis, the change in CRT (ΔCRT) induced by DZP was defined as the difference in the corresponding values between the DZP and placebo experiments at each point of measure.

Evaluation of Heat Loss After DZP Administration

Between −0800 and −0600 hours, skin temperature thermistors were attached to both wrist areas and to the instep of each foot. A rectal thermistor (polyethylene-covered thermoprobe, accuracy within 0.01°C) was inserted 10 cm into the subject's rectum. The thermistors were connected to an ambulatory temperature monitoring system (Kohden Medical Inc., Tokyo, Japan), and sampling occurred at 1-minute intervals. All temperature recordings taken on the day of the experiment were later collapsed into 20-minute bins from 1100 to 1600 hours. The data at 1200 hours were averaged for 10 minutes just before drug administration to control for acute drug effects. After that, bin data obtained from each subject were expressed relative to the data at 1200 hours. Proximal body temperature (p-BT, rectal temperature) and distal body temperature (d-BT, average skin temperature from 4 sites) were used to calculate the distal-proximal body temperature gradient (DPG, difference between d-BT and p-BT) as an indicator of blood flow in distal skin regions.[26,45] For further analysis, the changes in p-BT, d-BT, and DPG (Δp-BT, Δd-BT, and ΔDPG, respectively) induced by DZP were defined as the difference in corresponding values between the DZP and placebo experiments at each point of measure.

Data Analysis

Results are shown as mean ± SEM. $P < 0.05$ was considered statistically significant. One-way analysis of variance or two-way analysis of variance (factors: time and dose) followed by Student t test was used to examine differences in BT and CRT values obtained for placebo and for each dose of DZP. Relations between psychomotor performance and plasma DZP concentrations or the DPG were determined by Pearson correlation coefficient. Analysis of covariance was used to examine the difference in the slopes of the simple linear regression lines between the young and older subjects.

RESULTS

Plasma DZP Concentration

Disappearance of DZP was accompanied by formation of dmDZP. In both groups, mean plasma concentrations of DZP and dmDZP after administration of 5-mg DZP showed similar transitions over 4 hours. Pharmacological parameters after DZP administration are shown in Table 1. There were no significant differences between the 2 groups in any of these parameters after administration of 5-mg DZP.

Psychomotor Performance

Changes in the ΔCRT for the 5- and 10-mg DZP experiments in the young subjects and for the 5-mg DZP experiment in the older subjects are shown in Figure 1A. Average absolute CRT values before drug administration in the placebo, 5-mg, and 10-mg DZP experiments in the young subjects were 294.8 ± 16 milliseconds, 300.2 ± 18 milliseconds, and 314.5 ± 15 milliseconds, respectively. Average absolute CRT values before drug administration in the placebo and 5-mg DZP experiments in the older subjects were 398.3 ± 24 milliseconds and 385.5 ± 17 milliseconds, respectively. There were no significant differences in baseline CRT values between each experiment in either the young or older subjects. DZP, 10 mg, in the young subjects ($F = 3.162$, $df = 15$, $P < 0.001$) and 5-mg DZP in the older subjects ($F = 2.157$, $df = 15$, $P < 0.02$) induced a significant increase in the ΔCRT, whereas 5-mg DZP in the young subjects did not induce a significant change in the ΔCRT. There was no significant difference in the ΔCRT between the 10-mg DZP experiment in the young subjects and the 5-mg DZP experiment in the older subjects. Area under the ΔCRT curve was significantly greater with 10-mg DZP than with 5-mg DZP in the young subjects ($P < 0.02$) but did not differ significantly from area under the ΔCRT curve with 5-mg DZP in the older subjects (Fig. 1B).

TABLE 1. Pharmacological Parameters After DZP Administration

	Young Subjects		Elderly Subjects
Parameter	**10 mg (n = 8)**	**5 mg (n = 8)**	**5 mg (n = 8)**
C_{max} of DZP (ng/mL)	484.7 ± 31.1	234.6 ± 23.1	219.7 ± 31.6
T_{max} of DZP (minutes)	45.0 ± 9.1	50.0 ± 7.6	55.0 ± 13.5
4hr-AUC of DZP (ng/mL-hours)	2492.2 ± 134.3	1136.1 ± 63.7	1051.2 ± 96.8
4hr-AUC of dmDZP (ng/mL-hours)	36.4 ± 11.3	35.3 ± 7.5	82.4 ± 13.9

Abbreviations: C_{max}, peak plasma concentrations; T_{max}, time to C_{max}; 4hr-AUC, area under the curve for 4 hours after administration.

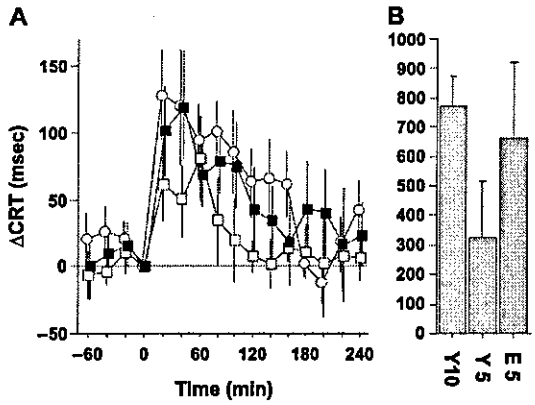

FIGURE 1. Changes in ΔCRT. A, Horizontal bars indicate time of DZP administration relative to 1200 hours. Mean ± SEM values are shown. ΔCRT is defined as difference in corresponding values between placebo and DZP experiments at each point of measure and is expressed relative to value at 1200 hours. White circles and white squares represent data for 10-mg and 5-mg DZP experiments in young subjects; black squares represent data for 5-mg DZP experiment in older subjects. B, Area under curve values for ΔCRT 20 to 240 minutes after DZP administration in 5-mg (Y5) and 10-mg (Y10) DZP experiments in young subjects and 5-mg DZP experiment in older subjects (E5).

Relation Between Plasma DZP Concentration and Psychomotor Performance

There was a strong positive correlation between mean plasma DZP concentration and mean ΔCRT at each point of measure in both the young subjects ($r = 0.876$, $P < 0.0001$) and the older subjects ($r = 0.878$, $P < 0.001$) (Fig. 2). The slopes of the regression lines differed significantly between the 2 groups ($F = 61.4$, $P < 0.001$).

Proximal Body Temperature

Mean changes in the p-BT for placebo, 5-mg, and 10-mg DZP experiments in both groups are shown in Figure 3A and B. The young subjects showed significant decrease in p-BT in the 10-mg DZP experiment compared to that in the placebo experiment ($F = 4.14$, $df = 15$, $P < 0.0001$), whereas no statistically significant change in p-BT was observed in the 5-mg DZP experiment. The older subjects showed a significant decrease in p-BT in the 5-mg DZP experiment compared to that in the placebo experiment ($F = 2.31$, $df = 15$, $P < 0.005$). Mean changes in the Δp-BT for the 5- and 10-mg DZP experiments in both groups are shown in Figure 4A. The maximum p-BT suppression (0.17°C ± 0.04°C) induced by 10-mg DZP in the young subjects occurred 60 minutes after administration followed by gradual recovery to the placebo level, whereas a 2-phase thermoregulatory effect, that is, short-term hypothermia followed by a hyperthermic phase, was observed in the 5-mg DZP experiment in this group.

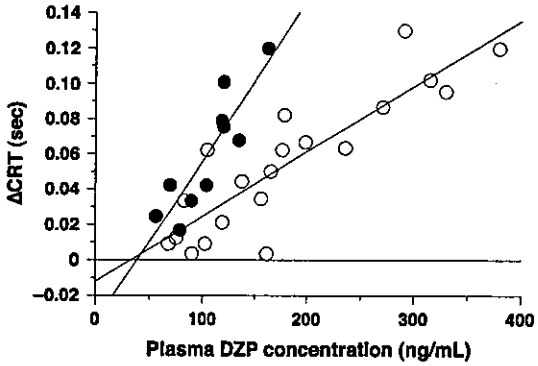

FIGURE 2. Relation between ΔCRT and plasma DZP concentrations at each point of measure in young (white circles) and older (black circles) subjects. Pearson correlation coefficient was determined for mean plasma DZP concentrations (*x*-axis) or ΔCRT (*y*-axis) at corresponding points of measure. Data in 5- and 10-mg experiments for young subjects were combined.

The maximum p-BT suppression (0.14°C ± 0.04°C) induced by 5-mg DZP in the older subjects occurred 100 minutes after administration and continued somewhat longer than suppression for the 10-mg DZP experiment in the young subjects.

Distal Body Temperature

Mean changes in the d-BT for the placebo, 5-mg, and 10-mg DZP experiments in both groups are shown in Figure 3C and D. The young subjects showed a significant increase in d-BT in the 10-mg DZP experiment compared to that in the placebo experiment ($F = 1.911$, $df = 15$, $P < 0.03$) such that the maximum d-BT elevation (1.07°C ± 0.29°C) occurred 40 minutes after administration. This elevation was followed by gradual recovery to the placebo level by 120 to 180 minutes after administration. No statistically significant change in d-BT was observed in the 5-mg DZP experiment in the young subjects. The older subjects showed no statistically significant change in d-BT in the 5-mg DZP experiment compared to that in the placebo experiment.

Distal-Proximal Body Temperature Gradient

Mean changes in the ΔDPG for the 5- and 10-mg DZP experiments in young subjects and the 5-mg DZP experiment in older subjects are shown in Figure 4B. DZP, 10 mg, for the young subjects induced a significant increase in the ΔDPG ($F = 2.677$, $df = 15$, $P = 0.0018$). DZP, 5 mg, induced a transient but not statistically significant increase in the ΔDPG in both groups.

Relation Between DPG and Psychomotor Performance

Significant positive correlation was observed between mean ΔDPG and mean ΔCRT at each point of measure in both

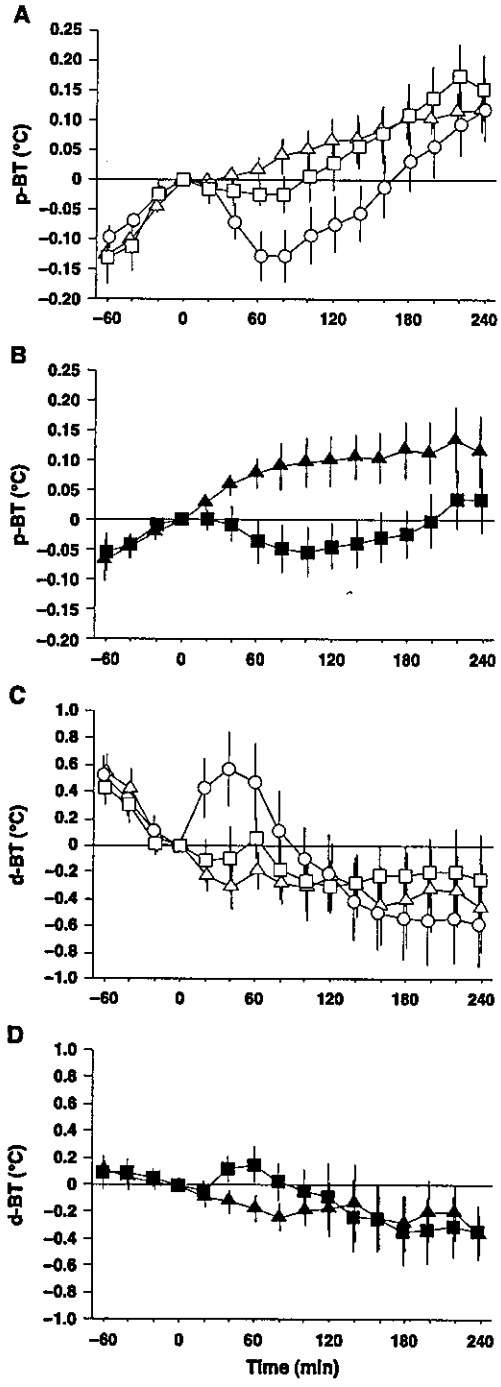

FIGURE 3. Changes in p-BT in young subjects (A) and older subjects (B) and changes in d-BT in young subjects (C) and older subjects (D) after administration of placebo (triangles), 5-mg (squares), and 10-mg (circles) DZP, respectively. Horizontal bars indicate time of drug administration relative to 1200 hours. Mean ± SEM values are shown. BT values are shown as data relative to value at 1200 hours.

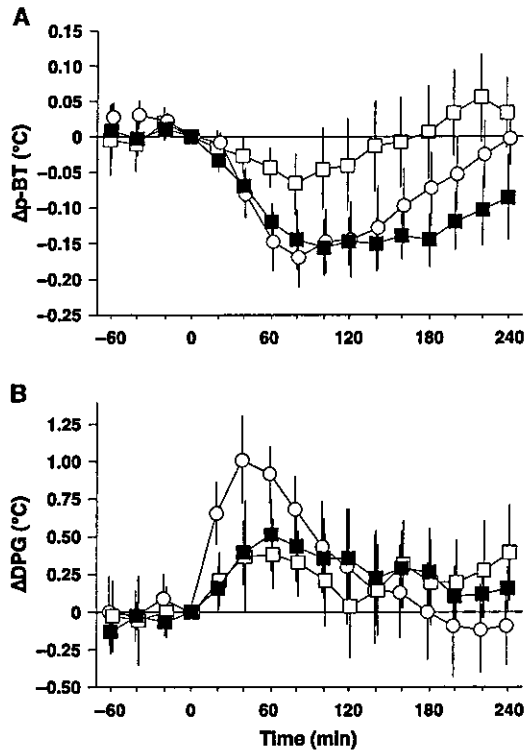

FIGURE 4. Changes in Δp-BT (A) and ΔDPG (B) in young and older subjects after DZP administration. White circles and white squares represent data for 10- and 5-mg DZP experiments in young subjects; black squares represent data for 5-mg DZP experiment in older subjects. Horizontal bars indicate time of drug administration relative to 1200 hours. Mean ± SEM values are shown. Δp-BT and ΔDPG were defined as difference in corresponding values between placebo and DZP experiments at each point of measure. Δp-BT and ΔDPG values are shown as data relative to value at 1200 hours.

the young ($r = 0.799$, $P < 0.0001$, Fig. 5A) and the older subjects ($r = 0.692$, $P < 0.01$, Fig. 5B).

DISCUSSION

In this study, we aimed to clarify whether older persons possess hypersensitivity to BZPs. We used frequently recorded CRT and body temperature data as physiologic markers for psychomotor performance and thermoregulation under strict control of potential masking effects. Under the conditions of the present study, 4-hour plasma DZP concentration profiles were very similar between young and older subjects. Neither group showed significant differences in C_{max}, T_{max}, or area under curves for DZP and dmDZP after oral administration of DZP at 5 mg, suggesting that older subjects in the present study experienced no significant age-related changes in PK responses, at least after a single administration of the drug. Nevertheless, older subjects, in comparison to young subjects, showed significantly greater impairment of psychomotor performance after DZP administration. DZP at 5 mg in older subjects induced a significantly greater prolongation of CRT compared to that in the young subjects, resulting in decline in psychomotor performance similar to that induced by 10-mg DZP in the young subjects. Furthermore, analysis of covariance between plasma DZP concentration and increase in CRT (Fig. 2) revealed that the same degree of psychomotor performance impairment was induced by lower plasma DZP concentration in older subjects than in young subjects, at least within clinical dosage. Contrary to the present study, some previous studies have shown an age-related reduction in DZP clearance after single intravenous administration.[11,13] The lack of change in PK responses in our older subjects seemed to be caused partly by the smaller study group, shorter observation period (4 hours after administration), and lower mean age (60.9 years, with some subjects in middle age) compared to that of previous studies. Despite these limitations, our findings indicate increased sensitivity to DZP in older subjects and that the decline in psychomotor performance induced by DZP was due mainly to hypersensitivity to DZP.

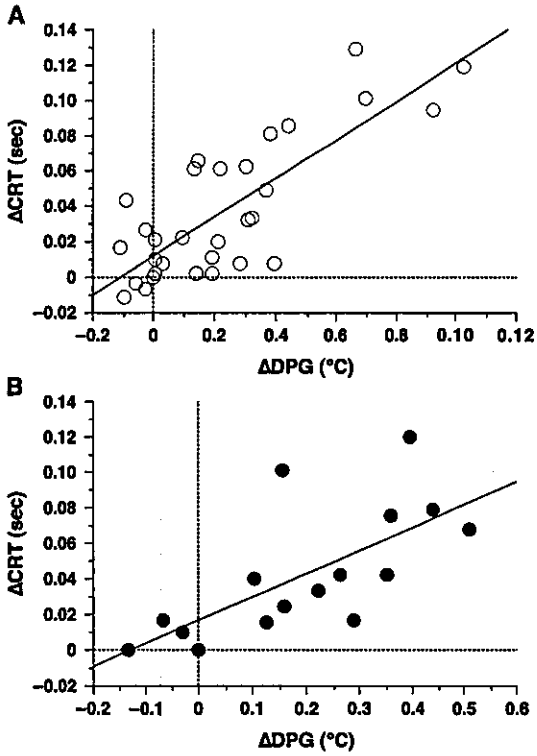

FIGURE 5. Relation between ΔDPG and ΔCRT in young (A) and older (B) subjects. Pearson correlation coefficient was determined for mean ΔDPG (x-axis) or ΔCRT (y-axis) at corresponding points of measure. Data in 5- and 10-mg experiments for young subjects were combined.

We showed that heat loss might be a reliable physiologic marker of increased age-related sensitivity to BZPs and that it may to some extent explain the mechanism underlying increased impairment of psychomotor performance induced by DZP in elderly persons. Growing evidence suggests that thermoregulation, especially the process of heat loss, is a key generator of sedative effects in the brain, such as those producing sleepiness and impaired psychomotor performance, in humans. Recent studies have shown that the sleep-producing[21,24–26] and psychomotor performance-suppressing[31,36] mechanism in humans is preceded by increased heat loss. Various manipulations accelerating sleepiness or impaired psychomotor performance act, at least in part, by enhancing the heat loss mechanism.[23,24,26,29,31,36] Our study confirmed that a standard hypnotic drug, DZP, also induces an acute and transient decrease in p-BT (rectal temperature), as was previously reported in humans[24,32–35] and animals[46,47] for some other kinds of BZPs. The decrease in p-BT after administration of DZP at 10 mg was preceded by a prominent increase in distal skin temperature in the young subjects. Why the heat loss induced by 5-mg DZP in the young subjects did not last long and did not reach statistical significance remains unclear. As was discussed elsewhere,[36] it is possible that there is a threshold for the plasma concentration of DZP to cause an obvious heat loss reaction in humans. Additionally, some of the young subjects participated in the 5-mg DZP experiment only 2 weeks after participating in the 10-mg DZP experiment, and these subjects might therefore have developed a tolerance for DZP. By contrast, the older subjects exhibited significant decrease in p-BT, even with 5-mg DZP. This decrease was equal to that induced by 10-mg DZP, but not equal to that induced by 5-mg DZP in the young subjects; this supports the assumption that older subjects possessed thermoregulatory hypersensitivity to BZPs. Maximum suppression of mean p-BT (maximum difference between BT with placebo and BT with DZP) was 0.17°C ± 0.04°C for young subjects with 10-mg DZP and 0.14°C ± 0.04°C for older subjects with 5-mg DZP. Heat loss to this extent could significantly affect brain function because rapid decline in p-BT rather than the absolute value of the change in p-BT appears to be the critical factor in generating physiologic sleepiness in humans.[21] Our young and older subjects showed prominent daily p-BT rhythms with amplitudes of 0.77°C and 0.61°C in the placebo experiment (not shown in RESULTS), in which physiologic heat loss from peak (late afternoon) to nadir (early morning) took 12 hours 15 minutes and 11 hours 30 minutes, respectively. By contrast, the magnitudes of heat loss reactions with DZP, which were equivalent to approximately 22% of the amplitudes of the daily p-BT rhythms, occurred acutely within 60 to 100 minutes after DZP administration. It is interesting that our older subjects showed a lower magnitude of DPG after administration of 5-mg DZP despite their marked drop in p-BT. We assume that this was due to their diminished ability to produce heat. P-BT is determined by the balance between heat production and heat loss. Humans produce heat steadily by means of basal metabolism. When exposed to cold stress or heat loss stimulation, compensatory heat production is achieved mainly through shivering thermogenesis, in which the activity of skeletal muscles leads to involuntary contractions. Aging is associated with a decrease in the amount of skeletal muscle, diminishing both the capacity for shivering thermogenesis and basal metabolism.

The present study revealed that the heat loss induced by DZP might be intimately related to delayed CRT in humans. We found a strong positive relation between the magnitude of DPG, difference between distal and proximal BT, and impaired psychomotor performance induced by DZP in both young and older subjects. DPG has been shown to accurately reflect blood flow in distal skin regions regulated by anastomosis arteriovenosa.[45] This means that the greater the distal vasodilation after DZP administration, the greater the impairment of psychomotor performance. Krauchi et al[26] applied DPG as an indicator of distal heat loss and showed that it successfully predicts the reduction in sleep onset latency induced by various sleep-promoting modifications. Our findings show that the DPG induced by DZP might also be a potent physiologic marker to predict sedative effects on human brain function. We did not obtain enough data to determine to what extent the enhanced heat loss reaction observed in the older subjects contributed to their DZP-induced impaired psychomotor performance. We realize that our findings do not necessarily indicate a causative link between the heat loss process and impaired psychomotor performance. It is likely that at least some of the sedating effects of BZPs are due to their effect on several central activating systems that are likely not linked directly to thermoregulation. Additionally, whether the heat loss process could contribute to impairment of other cognitive functions including memory remains unresolved. To obtain more convincing evidence of an etiologic link, future study should focus on whether prevention of heat loss induced by BZPs results in attenuation of the sedative effect determined by various cognitive markers. Nevertheless, the present study provides a useful physiologic marker for predicting the magnitude of suppression of psychomotor performance with BZP in humans and important insight into the physiologic pathway of sedative/hypnotic effects induced by BZPs and the mechanisms underlying age-related hypersensitivity to BZPs.

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Larger Phase Angle Between Sleep Propensity and Melatonin Rhythms in Sighted Humans with Non-24-Hour Sleep-Wake Syndrome

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Study objectives: This study was aimed to clarify phase angle between sleep propensity and the circadian pacemaker in patients with non-24-hour sleep-wake syndrome (Non-24).
Design and Setting: A case-control study was undertaken.
Participants: Sighted patient with Non-24 (4 males and 1 female, aged 16 to 39 y), and sex- and age-matched healthy controls (12 males and 3 females, aged 19 to 35 y) participated the study.
Measurement and Intervention: Following an actigraphic assessment of the sleep–wake cycle in their homes, the participants entered an ultra-short sleep-wake schedule together with simultaneous measurement of dim light melatonin rhythm after 24-hour sleep deprivation.
Results: The period of the sleep-wake cycle observed at home was longer in the Non-24 patients (25.12 hours) than in the controls (24.02 hours, $p<0.0001$). The interval from sleep propensity (SP) onset to the melatonin midpoint (MLmid) was significantly shorter in the Non-24 patients than in the controls. The interval from the MLmid to the SP offset was significantly longer in the Non-24 patients than in the controls.
Conclusions: It was postulated that Non-24 sufferers' delayed SP onset relative to the circadian pacemaker may accelerate the light-induced phase-delay, leading to sleep-wake cycle that is longer than 24 hours.
Key words: Non-24-hour sleep-wake syndrome; circadian rhythm, sleep; melatonin; sleep propensity; light

INTRODUCTION

SYNCHRONIZATION OF THE CIRCADIAN SYSTEM IN ANIMALS AND HUMANS IS MOST STRONGLY INFLUENCED BY THE LIGHT-DARK CYCLE.[1] Individuals living in isolation without a normal 24-h light-dark cycle have a sleep-wake cycle longer than 24 hours.[2-5] This cycle results in progressively later bedtimes and wake times. Non-24-hour sleep-wake syndrome (Non-24) is a rare condition which causes a chronic steady pattern of one- to two-hour delays in sleep onset and wake times in an individual living in normal environmental conditions;[6] the period of the sufferer's sleep-wake cycle is longer than 24 hours. *The International Classification of Sleep Disorders* (ICSD)[6] provides following criteria for diagnosing Non-24 in clinical setting; 1) difficulty initiating sleep or difficulty in awakening, 2) progressive delay of sleep phase with inability to maintain entrainment to 24 hour-day, and 3) presence of the sleep pattern for at least six weeks.

Based on the characteristics of previously documented cases, blindness is a strong predisposing factor for development of the disorder.[7-10] In blind patients with Non-24, light stimulus is unlikely to be conveyed from the retina to the circadian pacemaker, so their biological clock may not be regulated by normal environmental light-dark cycles. Subsequent reports have described Non-24 in sighted subjects living in normal environments.[11-19] In some of these reports, other pathological factors may have had an influence. For example, a patient's extreme social withdrawal due to psychopathology[19] or deviated personality[11] may result in less exposure to the regulatory influences of the normal light-dark cycle, and, therefore, a Non-24-hour sleep-wake cycle.

In animal studies, mutations with long tau or short tau (endogenous period length) have been shown to be responsible for alteration of the phase angle between the rest-activity cycle and the 24-hour environmental light-dark cycle.[20-22] However, in human Non-24 there is no evidence for such a tau abnormality mutation nor have there been any observations of sleep-wake problems in the early developmental stages that would be an expected consequence of this mutation.[6] In other single case reports, the investigators hypothesized that a blunted response to light stimulus or a limited phase advance capacity may have etiological significance for the prolonged sleep-wake period in sighted Non-24 patients.[19,23] These hypotheses have not been tested. There have been suggestions that changes in the phase relationship between sleep timing and the circadian pacemaker could be involved in Non-24.[11,15,17,18] However, the etiological significance of such abnormal phase relationships has not been elucidated.

These studies indicate that there may be systematic changes of period in the sleep-wake cycle of sighted patients with Non-24.[11,14,15,17,18] However, there have been no explanations about

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Table 1—Clinical features of the patients

Subject	Sex	Age at study	Age at onset	Sleep-wake patterns or habits prior to the onset of Non-24
1	F	39	30	Irregular sleep-wake habit following job loss
2	M	20	16	Strong evening-type lifestyle preference prior to the onset of Non-24
3	M	16	14	Strong evening-type lifestyle preference prior to the onset of Non-24
4	M	28	26	Engaged in shiftwork for 2 years prior to the onset of Non-24
5	M	30	22	Irregular sleep-wake habit following job loss

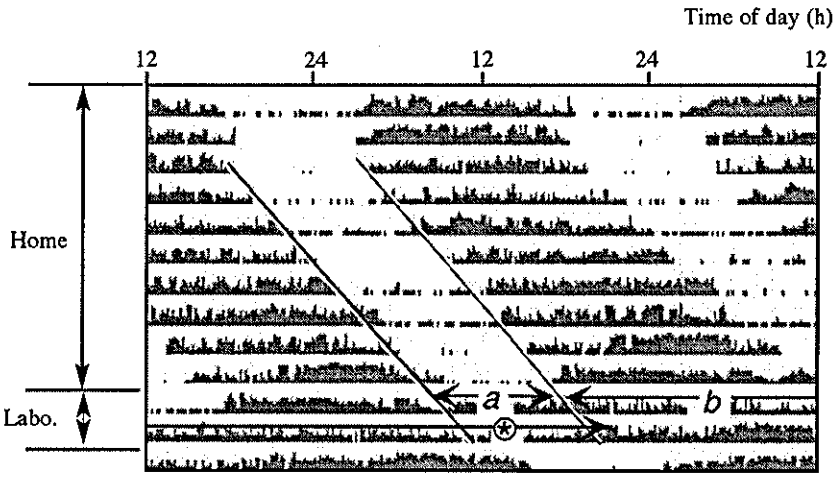

Figure 1—Wrist activity measurements (Actigraph, AMI, New York, USA) throughout the study obtained from a representative Non-24 subject (subject 5, 30-year-old male). Sleep onset and offset times were determined by using actigrams and automatically generated data (Action3 software, AMI, New York, USA), together with detailed sleep logs. To determine changes in sleep timing, regression lines were fitted through the sleep onset and offset times obtained during 8–10 consecutive days prior to the laboratory investigation. Linear regression functions in this subject were computed with respect to sleep onset (Y = 20.99 - 1.73 * X; R=0.99, 95%CI (1.54-1.92)), and sleep offset (Y = 4.94 - 1.82 * X; R=0.98, 95%CI (1.53-2.11)). The encircled asterisk represents MLmid. a: Sleep deprivation. b: Ultra-short sleep-wake schedule.

why sighted and otherwise healthy individuals, who previously had been entrained to a 24-hour day, started to experience prolongation of the sleep-wake cycle and lost the ability to reset it. Here we report a study on sighted Non-24, in which the melatonin rhythm and diurnal sleep propensity fluctuation were investigated using an ultra-short sleep-wake schedule together with simultaneous measurement of dim light melatonin rhythm and compared with those in healthy control subjects. To testify phase angle abnormality in Non-24 was of our particular interest.

METHODS

Subjects

We studied five patients with Non-24 and 15 age- and sex-matched healthy controls. The patient group consisted of four males and one female, aged 16 to 39 years, in whom Non-24 was diagnosed according to ICSD criteria.[6] Ophthalmologic specialists found no ophthalmologic abnormalities, except for myopia. The study was conducted before the patients had undergone any therapeutic interventions. No patients had been given any medication, drank alcohol before bedtime habitually, or abused alcohol or other psychotropic agents. We conducted a semi-structured psychiatric interview[24] and found no Axis I or II disorders of DSM-IV.[25] Interviews and examinations by general physicians of our hospital, together with blood counts, urine examinations, serum biochemistry, electrocardiography and routine electroencephalography, confirmed that no patient had a diagnosed medical disorder, or a history of developmental problems or severe physical disorders. The patients had experienced a persistent longer-than-24-hour sleep-wake cycle for 2-9 years. Prior to onset of Non-24 no subjects had any history of any sleep disorders and all had been entrained normally to a 24-hour day. Symptoms started when the subjects changed their sleep habits due to night-shift work, job loss, or an evening-type lifestyle preference (Table 1). None of the patient's repeated attempts to advance or correct their sleep timing by retiring and arising at conventional social times had been successful.

The controls were healthy, paid volunteers (12 males and 3 females, aged 19 to 35 years) without any known sleep, physical or psychiatric disorders, or any histories of using psychoactive drugs. They had regular sleep-wake habits, and no marked weekday-weekend differences in sleep length or remarkable irregularity due to work schedules. None of the controls had difficulty in waking up at the desired time. The basal body temperature of the female participants was used to determine the phases of their

menstrual cycles and we carried out the study during the follicular phase of their cycle. The study protocol was approved by the Intramural Research Board of National Center of Neurology and Psychiatry, and each subject gave his or her informed consent after the procedures and the possible risks of the experiment had been explained in detail.

Study Design

Each participant was asked to keep a detailed sleep log for more than four weeks prior to the laboratory investigation and to wear a wrist activity-monitoring device (Actigraph, AMI, New York, USA) for the last 14 days (Figure 1). During this period, we found that delayed phase jumps in sleep onset (larger-than-four-hour delay per day[17]) occurred in two patients. The procedure was repeated in those patients. In all the Non-24 patients we obtained records of regular sleep-wake cycles[17] that were longer than 24 hours for at least eight consecutive days prior to the laboratory investigation. In the controls, we confirmed that no marked irregular day-to-day variations in their bed and wake times occurred and that their sleep schedules were not remarkably constrained by their work schedules.

Sleep onset and offset times, as well as sleep length, were determined by two raters who were unaware of each subject's status; actigrams and automatically generated data (Action3 software, AMI, New York, USA) in five-minute bins and sleep logs were inspected. Regression lines were fitted through the sleep onset times obtained during the 8–10 days prior to the laboratory investigation (Fig. 1).[26] The estimated sleep onset time on the day of laboratory investigation was obtained by adding the slope of the regression line of sleep onset times to that on the prior day. The periods of the sleep-wake cycles were computed by adding the slope of the regression line of SW period onset times to 24 hours. A rhythm was considered to be entrained to a 24-hour day when the 95% confidence intervals of the SW period crossed 24 hours.[26] We confirmed that both the sleep onset and offset times of the control subjects were entrained to a 24-hour day[26] and that those of the Non-24 patients were not entrained to a 24-hour day (Table 2).

In the Non-24 patients, the laboratory investigation was scheduled when the patient's sleep phase was not completely out of phase, in order to avoid irregular changes of phase angle between sleep timing and the circadian pacemaker due to delayed phase jumps.[17,27] In all the subjects, the means of sleep length obtained during the 8–10 days prior to the laboratory investigation was computed for statistical analyses (HSlength).

On the day before the investigation, the subjects were instructed to rise at their normal time, to refrain from napping and to come to the laboratory after sunset. These instructions were verified with actigraph data. The subjects sat on a semi-upright sofa except for brief periods (less than five minutes) to go to the bathroom, and were enforced to stay awake under dim light conditions (<10 lux) until the estimated sleep onset time on the day of the laboratory investigation. There were technicians with the subjects through the entire time to maintain wakefulness. The length of sleep deprivation ranged from 23.9 to 24.1 hours in the controls, and from 24.8 to 26.5 hours in Non-24 patients. During the last hour of sleep deprivation, patients were attached to electrodes for standard polysomnography. Following the sleep deprivation, subjects entered nap trials with an ultra-short sleep-wake cycle originally developed by Lavie and Scherson[28] and later modified by Lack and Lushington[29] This cycle consisted of 10 minutes of standard sleep polygraphic recording on a bed in a dark room (<1 lux) and 20 minutes of enforced wakefulness sitting on a semi-upright chair under dim light (<10 lux). The ultra-short sleep-wake cycle was repeated 52–60 times for 26–30 hours. Polysomnograms obtained during the nap trials were scored in epochs of 30 seconds according to standard criteria.[30] Sleep propensity for each cycle was defined as the summed duration in minutes of the sleep stages 2, 3, 4, and REM. The sleep propensity values were smoothed with a three-point moving average (given nap trial and the next two). Timing of the circadian rise and fall of sleep propensity were determined as upward and downward crossings of the smoothed sleep propensity curve and five-minute sleep propensity onset (SPon) and offset (SPoff).[31] The interval between SPon and SPoff was defined as the duration of high sleep propensity (SPduration). Throughout the laboratory investigations, room temperature was controlled at 24°C, and subjects took a light snack (150 kcal) and water (200 mL) every two hours.

In the 10–20 routine, blood samples were taken every hour from the cubital vein through indwelling catheters with a heparin lock. Serum melatonin was measured using a radioimmunoassay kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland). The sensitivity, inter-assay and intra-assay coefficients of variation were 2.7pg/ml, 4.3% and 7.5%, respectively. The melatonin onset and offset times were defined as the time at which the raw data crossed the mid-range of peak concentration. The duration of melatonin secretion (MLduration) was defined as the period between melatonin onset and offset. The melatonin midpoint time (MLmid) was defined as a midpoint time of melatonin onset and offset. To investigate the timing of sleep propensity bout relative to melatonin rhythm, we subtracted the MLmid from SPon and SPoff. To determine circadian fluctuations in sleep propensity, sleep propensity data were average-accumulated, time-locked to the MLmid and further averaged for the hour.

The two groups were compared using a two-tailed t-test. A repeated ANOVA with a correction of Greenhouse-Geisser epsilon was used to assess time and group differences in sleep propensity and melatonin rhythms. When a significant interaction was obtained, the data were submitted to the Bonferroni/Dunn post-hoc test. All statistical analyses were performed using Systat 5.2 for Macintosh (Systat Inc., Evanston, IL).

RESULTS

The results are summarized in Table 2. The period of the sleep-wake cycle observed at home was longer in the Non-24 patients (25.12±0.18 hours, mean±SE) than in the controls (24.02±0.02 hours, $p<0.0001$). The HSlength during normal routines prior to the laboratory investigations was significantly longer in the patients (9.58±0.60 hours) than in the controls (7.33±0.31 hours, $p=0.002$). There was no significant difference in MLduration nor SPduration under experimental conditions between patients and controls (Table 2).

A repeated ANOVA for the melatonin data revealed that there was no difference between the two groups in the shape of the melatonin rhythm (Figure 2). The effect of time course was significant ($df=23$, $e=0.24$, $p<0.0001$), but the effects of the groups ($df=1$, $p=0.18$), and the interactions ($df=23$, $e=0.24$, $p=0.71$) were

Table 2—Sleep, sleep propensity, melatonin and phase-angle measures in patients and controls

					Intervals	
Non-24	SWperiod [95%CI]	HSlength	SPduration	MLduration	SPon-MLmid	MLmid-SPoff
1	25.09 [24.91-25.27]	9.39	8.56	8.25	3.52	5.03
2	25.23 [25.07-25.39]	9.27	6.38	8.12	1.00	5.04
3	24.63 [24.51-24.75]	8.92	7.45	8.80	2.44	4.98
4	24.91 [24.75-25.07]	8.41	10.11	7.96	4.01	6.32
5	25.73 [25.54-25.92]	11.89	6.99	7.88	3.53	3.64
Mean	25.12	9.58	7.90	8.20	2.90	5.00
SE	0.18	0.60	0.66	0.16	0.54	0.42
Controls (n=20)						
Mean	24.02	7.33	8.37	8.01	4.77	3.61
SE	0.02	0.31	0.21	0.23	0.30	0.28
p	<.0001	.002	.35	.64	.007	.02

SWperiod = period of sleep-wake cycle (h) ; HSlength = habitual sleep length (h); SPduration = duration of high sleep propensity (h); MLduration = duration of melatonin secretion (h); SPon-MLmid = interval between sleep propensity onset and melatonin midpoint (h); MLmid-SPon = interval between melatonin midpoint and sleep propensity offset (h)

not significant. A repeated ANOVA for sleep propensity data revealed a significant main effect of the groups (Non-24, 3.59±0.30 vs. controls, 4.67±0.13 hours) (df=1, p=0.04), a significant effect of time course of the nap trials (df=23, e=0.25, p<0.0001) and interaction (df=23, e=0.25, p=0.001) (Figure 2). A post-hoc test revealed that the hourly sleep propensity values in the patients were significantly smaller during the period before the patients' MLmid in comparison with those in controls (Figure 2).

The interval from SPon to the MLmid was 1.87 hours shorter in the Non-24 patients than in the controls (p=0.007). The interval from the MLmid to the SPoff was 1.39 hours longer in the Non-24 patients than in the controls (p=0.03) (Table 2) (Figure 2).

DISCUSSION

In the present study, the interval from sleep propensity onset to melatonin midpoint was shortened and that from melatonin midpoint to sleep propensity offset was prolonged in the Non-24 patients compared as that in the controls. Light-induced phase-delay and phase-advance of the circadian pacemaker in normal humans occurs over several hours in the evening (phase delay portion of phase response curve (PRC)) and in the early morning (phase advance portion of PRC).[4-5,32] It has been postulated that the delayed sleep onset and offset times relative to the circadian pacemaker observed in Non-24 sufferers differentially gated the light-induced phase-advance or phase-delay.[15,17,18] The later bedtime relative to the circadian pacemaker may increase the time during which the delay portion of PRC is exposed to light, whereas the later wake time relative to the circadian pacemaker prevented the advance portion of PRC from being properly exposed to morning light. However, the bedtime and wake time may be confounded by psychological status and/or social constraints, which had weakened the hypothesis. We minimized these confounding factors by using the ultra-short sleep-wake schedule, and found that the sleep propensity rhythm of Non-24 sufferers was more delayed relative to the circadian pacemaker in comparison with that of controls. These provided the first evidence of fundamental biological differences between sighted persons with Non-24 and healthy controls.

Recently, the authors, using ultra-short sleep-wake regimen identical to the present study, have revealed that sleep propensity onset was not delayed, but that the sleep propensity offset was delayed relative to the melatonin rhythm in DSPS patients compared as controls.[31] This confirmed DSPS patients' similar phase angle features observed in less controlled clinical settings.[18,24,33] These previous findings on DSPS sufferers made clear contrast to the present results with respect to circadian timings of sleep and sleep propensity onsets, but were comparable to the present results in respect with those of the offsets. These may provide an explanation for differences in clinical manifestations of these two phase-delay syndromes; DSPS sufferers can entrain to a 24-hour day, but Non-24 sufferers cannot. In addition, Non-24 patients slept less during their circadian day than controls, but had an adequate ability for sleep during the circadian night determined by melatonin secretion. This finding, together with our previous report on DSPS,[31] suggests that patients suffering from these sleep phase delay syndromes lack flexibility to sleep during their circadian day even after sleep deprivation.

The present laboratory findings on phase relation between sleep propensity rhythm and the circadian pacemaker may explain clinical observations reported previously. Oren et al.[34] have reported that Non-24 was provoked after chronotherapy, suggesting that an enforced sleep phase delay, irrespective of the circadian pacemaker, may trigger a prolongation of the sleep-wake cycle. A similar prolongation of the period of the sleep-wake cycle was reported in animals as a physiologic after effect following forced lengthening of the activity period.[35] Recent studies demonstrated that normal artificial illumination as much as,[36] and even less than[37] 200 lux can phase-shift a human circadian rhythm. In our study, artificial illumination during the nighttime might have caused a similar delayed phase response, leading to a sleep-wake cycle that was longer than 24 hours. In sighted Non-24 patients living in a normal day-night cycle, the sleep-wake cycle longer than 24 hours may be a consequence of delayed phase response triggered by artificial and/or natural illu-

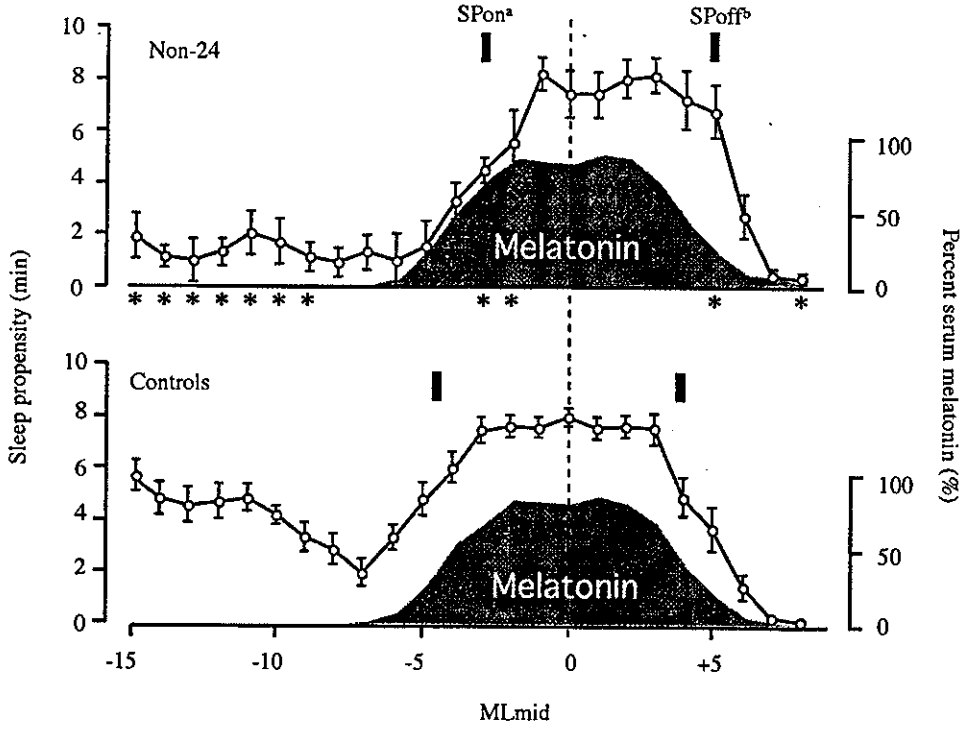

Figure 2—Sleep propensity values, percentage serum melatonin values (using each subject's maximum value as 100%), SPon and SPoff are shown for the Non-24 patients and the controls, time-locked to MLmid. The open circles and the gray areas represent sleep propensity and percentage serum melatonin values, respectively. The thick vertical bars represent SPon and SPoff. a: p=0.007, and b: p=0.02 (t-test, vs. controls). A repeated ANOVA revealed that shape of sleep propensity rhythm was different between two groups, while shape of melatonin rhythm did not differ. The asterisks indicated differences of a post-hoc test (vs. controls).

mination rather than a manifestation of the endogenous free-run period that is likely in blind Non-24 sufferers.[7-10,26]

Since mutations in rodents with short or long tau are associated with phase-advanced or phase-delayed sleep-wake cycles under normal LD conditions[20-22] it could be postulated that a human tau (endogenous period length) mutant also exists in Non-24 sighted humans. The present laboratory experiment provides only limited information about tau because of the short period of the investigation (1-1.5 circadian cycle), but our findings that, under strictly controlled conditions, neither the MLduration nor SPduration differ significantly between Non-24 patients and controls indicate that a period mutation may be unlikely in these patients. The clinical history of the patients also suggested that a profound inborn period mutation was unlikely. All the subjects had been entrained normally to a 24-hour day until they changed their sleep habits due to night shift work, an altered vacation schedule, or an evening-type lifestyle preference and no unusual features were observed with respect to sleep-wake habits during childhood in the patients. Recent reports demonstrated that some Non-24 patients had an abnormal melatonin receptor gene,[38] which expressed low receptor affinity in transfected cells.[39] These observation may indicate that coupling between the circadian pacemaker and sleep could have pathogenic significance in Non-24.

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Circadian fluctuation of time perception in healthy human subjects

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Abstract

Previous studies suggested that various psychophysiological factors have influences on human time perception. In particular, working memory loads, time of day, body temperature, and mood were known as important modifiers of time perception. The purpose of this study is to elucidate factors affecting the short-term time perception under controlled condition. Fourteen healthy young male adults participated in this study. Time perception sessions (TPS) were conducted 4 times at 0900, 1300, 1700 and 2100 h. The TPS consisted of five 10-s time production trials under five different conditions (control trial, those with reward, and 3 different dual-load working memory tasks). Subjective status was assessed using visual analogue scales (VAS). To verify a participant's vigilance state, an alpha attenuation coefficient (AAC) was calculated. Two-way repeated measures ANOVA for produced time revealed a significant main effect of session, but no effect of task or interaction. Although produced time was not correlated with AACs or VAS scores, there was a significant negative correlation between produced time and core body temperature. These results suggest that human short-term time perception may be more influenced by circadian rhythm than working memory load or psychophysiological status.

Keywords: Time perception; Circadian rhythm; Interval timing clock; Working memory; Mood; Core body temperature; Time production test

1. Introduction

Animal studies have suggested that organisms have two endogenous timekeeping systems; a circadian clock that provides time of day and an interval timing clock that measures passage of time like a stopwatch (Virginia, 1996). The circadian clock is driven by self-sustaining oscillator with a period of about 24 h, which is located in the suprachiasmatic nuclei of the hypothalamus and synchronized to the light–dark cycle (Moore-Ede et al., 1983). In contrast to the circadian clock, the interval timing clock counts the number of signals that generated in certain intervals (Gibbon and Church, 1981; Roberts and Holder, 1984). These clocks play a crucial role in long lasting natural selection because the ability to anticipate risks and opportunities that occur in the environment could increase the chance for the animal to survive.

It has been well-documented that humans can perceive the passage of time without referring to an external clock or stopwatch, whether duration to be perceived

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was longer (long-term time perception) or shorter (short-term time perception). Previous studies on long-term time perception, duration to be perceived was widely ranged from minutes to hours (Mischel et al., 1969; Watts and Sharrock, 1984; Zakay, 1992; Campbell et al., 2001; Conti, 2001; Rammsayer et al., 2001), whereas those on short-term time perception concerned a perceived period of less than a minute (Treisman, 1963; Rammsayer and Vogel, 1992; Fortin et al., 1993; Fortin and Breton, 1995; Ivry, 1996; Fortin and Rousseau, 1998; Fortin and Masse, 1999; Casini and Ivry, 1999; Rammsayer et al., 2001). Though various mechanisms of long-term time perception have been postulated, there was significant controversy on the nature and functions because definitions of long-term time perception were apparently diverse from study to study, and because different study protocols were used in the previous studies (Mischel et al., 1969; Watts and Sharrock, 1984; Zakay, 1992; Campbell et al., 2001; Conti, 2001; Rammsayer et al., 2001). In contrast, the experimental settings in which short-term time perception was investigated were similar and comparable in the previous studies (Treisman, 1963; Rammsayer and Vogel, 1992; Fortin et al., 1993; Ivry, 1996; Fortin and Rousseau, 1998; Rammsayer et al., 2001). Moreover, recent progresses in functional imaging technique have suggested that certain regions of the brain were activated when short-term time perception tasks were loaded (Maquet et al., 1996; Harrington et al., 1998; Pouthas et al., 1999; Schubotz et al., 2000), though variations and changes of perceived time were not monitored in these studies because of the methodological limitations.

In the previous reports on short-term time perception, it was indicated that psychological or cognitive factors influenced human time perception. Some researchers have reported that short-term memory or working memory tasks that were loaded simultaneously with a short-term time perception task had an effect on perceived time (Fortin et al., 1993; Fortin and Breton, 1995; Fortin and Rousseau, 1998; Fortin and Masse, 1999). Enhancement of subjects' attention to temporal information has been reported to give more accurate short-term time perception (Zakay, 1992; Casini and Ivry, 1999; Rammsayer et al., 2001).

In chronobiological studies, it was reported that short-term time perception seems to fluctuate across the day (Aschoff, 1998; Aschoff and Daan, 1997; Morofushi et al., 2001). Under the free-running condition, produced time (10-s) has been reported to change along with core body temperature, which is a possible marker of circadian rhythm. During the subjective day, produced time (10-s) decreased from the morning into the night (Aschoff, 1998). These findings might suggest that short-term time perception may be modulated by a circadian oscillator.

However, there may be another possible explanation. That is, psychophysiological status such as mood, alertness, or tiredness, or changes in the environment such as light, posture or exercise may produce diurnal fluctuation in perceived time, providing that diurnal fluctuation of the time perception documented in the previous studies may have been a consequence of non-circadian origin.

Here we investigate short-term time perception in healthy human and study factors affecting it. In the present study, we focused on the effects of working memory load and psychophysiological status on time perception and those of diurnal fluctuations, as well as the possible interaction among those factors. To differentiate the influence of these factors clearly, we utilized a regimen, in which a constant resting wakefulness with semi-recumbent position was kept for 17 h, so that we excluded potential confounding factors that may have influences on the participant's psychological and physical status and may mask the differences of the experimental setting.

2. Methods

2.1. Participants

Fourteen healthy young male volunteers aged 18–24 years (mean age $\pm$S.D., 20.9 $\pm$ 1.8 yr) participated in the present study. A physician and a psychiatrist examined them and found that no participants had a history of, or suffered from, neurological or psychiatric disorders, or had a history of using any psychoactive drugs. They were asked to abstain from caffeine, nicotine, and alcohol for a week prior to the experiment. They were instructed to keep to a regular sleep–wake schedule and to record sleep logs for 2 weeks. We confirmed the validity of their sleep logs by using an ambulatory wrist activity recorder (Actiwatch-L, Mini-Mitter co., Inc. Bend, OR) for a week prior to the experiment, and found that the participants had regular sleep–wake habits without marked weekday–weekend differences in sleep schedule. The experimental protocol was approved by the Intramural Research Board of National Center of Neurology and Psychiatry, and each participant gave his informed consent after the nature, the purpose, and possible risks of the experiment had been explained in detail. All the experiment was performed at the time isolation laboratory of National Center of Neurology and Psychiatry.

2.2. Experimental design

2.2.1. Overview

Experimental schedule is illustrated in Fig. 1. The participant entered a two-day laboratory experimental

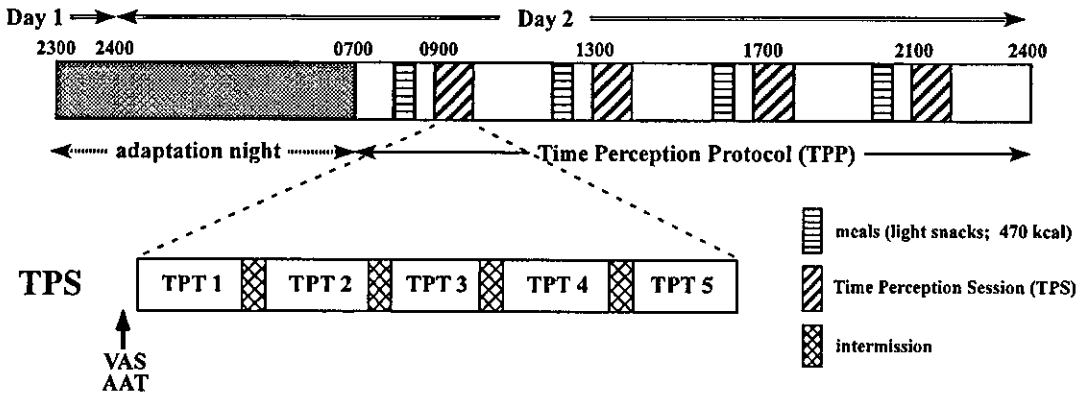

Fig. 1. A schematic schedule of the two-day study is shown. The participant was instructed to retire to temporal isolation facilities at 2300 h (Day 1) and to stay in bed until 0700 h (Day 2) under complete darkness (< 0.1 lx). Thereafter, they were enforced to keep awake in a semi-recumbent position under room light conditions (180 lx) until 2400 h (Day 2). Time production sessions (TPS) were carried out 4 times (0900, 1300, 1700 and 2100 h) on day 2. The TPS consisted of VAS, AAT, and five TPTs under five different psychological conditions; TPT without any tasks (control task), that with a reward (5000 ¥) for accuracy of the perception, that with memory task of 8-digit numbers, that with memory task of 4 words, and that with memory task of a figure of Benton's memory test. Three memory tasks were designed as dual-load tasks. The tasks were presented to the participant in a randomized crossover manner. At the beginning to each TPS, VAS and AAT were administered.

session. On the first day (Day 1), the participant arrived at the laboratory at 1900 h. Setting of rectal temperature sensor (inserted 10–15 cm from the anus) and attachment of EEG electrodes were completed between 2000 and 2200 h. The participant was instructed to retire at 2300 h and to stay in bed until 0700 h on the second experiment day (Day 2) when he was awakened by the technician. During this period, polysomnographic recordings were conducted under complete darkness (< 0.1 lx). Thereafter, the participant was enforced to keep awake on a semi-recumbent bed under room light conditions (180 lx) until 2400 h. During this period on Day 2, time perception protocol (TPP) was carried out. During the TPP, TP session (TPS) was conducted 4 times at 0900, 1300, 1700 and 2100 h. Iso-caloric meals (470 kcal) were given an hour before every TPS (0800, 1200, 1600 and 2000 h). Non-sparkling mineral water was supplied at any time according to the participant's requests. During the study, the ambient temperature and humidity in the time isolation facility were controlled at 24.0±0.5 °C and 60±5%, respectively. Throughout the study, no time cues or information on the exact numbers of TPSs and meals were given to the participant. Rectal temperature measurement and polygraphic recordings were carried out continuously throughout the study. Temperature data were measured every 2 min and stored telemetrically in a computerized monitoring system (Vital Sense, resolution 0.02 °C, Mini-Mitter Co. Inc., Bend, OR). Polygraphic recordings were performed throughout the study by using a polygraphic recorder (Neurofax, Nihon Kohden, Tokyo, Japan). Polygraphic recordings consisted of C3–A1, C4–A2 and O1–A1 EEGs in conformity with the 10–20 electrode system, electrooculograms (left and right), chin surface electromyogram, and electrocardiogram. During TPP, the participant was enforced to keep awake quietly without any amusements. The investigators monitored the participant's status via a video monitoring system and polygraphic recordings, and enforced them to stay awake. The participant was restricted to meet people except for meals.

2.2.2. TP Sessions

The TPS consisted of psychological assessment by visual analogue scales (VAS), alpha attenuation tests (AAT), and five time production trials (TPT) under five different task conditions: (1) TPT without any tasks (control task), (2) one with reward (5000 ¥) for accuracy of the produced time, (3) one with a memory task consisting of 8-digit numbers, (4) one with a memory task consisting of 4 different words (common nouns written in three different font styles of the Japanese cursive (*hiragana*) syllabary), and (5) one with a memory task of a figure of Benton's Memory test. The memory task was designed as a dual-load regimen, so that each task load would require processing in working memory simultaneously with the TPTs (Baddeley and Hitch, 1974; Baddeley, 1982, 1986). These trials were given to the participant in a randomized crossover manner. All the tasks were performed on a laptop computer with a 14-in. color liquid crystal monitor. The participant was instructed to produce 10 s by pressing a space key on the computer. The answers of the memory tasks were provided by pressing a key on the numerical keyboard of the computer. The screen was placed at 50 cm from the participant's eyes. All the data were transmitted via

a local network system and were stored in the host computer. Prior to the study, they trained on TPTs to understand the operation of the PC-based test battery; between 1900 and 2000 h on Day 1, a TP session was carried out after the investigator gave a detailed explanation. Produced time in the training trials ranged from 9.40 to 14.04 s (11.45±1.14, mean±S.D.). For all performance tasks, participants were instructed to perform as quickly as possible, but without sacrificing accuracy.

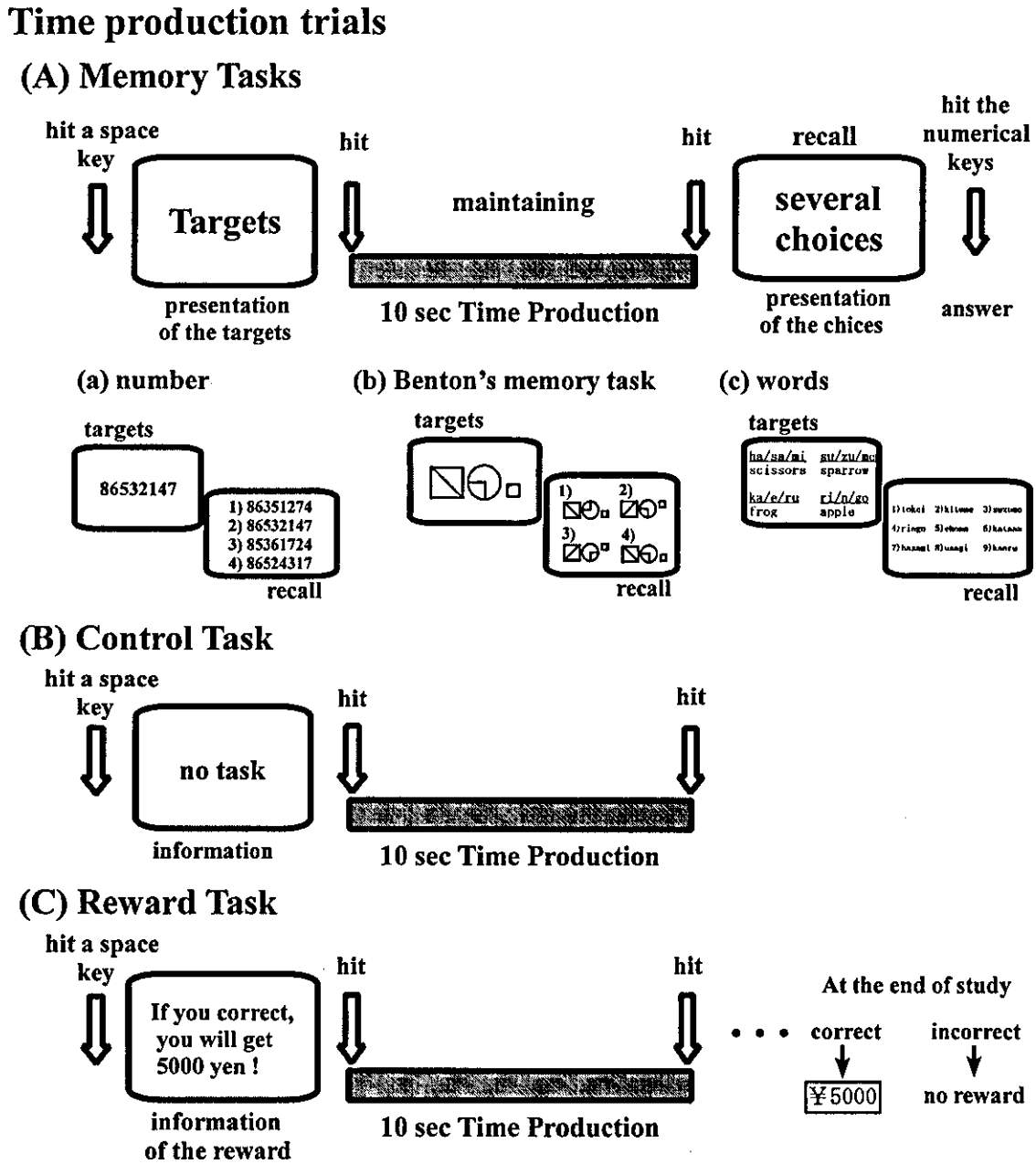

Fig. 2. TPS was conducted under five different conditions. Produced time was measured as the interval between keypress to begin and keypress to end. This procedure was operated under following conditions: (A) memory task condition. The participant hits a space key to start the trial, this keypress triggered the presentation of memory sets. It disappeared between 5 and 15 s to avoid itself influenced on the participant's judgment of a 10-s time production. After the TPT, several choices of the memory targets were presented and urged to select the correct answer. The memory task of numbers and figure of Benton's Memory test had four choices (a and b), and the task of words had nine choices (c). (B) Control task condition. TPT without any additional tasks was carried out. (C) Reward task condition. At the beginning of TPT, instruction of reward condition was presented to the participant. The reward was given to the participant when his produced time was within the range of 10±0.5 s. The results of time production were not announced until the end of all the study. In this trial, no additional task was carried out. Each condition was presented in a randomized manner. The intervals between trial conditions were set up 70–100 s to avoid being used by the participant as a ruler interval.

2.3. Measures

2.3.1. TP trials

Produced time was measured as the interval between keypress in each different condition. The TPTs are illustrated in Fig. 2. The memory tasks (Fig. 2A) was designed to be loaded simultaneously during time production. After pressing a space key to start the trial, the participant was instructed to memorize the targets displayed on the screen for 5–15 s and to retain them until the end of the trial. The participant was required to select the correct targets among several choices. The memory task for the numbers and for the figure of Benton's Memory test had four choices (Fig. 2A (a and b)), and the memory task for words had nine choices (Fig. 2A c). The inter-trial interval ranged from 70 to 100 s. In the control task (Fig. 2B), the participant was instructed directly to enter the TPT. In the reward task (Fig. 2C), the amount of reward for accurate produced time was presented prior to the TPT. A reward (¥5000) was given to the participant when his produced time was within the range of 10 ± 0.5 s.

Information concerning the accuracy of time production and the results of the memory tasks was not given to the subject until the end of the study.

2.3.2. Alpha attenuation test

At the beginning of each TPS, AAT was administered. In the AAT, three 1-min artifact-free EEG recordings with eyes-open and those with eyes-closed were obtained. The EEG data were digitized using a sampling rate of 200 Hz. A half-amplitude low-frequency filter was set at 0.5 Hz, and high frequency filter at 50 Hz. The digitized data was stored on an optical disk and analyzed offline with an EEG spectral analyses package (FOCUS; MEGIS software, GmbH). Power spectra of eyes-opened and eyes-closed EEG recordings derived from O1 to A1 were analyzed by using a fast Fourier transformation (FFT) within the alpha frequency band (8–12 Hz) using a bin size of 0.5 Hz. The FFT analyses were conducted under 5.120-s Welch tapered windows with 2.620-s overlap. This yielded 24 windows per 1-min epoch. The bins were averaged across the 8–12 Hz frequency range to produce an estimate of alpha power in squared microvolts. The ratio of the mean eyes-closed to mean eyes-opened alpha power was defined as the alpha attenuation coefficient (AAC).

2.3.3. Visual analogue scale

Subjective psychophysiological status was assessed using VAS following the AAT in each TPS. The VAS consisted of seven items, which were alertness, mood, energy, tiredness, tension, motivation, and irritability. The VAS was presented as a horizontal line 100 mm in length, labeled for example 'Very Alert' on the left and 'Very Sleepy' on the right. The participant was asked to draw a vertical mark on the line at the point corresponding to their present status. The VAS scores were obtained by measuring the distance of the mark from the left end of the line (higher scores being associated with more intense feelings of each state).

2.4. Statistical analyses

To reduce inter-individual covariance, AACs and VAS scores were standardized by using Fisher's Z-transformation, and core body temperature was transformed to the mean deviations.

Two-factor 4×5 (session-by-task) repeated measures ANOVAs were used to analyze the produced time. Greenhouse-Geiser's adjustment factor (epsilon) was calculated to adjust the degrees of freedom.

Endogenous circadian phase of the core body temperature data was assessed by fitting of dual-harmonic cosine curves (periods, 24 and 12 h) with a least squares method (Uchiyama et al., 1995; Kubota et al., 2002; Tagaya et al., 2002) using a software package (KaleidaGraph, Synergy Software, Reading, PA). The nadir of the fitted curve was regarded as the circadian phase marker. The nadir of the core body temperature was 0541 ± 0122 (mean $\pm$ S.D.), suggesting the participant's circadian phase markers were normal range. Pearson's correlation coefficients were calculated between produced time and other measures.

SPSS ver.10.0J for Windows (SPSS Inc., Chicago, IL) was used for all the statistical analyses. All statistical

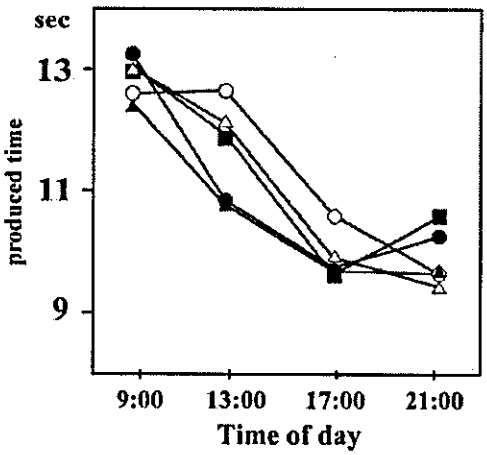

Fig. 3. The results of the TPTs of a representative participant are shown. The horizontal axis represents time of day. The vertical axis represents values of produced time. Black squares () represent produced time under control task condition. Black circles (●) are produced time with WM tasks of 8-digit numbers. Black triangles (?) are produced time with WM tasks of 4 words. Open circles (○) are produced time with WM tasks of figures. Open triangles (△) are produced time with rewards. The produced time decreased as time goes from 0900 to 1700 h regardless of presence of memory tasks or rewards condition.

analyses were two-tailed. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Overview

The results obtained from a representative participant are shown in Fig. 3. Produced time was longer than 10 s in the first two sessions and was decreased toward the evening (Fig. 3(a)). The difference in trial condition did not seem to influence the produced time.

The overall error rates for memory tasks obtained in all participants were 0.018% (1/56; at the fourth session) for number, 0.018% (1/56; at the second session) for Benton's figure, 0.018% (1/56; at the second session) for word, confirming that most of the dual-loaded memory tasks were properly performed.

3.2. Effect of trial condition and time of day

Two-way repeated measures ANOVA revealed a significant main effect of session ($F(3, 11) = 7.27$, $P = 0.005$), but no effect of trial condition ($F(4, 10) = 0.65$, $P = 0.56$) or interaction ($F(12, 2) = 1.70$, $P = 0.15$). Thus, produced times were averaged across condition. Produced times decreased with progression of the sessions (Fig. 4).

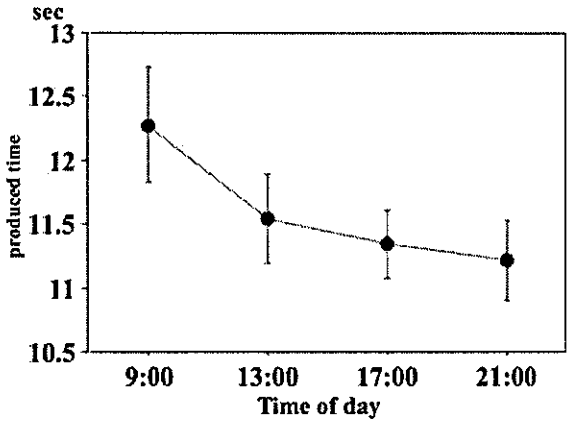

Fig. 4. Diurnal change of produced times are shown. The horizontal axis represents time of day. The vertical axis represents values of produced time. Two-way repeated measures ANOVA of produced time revealed a significant main effect of session ($F(3, 11) = 7.27$, $P = 0.005$). Each data point represents the mean produced time of all the conditions. Bars represent standard errors of mean. The produced time which were averaged across condition decreased from the morning till evening.

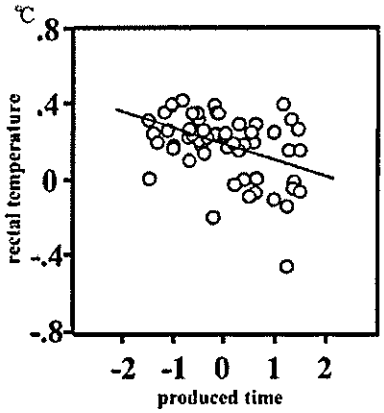

Fig. 5. Relation between produced time and core body temperature together with the regression line are shown. The horizontal axis represents standardized scores of produced time. The vertical axis represents mean deviated values of rectal temperature. Standardized produced time and standardized core body temperature were significantly negatively correlated ($r = -0.44$, $P < 0.001$).

3.3. Correlation with core body temperature, AACs and VAS scores

3.3.1. Core body temperature

A significant negative correlation was found between produced times and core body temperature ($r = -0.44$, $P < 0.001$; Fig. 5).

3.3.2. Alpha attenuation coefficients

No significant correlation was found between produced times and AACs ($r = -0.061$, NS).

3.3.3. Visual analogue scale scores

No significant correlation was found between produced times and VAS scores (*Alertness:* $r = -0.074$, *Mood:* $r = 0.283$, *Energy:* $r = 0.038$, *Tiredness:* $r = -0.236$, *Tension:* $r = -0.013$, *Motivation:* $r = 0.158$, *Irritability:* $r = 0.028$, NS).

4. Discussion

In the present study, short-term time production showed significant diurnal fluctuations, in which remarkable over-production in the morning decreased towards the evening. However, time production was not influenced by the working memory tasks that were simultaneously loaded to the participant. In addition, the vigilance level measured by using AAT or VAS scores did not affect the time production, whereas core body temperature at the TPS showed a significant negative correlation with produced time.