REGULAR ARTICLE

The effect of sleep restriction and psychological load on the diurnal metabolic changes in tryptamine-related compounds in human urine

Masayasu Minami · Hideyo Takahashi · Tsukasa Sasaki · Shun Matsumoto · Kazuhiro Sakai · Hirofumi Inagaki · Yuko Yamano · Hirotsugu Miyake

Abstract

Objective The effect of a severely stressful situation (sleep restriction and psychological load) on the diurnal changes in novel tryptamine-related compounds (hydroxy-diacetyltryptamine, sulphatoxymelatonin, and dihydromela-tonin) was evaluated in human subjects for 16 days.

Methods The subjects were allowed to sleep for 5 h on days three through 12 and for 8 h on the other days. On days three through 12, the subjects were asked to perform a psychological task. The first two and the last 4 days were viewed as control days. A performance test was administered to evaluate the extent of the subjects' fatigue. Total urine was sampled by collecting it into bottles three times a day [(1) during the sleeping period, (2) in the morning, and (3) in the afternoon]. Seven tryptamine-related compounds in urine were assayed using HPLC-fluorometry.

Results The urine melatonin level was high at night and low during the day. In contrast, urinary levels of hydroxydiacetyltryptamine and sulphatoxydiacetyltryptamine were low at night and high during the day. Dihydromelatonin was undetectable in urine during the sleeping period. Sleep restriction and psychological load did not affect diurnal changes in urinary melatonin, hydroxydiacetyltryptamine, sulphatoxydiacetyltryptamine, or *N*-acetylserotonin levels. The concentrations of hydroxymelatonin and sulphatoxyme-latonin in urine did not show diurnal changes and decreased gradually during the experimental days. A principal component analysis confirmed the diurnal changes and suggested two novel metabolic pathways: (1) *N*-acetylserotonin to sulphtoxydiacetyltryptamine via hydroxydiacetyltryptamine, and (2) melatonin to dihydromelatonin.

Conclusion Severely stressful situations did not affect diurnal changes in melatonin, hydroxydiacetyltryptamine, sulphatoxydiacetyltryptamine, or *N*-acetylserotonin levels in urine.

Keywords Hydroxydiacetyltryptamine ·
Dihydromelatonin · Melatonin · Diurnal change ·
Sleep restriction

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Introduction

Three novel indolamine compounds, hydroxydiacetyl-tryptamine (OH-diatry), sulphatoxydiacetyltryptamine (s-diatry), and dihydromelatonin (or reduced melatonin; red mel) have been detected in human urine [1]. Red mel was identified in human urine by our group, as well as

being detected in the brains of Goto-Kakizaki diabetic rats by Frese et al. [2], and it was tested pharmacologically by Gasparova et al. [3] and Ondrejickova et al. [4]. OH-diatry and s-diatry have diurnal rhythms of excretion in human urine; their concentrations are highest in the daytime and lowest at night. This evidence was obtained in an experiment with young human subjects in a non-stressful situation [1]; the OH-diatry level during the day was 189 ± 320 (μg/mg creatinine [cr]) and that at night was 71.9 ± 64.1 (μg/mg cr). The difference was statistically significant ($p < 0.01$). In that experiment, all of the subjects resided in special lodging rooms at our institute for 2 days [1]. The excretion rhythm of OH-diatry is opposite to the diurnal rhythm of melatonin [5–9]. The aim of our present study was to determine whether the diurnal rhythm of the urinary excretion of OH-diatry, s-diatry, and other indolamine compounds were unchanged under a highly stressful situation. Accordingly, we carried out the following human experiment, in which the subjects were placed in a stressful condition with sleep restriction and a psychological load. For 16 days, all of the subjects resided in special lodging rooms annexed to a large experimental space at our institute. For the first 2 days and on days 13–16, they were allowed 8 h of sleep per night without a psychological load (the control period) and for days 3–12, they were allowed 5 h of sleep per night and subjected to a psychological burden (the stress-exposed period). We also tried to elucidate the interrelationship among indolamine compounds in human urine by performing principal component analysis (PCA).

Human activity ordinarily increases during the day and declines at night, coinciding with the rhythm of OH-diatry excretion. OH-diatry may have a stress-alleviating action during the day. From the present work we report that: (1) the diurnal changes in the tested tryptamine-related compounds in human urine are detectable even in a stressful situation, and (2) some of these compounds are suitable as stress markers after sleep restriction and psychological task-loading.

Chronobiological studies that reported a diurnal change in plasma melatonin have already been published [10, 11], but no extensive study of the diurnal changes in tryptamine-related compounds in human urine has been reported prior to this study.

Subjects and methods

Eight healthy men aged 22–36 years (29.6 ± 4.7; mean $\pm$ SD) were studied. As stated above, all of the subjects resided in special lodging rooms at our institute. This study was approved by the ethics committee of the Institute for the Science of Labour, and informed consent was obtained from each volunteer.

Test schedules

The 16 days were divided into four terms. For the first 2 days the subjects were allowed 8 h of sleep (9:00 pm until 5:00 am on the next day); called term I. For the next 10 days (split evenly into terms II and III), they were allowed 5 h of sleep (1:00 am to until 6:00 am the next day). For the final 4 days, called term IV, the subjects were allowed 8 h of sleep (from 9:00 pm until 5:00 am on the next day). The control periods were the first 2 days (term I) and the last 4 days (term IV), because the subjects were not exposed to stressful conditions (sleep restriction and a psychological load) during those periods.

The experimental room was lit with 700 lux lamps from 5:00 am until the time immediately before the subjects went to bed.

Tasks for the subjects

The subjects were asked to void urine into three bottles per day, as follows: (1) during the sleeping period (between 9:00 pm and 6:00 am on the next day), for terms I and IV; or between 1:00 am and 6:00 am the next day for terms II and III; (2) in the morning (between 7:00 am and 12:00 pm); and (3) in the afternoon (between 12:30 pm and immediately before going to bed).

Each subject filled out a questionnaire, in Japanese, about their fatigue, three times a day, at 7:30 am, 12:30 pm, and 8:30 pm. The questionnaire details are shown in Table 1. This questionnaire is a revised version of the fatigue inventory established by the Industrial Fatigue Research Committee of the Japan Association of Industrial Health in 1967. The subject was asked to answer each question with "yes" or "no" and the number of "yes" responses indicated the degree of fatigue [12].

The subjects were requested to transcribe sentences in English that had been reprinted from current medical journals, using a personal computer, during terms II and III from 8:30 am to 9:20 pm, with breaks for lunch and dinner. They were permitted to take breaks freely during the transcription work. The accuracy and speed of the transcription were determined according to an individual's ability, based on the result of a 2-h transcription task performed on the second day of the experiment. This transcription work was the psychological load on the subject. During terms I and IV, the subjects were allowed to spend time freely listening to the radio, playing video games, and reading books, except for the period spent determining the accuracy and speed of the transcription task on the second day.

Table 1 Subjective symptoms of fatigue proposed by the Industrial Fatigue Research Committee of Japan Association of Industrial Health

I. Symptoms of drowsiness and dullness

  1. Feel heavy in the head

  2. Feel tired in the whole body

  3. Feel tired in the legs

  4. Yawning

  6. Becoming drowsy

  7. Feeling eyestrain

  8. Becoming rigid or clumsy in motion

  9. Feeling unsteady on standing

  10. Wanting to lie down

II. Difficulty in concentration

  11. Have difficultly thinking

  12. Become weary of talking

  13. Become nervous

  14. Unable to concentrate

  15. No interest in anything

  16. Become apt to forget things

  17. Lack of self-confidence

  18. Anxious about everything

  19. Unable to straighten posture

  20. Lack patience

III. Symptoms of physical exhaustion

  21. Have a headache

  22. Feel stiff in the shoulders

  23. Feel pain in the back

  24. Difficulty in breathing

  25. Feel thirsty

  26. Have a husky voice

  27. Feel dizzy

  28. Have spasms in the eyelids

  29. Have tremor in the limbs

  30. Feel ill

Performance test

A performance test or psychomotor vigilance task (PVT [13]), was administered to the subjects for 20 min twice a day; immediately after rising and immediately before going to bed.

The procedure was as follows: the subject was requested to push a button on a controller with the same number as that shown on a 14-inch TV connected to the controller. The numbers two, four, six, and eight were displayed one by one on the screen at random intervals, and in a random order for a total of 20 min. The subject had to respond to each number displayed. The time from when the number appeared on the display until the subject's reaction was measured in milliseconds and the mean values for approximately 80–90 trials were calculated.

Assay of tryptamine-related compounds

Seven tryptamine-related compounds were assayed in the sampled urine by a fluorometric HPLC method [1] and the concentrations were normalized to urinary creatinine levels to control for variations in urine flow and urinary concentrations due to the presence of salts [14].

Statistics

An analysis of variance (ANOVA) [15] was conducted to identify the statistical significance of differences in the results. To compare the differences between the means, Welch's *t*-test [16] was used. The seven tryptamine-related compounds we tested varied during the experiment, based on the following three factors: (1) sampling time, possibly indicative of a diurnal change in the tested compound, during the time allotted for sleep, the morning (7:00 am to 12:00 pm), or the afternoon (12:00 pm to going to bed); (2) the day of the experiment (the subjects would become more fatigued as the experiment continued from days 1–16); and (3) the person undergoing the experiment (individual variation).

The tryptamine-related compounds seemed to have mutual interrelationships according to their metabolism, so PCA [17] was used to clarify these relationships by classifying the compounds according to the principal component. Mutual standardized correlation coefficients were calculated using paired values of the urinary levels of the compounds determined from the PCA.

Results

Results of ANOVAs and differences in means

Effect of sampling time on the urinary excretion of the tested compounds

Figure 1 shows the levels of the tested compounds in the urine classified according to sampling time.

Sampling time was a statistically significant factor for all of the compounds, except for hydroxymelatonin and sulphatoxymelatonin. Red mel was detected only in the morning (9:00 am to noon) and afternoon (12:00 pm to bed time) and was not affected by sampling time during the daytime. The melatonin level during the sleep period was significantly higher than the level in the morning ($p < 0.02$) or that in the afternoon ($p < 0.01$). There was also a significant difference between the level in the morning and the level in the afternoon ($p < 0.01$). In contrast, the levels of hydroxymelatonin and sulphatoxymelatonin did not show diurnal fluctuations.

Fig. 1 Changes in the levels of the test compounds according to the time of sampling. The data are shown in the following order: A the sleep period (N = 128), B the time between 7:00 am and 12:00 pm (N = 128), and C the time between 12:30 pm and the time immediately before going to bed (N = 128). Each column and bar indicate the mean and standard error. Statistically significant differences in the means are shown (p < 0.02, p < 0.01, or p < 0.001), above the columns

A : during the sleeping time
B : the time between 7:00 and 12:00
C : the time between 12:30 and the time immediately before going to bed
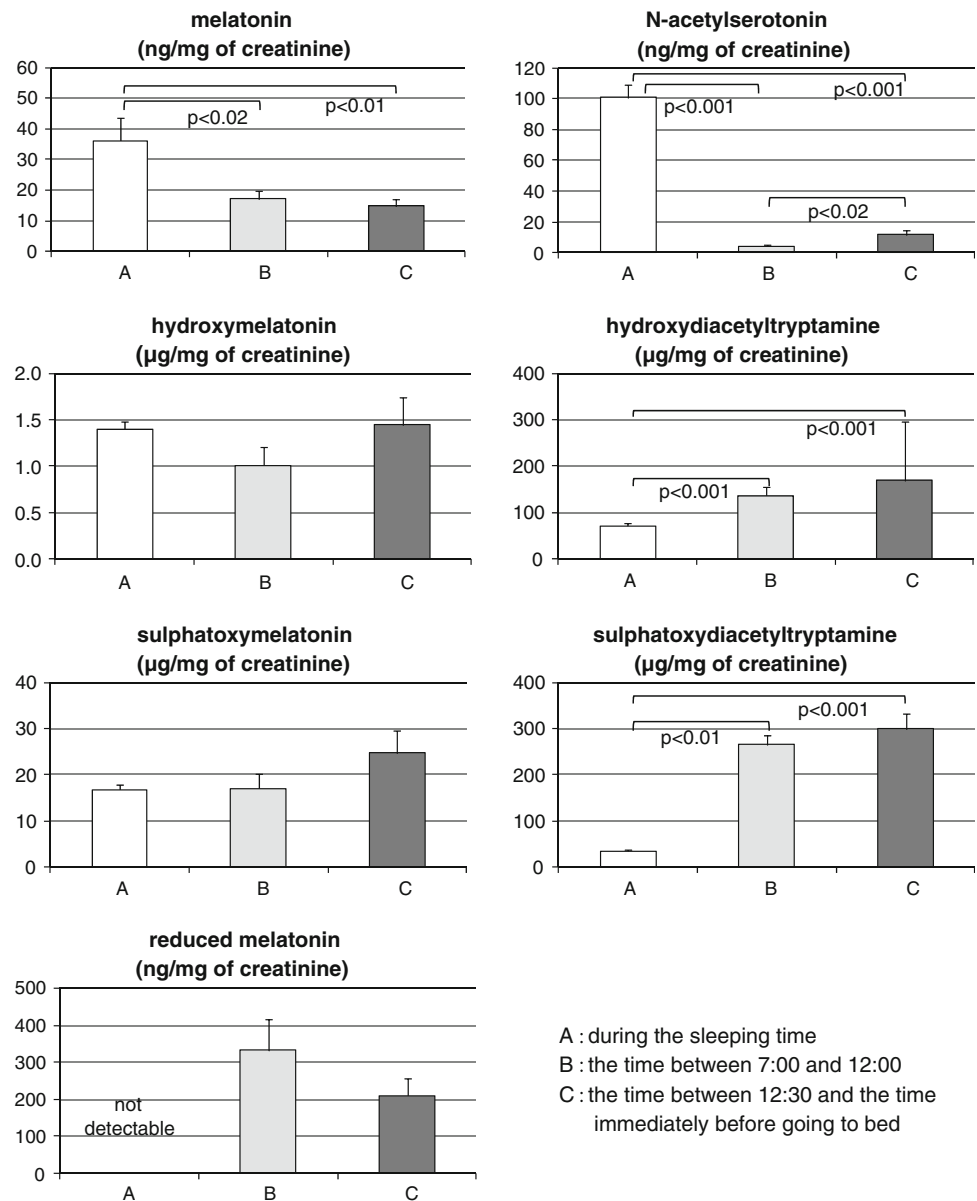
The level of OH-diatry during the sleep period was significantly different from the level in the afternoon ($p < 0.001$). There was also a statistically significant difference between the OH-diatry level in the morning and that during the sleep period ($p < 0.001$). There were statistically significant differences between the s-diatry level during the sleep period and in the morning ($p < 0.001$), as well as between the level during the sleep period and in the afternoon ($p < 0.001$). The levels of N-acetylserotonin differed significantly among the sleep period, the morning, and the afternoon [night vs. morning ($p < 0.001$), night vs. afternoon ($p < 0.001$), and morning vs. afternoon ($p < 0.02$)]. The melatonin level was high during the sleep period and low in the afternoon. OH-diatry and s-diatry levels were high in the afternoon and low at night. The

hydroxymelatonin and sulphatoxymelatonin levels did not fluctuate significantly with the sampling time. Red mel was also affected by the time; it was not detectable during the sleep period.

Effects of the hours of sleep and psychological load on urinary excretion of the compounds

Figure 2 shows the effect of sleep restriction and psychological stress on the levels of the compounds.

The 16 days were divided into four terms; terms I and II were viewed as the control terms, as described in the "Subjects and methods" section. The hydroxymelatonin levels were higher during term I than during term III or term IV, with the differences between term I and term III,

and between term I and term IV being statistically significant ($p < 0.001$ and $p < 0.02$, respectively). Sulphatoxymelatonin levels were higher during term I than during term III or term IV, with the differences being statistically significant ($p < 0.01$ and $p < 0.02$, respectively). The ratios of the levels in term I to those in term II were greater than the ratios of the levels in term III to term IV for hydroxymelatonin and sulphatoxymelatonin. Melatonin, red mel, OH-diatry, s-diatry, and *N*-acetyl serotonin did not show term-to-term variations. Three-way ANOVA revealed that individual variation affected all of the tested compounds, except for hydroxymelatonin.

The effect of sleep restriction and psychological load on diurnal changes in the urinary levels of the tested compounds

Diurnal changes were detected in the urinary levels of melatonin, OH-diatry, s-diatry, and *N*-acetylserotonin. We investigated whether the diurnal changes in the urinary levels of these compounds were affected by stressful conditions (sleep restriction and a psychological load). The urinary levels of the compounds were classified according to the control (terms I and IV) and the stress-exposed (terms II and III) periods (see Fig. 3). As shown in Fig. 3, diurnal changes in the compounds were apparent, irrespective of the control and the stress-exposed periods. The results show the robustness of the diurnal changes in these compounds.

The diurnal changes in these compounds were not affected by stress, and therefore PCA could be applied to all of the data, irrespective of the control or the stress-exposed periods.

Results of the PCA

Principal component analysis (PCA) is a statistical technique that linearly transforms an original set of variables into a statistically smaller set of variables that represents most of the information in the original set of variables [17]. The biological mean of the smaller set of variables, which is represented by the principal component, is considered by the researcher or the user of the PCA. Through PCA calculation in this study, we obtained 2 different sets of principal components, concerning the data obtained during the sleep period and concerning the data obtained during the daytime. The procedure used to detect the means of the principal components was as follows. Concerning component {1}, which was calculated using the data obtained during the sleep period, statistically significant correlation coefficients of the compounds with component {1} were arranged in a row: *N*-acetylserotonin (0.300), melatonin (0.489), hydroxymelatonin (0.727), and sulphatoxymelatonin (0.801). This

row seemed to follow the metabolic route of *N*-acetylserotonin → melatonin → hydroxymelatonin → sulphatoxymelatonin, as shown in the metabolic maps (Figs. 4, 5a), which could indicate melatonin metabolism. Component {2} was calculated using the data obtained during the sleep period, and statistically significant correlation coefficients of the compounds with component {2} were arranged in a row; *N*-acetylserotonin (0.581), OH-diatry (0.287), and s-diatry (0.801). This arrangement suggests that *N*-acetylserotonin is metabolized to s-diatry via OH-diatry, as shown in the metabolic maps (Figs. 4, 5a). A similar procedure was used to arrange the statistically significant correlation coefficients of the data obtained during the day, and this procedure revealed the pathway of indolamine metabolism during the day. Thus, it seemed possible that, after the PCA treatment of the data, the statistically significant correlation coefficients for each component could be classified according to the metabolic flow of the compounds. The metabolic flow of tryptamine-related compounds proposed by Minami et al. [1] is shown in Fig. 4.

The PCA method was applied separately to the two following groups of data: (1) the data sampled during the sleep period ($N = 128$) and (2) the summed data sampled between 7:30 am and 12:00 pm ($N = 128$) and the summed data sampled between 12:30 pm and going to bed ($N = 128$); thus, the total number of samples for this group was 256. The calculated variance and the correlation coefficient were given according to each component during each time section, as shown in Fig. 5a, b with metabolic maps; in this Fig., the number under the name of the compound is the correlation coefficient of the compound related to the indicated component at the time of sampling. In Fig. 5, the contribution of a component to the total variance is expressed as a percentage under the name of each component.

During the sleep period, component {1} had the largest contribution (28.6%) to the variance (six variables; red mel was not detectable at this time). The correlation coefficients for hydroxymelatonin (0.727) and sulphatoxymelatonin (0.804) were high and statistically significant, whereas low, but statistically significant, correlation coefficients were found for *N*-acetylserotonin (0.300) and for melatonin (0.489). The compounds having statistically significant and/or high correlation coefficients were ranked as follows, suggesting a metabolic flow: *N*-acetylserotonin → melatonin → hydroxymelatonin → sulphatoxymelatonin. The second largest component was component {2} and the contribution to the variance was 24.2%. The correlation coefficient for *N*-acetylserotonin was 0.581 and that for s-diatry was 0.786. These correlation coefficients were both high and statistically significant, whereas the correlation coefficient for OH-diatry, 0.287, was low, but statistically significant. The compounds with statistically
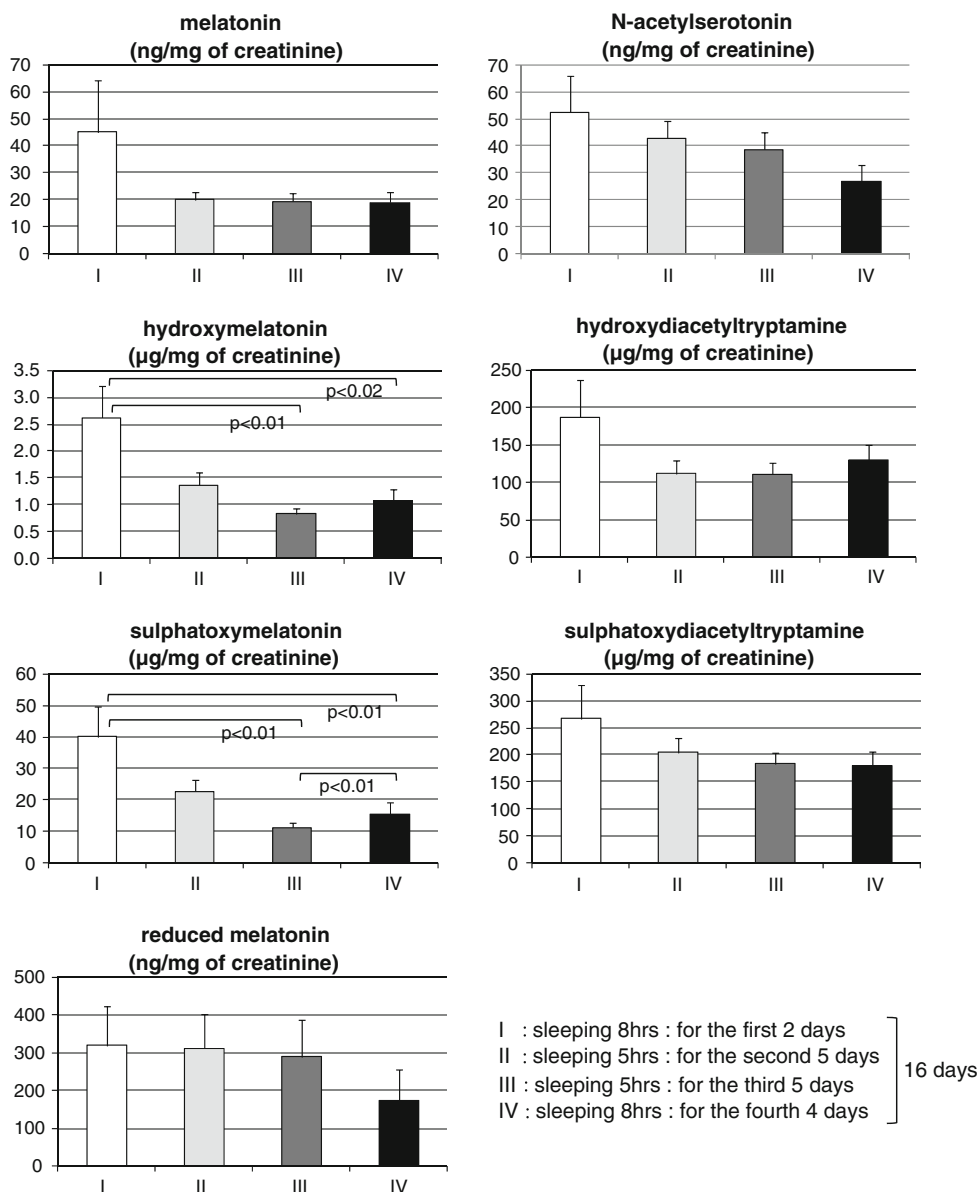
significant and/or high correlation coefficients were ranked
as follows, and the ranking also seemed to suggest a
metabolic pathway: *N*-acetylserotonin → OH-diatry →
s-diatry.

Between 9:00 am and bed time, component {1} was the
greatest contributor (25.0%) to the variance (7 variables);
its correlation coefficients for OH-diatry (0.784) and
s-diatry (0.736) were high and statistically significant.
Again, the compounds with statistically significant corre-
lation coefficients were ranked, and their ranking suggested
a metabolic flow: OH-diatry → s-diatry. Concerning the
correlation coefficient for melatonin with component {1}, a
statistically significant correlation coefficient was calcu-
lated (0.357). Additionally, a statistically significant cor-
relation coefficient was found for red mel with component

{1} (0.636). Statistically significant and/or high correlation
coefficients were ranked as follows and were regarded as
possibly metabolically significant; melatonin → red mel.
Component {2} accounted for 20.5% of the variance and
its correlation coefficients for melatonin (−0.368),
hydroxymelatonin (0.672), and sulphatoxymelatonin (0.773)
were statistically significant, and were ranked according to
a metabolic flow. Melatonin correlated negatively with
component {2}, and seemed to be excluded from the
metabolic route to sulphatoxymelatonin via the interme-
diate hydroxymelatonin. This finding was an important
point considering the relationship between components {1}
and {2} during the daytime. Concerning component {2},
the correlation coefficient for melatonin was 0.357 and that
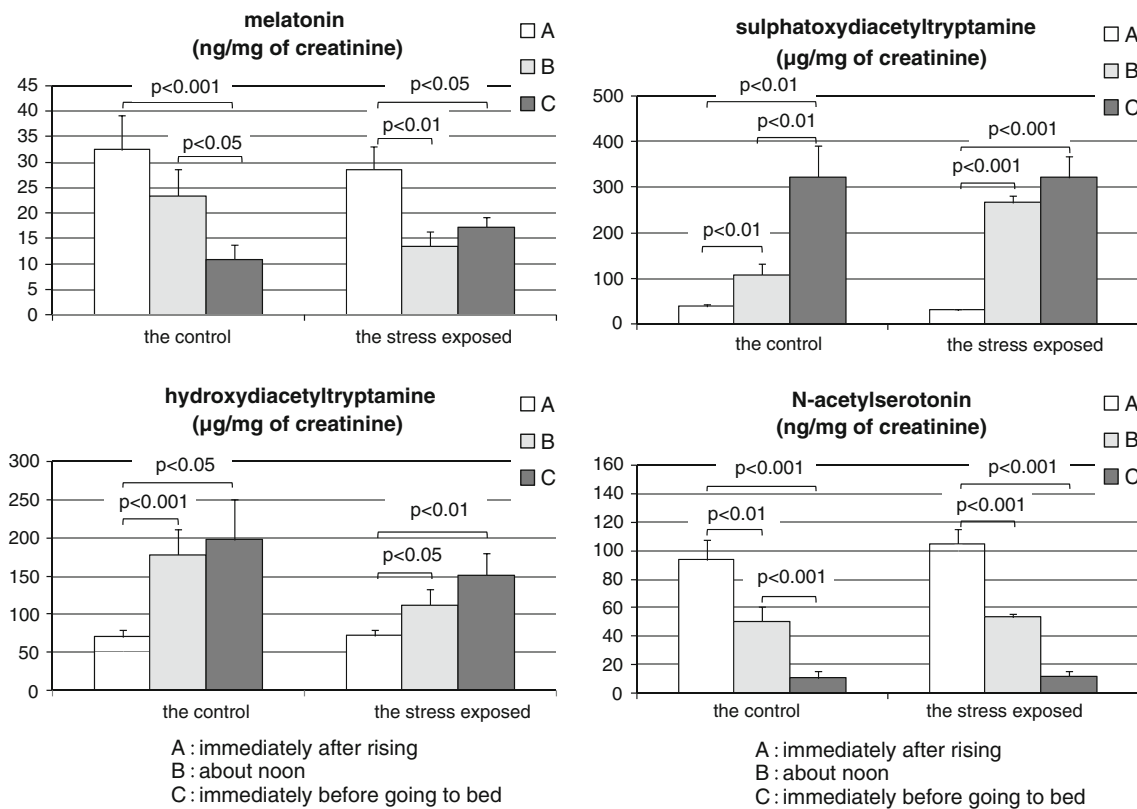for red mel was 0.636; both were statistically significant.

A : immediately after rising
B : about noon
C : immediately before going to bed

A : immediately after rising
B : about noon
C : immediately before going to bed

Fig. 3 Changes in compound levels according to the sampling times in data classified as the control (terms I and IV) and the stress-exposed (terms III and IV) periods were evaluated, to determine how sleep restriction and psychological load affected the urinary level of the compound. The data are shown in the following order: *abscissa*, *A* during the sleep period, *B* the time between 7:00 am and 12:00 pm, and *C* between 12:30 pm and the time immediately before going to bed. The data are classified as stress-exposed and control periods, for the total of 16 days of the experiment *Ordinate*, the urinary level/mg of creatinine. *Each column and bar* indicate the mean and standard error. Statistically significant differences of means are shown ($p < 0.05$, $p < 0.01$, or $p < 0.001$), *above the columns*

This suggested, as noted above, another metabolic pathway of melatonin. The metabolism of hydroxymelatonin to sulphatoxymelatonin showed statistically significant correlation coefficients with component {1} during the sleep period and with component {2} between 7:30 am and the time before going to bed.

Fatigue-related indicators

Figure 6 shows the time difference and the effect of stress (sleep deprivation and the psychological task) on the subjective symptoms of fatigue and the speed of the performance test.
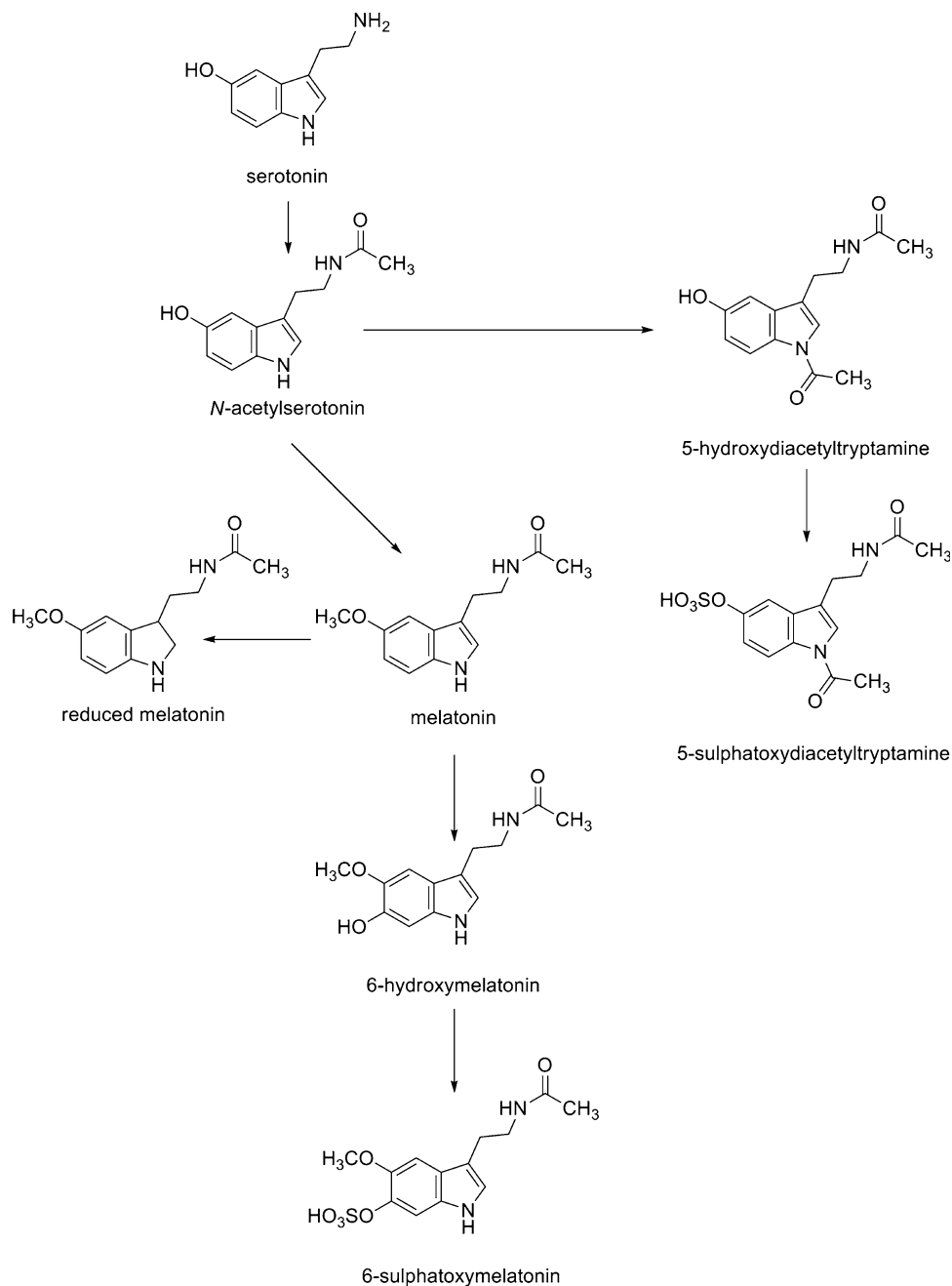
The fatigue-related factors that we measured were under the control of: (1) the time during which the data were obtained, (2) the days the experiments were conducted, and (3) the persons from whom the data were obtained. A three-way ANOVA was applied to fatigue-related factors, i.e., fatigue-related subjective symptoms and the results of the PVT. All of the factors were statistically significant for both of the fatigue-related indicators (subjective symptoms and PVT). A comparison of means with Welch's *t*-test

indicated that there were statistically significant differences between the subjective symptom data in term I and term III ($p < 0.01$), between the data in terms II ($p < 0.05$) and IV ($p < 0.01$), and between the data in terms III and IV ($p < 0.001$). The levels of fatigue-related subjective symptoms during the control periods (terms I and IV) were significantly lower than those in the stress-exposed period (terms II and III). PVT did not reveal any statistically significant differences concerning these factors.

Discussion

Three novel tryptamine-related compounds; red mel, OH-diatry, and s-diatry were found in human urine [1]. The urinary levels of the newly found compounds were high in the daytime and low at night [1], in contrast to findings for melatonin [5–9]. Considering the diurnal changes in melatonin levels, its physiological actions appear to occur mainly at night. It seems likely that melatonin's counterpart might show similar physiological actions during the

Fig. 4 The metabolic flow of the tryptamine-related compounds. Minami et al. [1] proposed the metabolism of novel 3-substituted indolamine compounds, hydroxydiacetyltryptamine, sulphatoxydiacetyltryptamine, and reduced melatonin, expanding the ordinary melatonin metabolic pathway

serotonin

N-acetylserotonin

5-hydroxydiacetyltryptamine

reduced melatonin

melatonin

5-sulphatoxydiacetyltryptamine

6-hydroxymelatonin

6-sulphatoxymelatonin

daytime; because more physico-chemical reactions would be expected to occur during the day than at night.

The metabolic conversion of hydroxymelatonin to sulphatoxymelatonin does not change with the time of day. This observation was confirmed by the ANOVA and t-tests in the present study. ANOVA revealed that individual variance did not affect the hydroxymelatonin level. Hydroxymelatonin and sulphatoxymelatonin levels decreased with increasing lack of sleep and psychological load. Thus, these two compounds, especially hydroxymelatonin, could be used as stress markers.

In our study, the number of fatigue-related symptoms was significantly greater during the period of sleep restriction with the psychological load (terms II and III) than during the control period (terms I and IV).

When the data were classified according to control and stress-exposed periods, the diurnal rhythm of the levels of melatonin, OH-diatry, s-diatry, and N-acetylserotonin did not change according to whether period was the control or the stress-exposed period, as shown in Fig. 3. The diurnal rhythm was robust even in a stressful situation.
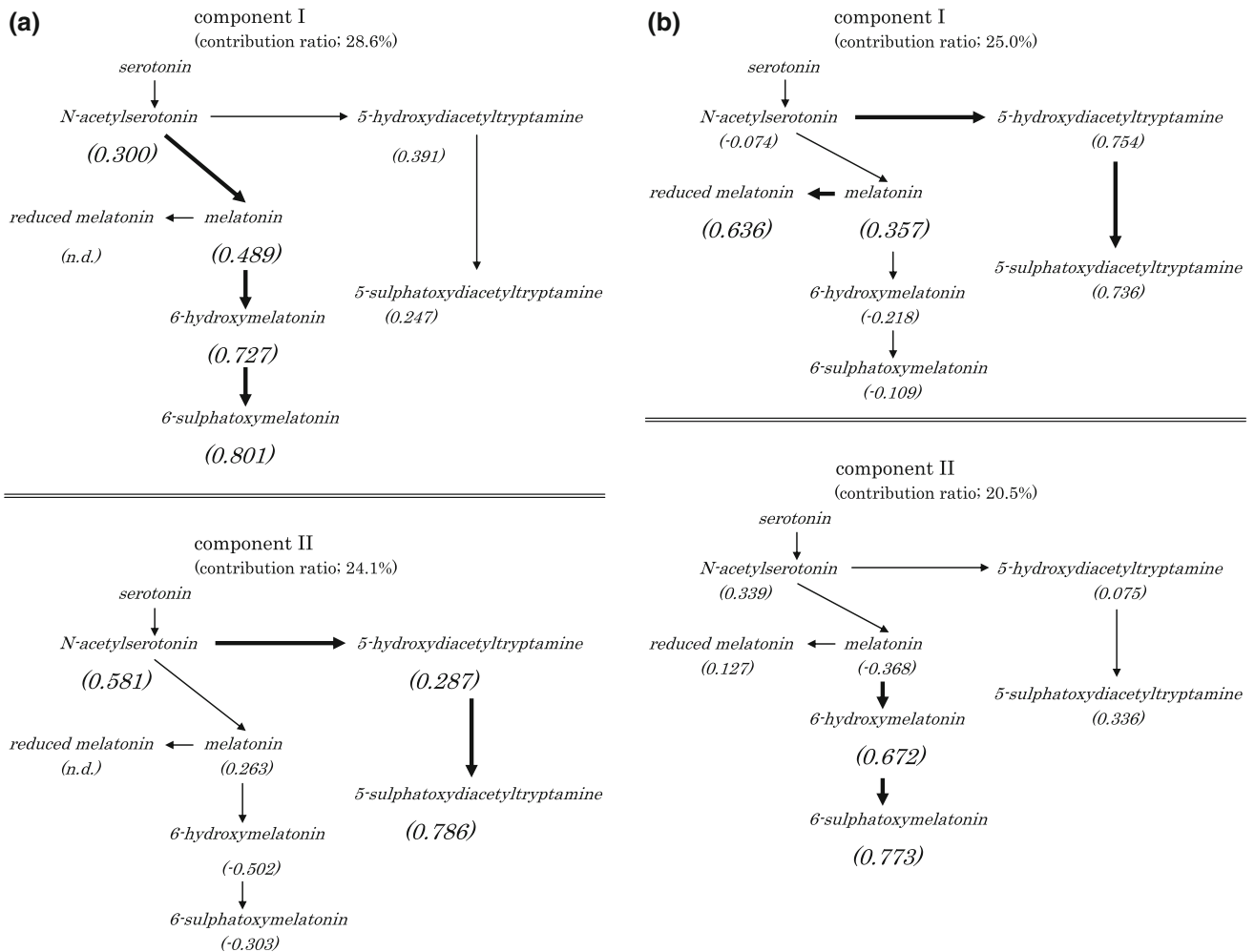
(a)

component I
(contribution ratio; 28.6%)

serotonin

↓

N-acetylserotonin ——————→ *5-hydroxydiacetyltryptamine*

(0.300)   *(0.391)*

reduced melatonin ← *melatonin*

(n.d.)   *(0.489)*

↓   *5-sulphatoxydiacetyltryptamine*

6-hydroxymelatonin   *(0.247)*

(0.727)

↓

6-sulphatoxymelatonin

(0.801)

component II
(contribution ratio; 24.1%)

serotonin

↓

N-acetylserotonin ——————→ *5-hydroxydiacetyltryptamine*

(0.581)   *(0.287)*

reduced melatonin ← *melatonin*

(n.d.)   *(0.263)*

↓   *5-sulphatoxydiacetyltryptamine*

6-hydroxymelatonin   *(0.786)*

(-0.502)

↓

6-sulphatoxymelatonin

(-0.303)

(b)

component I
(contribution ratio; 25.0%)

serotonin

↓

N-acetylserotonin ——————→ *5-hydroxydiacetyltryptamine*

(-0.074)   *(0.754)*

reduced melatonin ← *melatonin*

(0.636)   *(0.357)*   *5-sulphatoxydiacetyltryptamine*

↓   *(0.736)*

6-hydroxymelatonin

(-0.218)

↓

6-sulphatoxymelatonin

(-0.109)

component II
(contribution ratio; 20.5%)

serotonin

↓

N-acetylserotonin ——————→ *5-hydroxydiacetyltryptamine*

(0.339)   *(0.075)*

reduced melatonin ← *melatonin*

(0.127)   *(-0.368)*   *5-sulphatoxydiacetyltryptamine*

↓   *(0.336)*

6-hydroxymelatonin

(0.672)

↓

6-sulphatoxymelatonin

(0.773)

Fig. 5 Results of the principal component analysis (PCA). Each component was calculated using a matrix consisting of standardized correlation coefficients for the data classified according to sampling time; **a** data obtained during the sleep period and **b** data obtained between 9:00 am and the time immediately before going to bed. The correlation coefficients for the components appear to be closely related to the metabolic flow of the compounds; for further details see text. The presumed metabolic flow of tryptamine-related compounds is shown in Fig. 4. The calculated variance and the correlation coefficient are given according to each component and in each time section, shown in **a** and **b** along with the metabolic maps. The *number under the name of the compound* is the correlation coefficient of the compound with the indicated component at the time of sampling. The contribution of a component to the total variance is expressed as a percentage *under the name of each component*. The correlation coefficients could be indicative of the relationship of the compound to the component in the metabolic pathway. The metabolism seemed to differ at the various times when the samples were obtained. *n.d.* means not detectable

The results of our PCA suggested differential control of indolamine metabolism according to the time of day. During the sleep period, melatonin metabolism proceeds as *N*-acetylserotonin → melatonin → hydroxymelatonin. However, when considering melatonin with both components {1} and {2} during the day, the metabolism of melatonin proceeded to red mel (component {1}) rather than proceeding to the metabolic route from hydroxymelatonin to sulphatoxymelatonin (negative correlation coefficient of melatonin with component {2}). The metabolism of melatonin proceeds to red mel, suggesting a novel metabolic pathway; melatonin to red mel during the day.

In the daytime, the correlation coefficient of *N*-acetylserotonin for component {1} (−0.074) was neither statistically significant nor high. This finding can be explained by the increased demand for *N*-acetylserotonin for the synthesis of OH-diatry and s-diatry in the daytime, when the level of OH-diatry was 153 ± 256 µg/mg cr and that of s-diatry was 281 ± 319 µg/mg cr. The respective levels at night were 71.8 ± 63.8 µg/mg cr and 34.5 ± 27.1 µg/mg-cr; the latter two levels at night were significantly lower than the former two in the daytime (both, $p < 0.001$). The level of *N*-acetylserotonin was significantly lower during the daytime (7.83 ± 24.7 ng/mg cr) than at night (100 ± 91.2 ng/mg cr, $p < 0.001$). This phenomenon supports the hypothesis that the metabolic demand for *N*-acetylserotonin required to synthesize OH-diatry and s-diatry is lower at night than in the daytime. The supply of

(a)

subjective symptoms (points)

performance test (millisecond)

not administered the test

A : immediately after rising
B : about noon
C : immediately before going to bed

(b)

subjective symptoms (points)

p<0.001

p<0.05

performance test (millisecond)

I : sleeping 8hrs : for the first 2 days
II : sleeping 5hrs : for the second 5 days
III : sleeping 5hrs : for the third 5 days
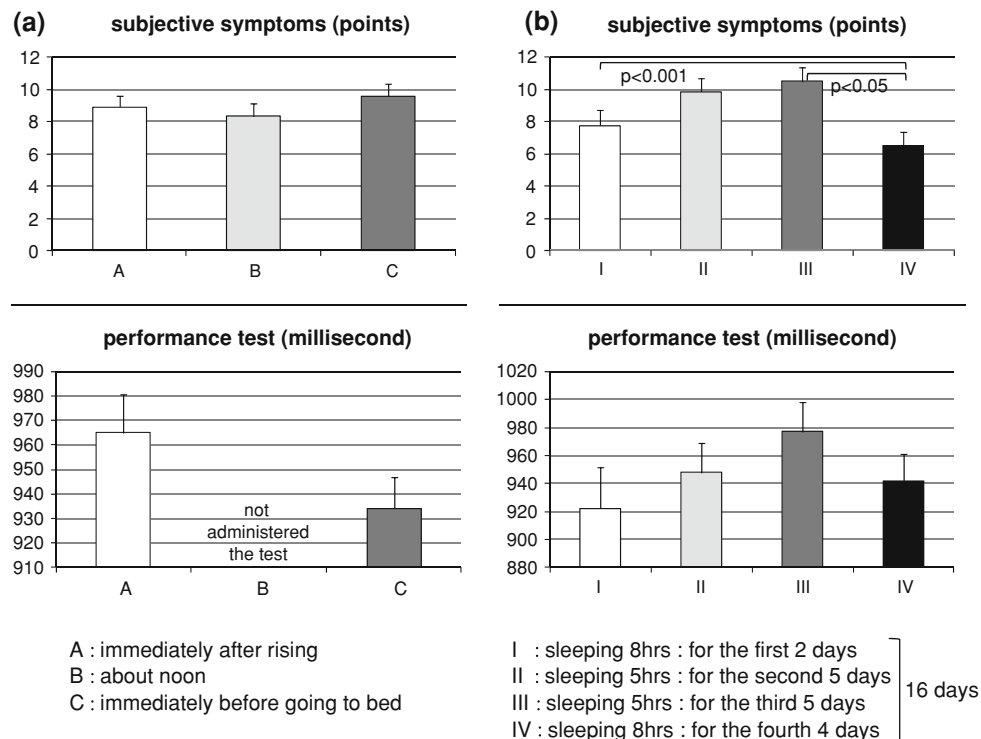IV : sleeping 8hrs : for the fourth 4 days
16 days

Fig. 6 Symptoms of fatigue and the results of performance test (psychomotor vigilance task; PVT). **a** The data were classified according to the time of the test. *Each column and bar* indicate the mean and standard error; *A* immediately after rising ($N = 128$, for the subjective symptoms and the performance test), *B* approximately noon ($N = 128$ for the subjective symptoms, no performance test was administered at this time), *C* immediately before going to bed ($N = 384$ for the subjective symptoms; $N = 256$ for the performance test). **b** The data were classified according to the duration of the sleep period. Each *number* indicates the mean and standard error. *I* indicates the first 2 days with 8 h of sleep ($N = 48$ for subjective symptoms; $N = 36$ for the performance test), *II* the next 5 days with 5 h of sleep ($N = 120$ for subjective symptoms; $N = 80$ for the performance test), *III* the next 5 days with 5 h of sleep ($N = 120$ for subjective symptoms; $N = 80$ for the performance test), and *IV* the last 4 days with 8 h of sleep ($N = 96$ for subjective symptoms; $N = 64$ for the performance test). Statistically significant differences in means are shown ($p < 0.05$ and $p < 0.001$)

N-acetylserotonin required for the syntheses of OH-diatry and s-diatry is sufficient at night, but not during the daytime. Thus, the correlation coefficient of N-acetylserotonin for component {2} at night was statistically significant and high (0.581). However, during the day, the demand increases for N-acetylserotonin. The supply of N-acetylserotonin seems to be a rate-limiting step for the synthesis of OH-diatry and s-diatry. The correlation coefficient of N-acetylserotonin was exceedingly low during the day due to increased demand. These results suggest another novel metabolic pathway: N-acetylserotonin → OH-diatry → s-diatry.

As expected, the levels of fatigue-related subjective symptoms during the control period (terms I and IV) were significantly lower than those in the stress-exposed period (terms II and III).

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