nia and all symptoms of insomnia among both junior and senior high school students. It is known that although alcohol promotes sleepiness immediately after its consumption, its hypnotic effect persists for only a short time, and that it disturbs the later part of sleep at night.[35] Furthermore, Wetter et al. in an epidemiological study that targeted 3,516 adults reported an association between smoking and sleep disturbance.[36] They inferred that sleep disturbances were due to the stimulant effects of nicotine, followed by withdrawal of nicotine at night, although they stated that a specific causal relationship was not proven. Such pharmacodynamics of alcohol or nicotine may affect sleep patterns among Japanese adolescents.

Associations between various sleep disorders and skipping breakfast have been reported among Japanese adolescents.[8,37] Arakawa et al. in a study of 3,754 junior high school students indicated that the percentage of students who skipped breakfast was significantly high among students who went to sleep at 00:00 or later.[37] They also warned against the increase in active night life culture in Japanese society. In a previous study, we observed associations between: (1) DIS, short sleep duration, subjective sleep insufficiency, and excessive daytime sleepiness; and (2) skipping breakfast.[8] In the present study, an association was newly recognized between insomnia and skipping breakfast. These findings indicate the need for future health education regarding eating habits of adolescents in Japan.

Among senior high school students who intended to study at university, we observed a significantly decreased AOR for insomnia. It is suggested that senior high school students who study for university entrance examinations rarely suffer from insomnia. Participating in extracurricular activities significantly decreased the AORs for insomnia among both junior and senior high school students. The risk of insomnia onset was high among those who did not intend to enter university, did not participate in extracurricular activities, or both. In the future, measures should be taken to improve sleep patterns in these groups.

In the present study, AORs for DIS and insomnia were significantly higher among those whose bedtime was after 00:00. As this study was a cross-sectional survey, a causal relationship cannot be discussed. However, it can be assumed that DIS leads to late bedtime. Among previous studies on sleep among adolescents, some included insomnia symptoms and bedtime as survey items,[5,7] but none of them scrutinized possible associations between each insomnia symptom and bedtime. In the present study, associations between each insomnia symptom and bedtime were clarified, by entering bedtime as a covariate in the logistic model. This method of analysis also helped us to distinguish delayed sleep phase syndrome from insomnia when we translated the results of the study. We expect that this method of analysis will be used in future epidemiological studies on sleep.

There were some limitations in our study. First, since this was a cross-sectional survey, a causal relationship could not be determined. When examining a causal relationship, a longitudinal study such as a cohort study is required, and such a study will be required in the future. Second, physiologic measurements such as electroencephalography could not be employed to obtain objective data for evaluation of sleep habits; such measurements, although desirable, are not normally included in epidemiological studies because such studies involve many subjects. Furthermore, several reports have stated that self-reported data on sleep status were consistent with the physiologic data to a certain degree.[38,39] Third, in the present study, questions concerning underlying sleep disorders were not posed in the questionnaire. Because insomnia symptoms may be caused by underlying sleep disorders, questions concerning underlying sleep disorders must be posed in the questionnaires of future studies. Fourth, the questions included in our questionnaire did not include all the factors that might affect sleep. For example, noise levels at night, the person/s with whom a subject sleeps, and commuting time to school are factors that could affect a subject's sleep. However, we could not include them in the questionnaire because of space limitations. These items must be examined in future. Fifth, there may have been a nonresponse bias. The rate of response to the questionnaire in this study was 64.8%; therefore, approximately 35% of the subjects did not participate in the survey. In Japan, people below 20 years of age are prohibited by law from smoking and drinking alcohol. Therefore, schools and individual students tend to be noncooperative in responding to a survey that includes questions on smoking and drinking alcohol. This may be the main reason for the nonresponsiveness. However, there is a possibility that the effect of the non-response bias on the results of our analysis is present.

In conclusion, this study revealed that the prevalence of insomnia among Japanese adolescents was similar to that among the general adult population of Japan. However, the prevalence of DIS, in comparison with the other symptoms of insomnia, was conspicuously high. The positive factors associated with insomnia among the junior high school students were male sex, poor mental health, skipping breakfast, drinking alcohol, smoking, and not participating in extracurricular activities. Positive factors in the senior high school students, besides the above-mentioned factors, was having no intention to enter university. The results of this study suggest that education on sleep hygiene must be promoted among Japanese adolescents.

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ABBREVIATIONS

CI, confidence interval
DIS, difficulty initiating sleep
DMS, difficulty maintaining sleep
EMA, early morning awakening
AOR, adjusted odds ratio

The Relationship Between Depression and Sleep Disturbances: A Japanese Nationwide General Population Survey

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Objective: Among the existing epidemiologic studies that have examined the relationship between depression and sleep disturbances, there are few nationwide studies that have been conducted on subjects representing the general population. The present study was therefore conducted to clarify the relationship between depression and sleep disturbances, in particular the relationship between depression and both sleep duration and subjective sleep sufficiency, using a large sample representative of the general population.

Method: The survey was conducted in June 2000, using self-administered questionnaires, targeting a population that was selected randomly from among 300 communities throughout Japan. Among the respondents, data from 24,686 individuals aged 20 years or older were analyzed. The Center for Epidemiologic Studies Depression Scale was used to assess the presence of depression. Sleep status, including sleep duration, subjective sleep sufficiency, and the presence or absence of insomnia symptoms, was evaluated.

Results: Those whose sleep duration was less than 6 hours and those whose sleep duration was 8 hours or more tended to be more depressed than those whose sleep duration was between 6 and 8 hours. Thus, sleep duration exhibited a U-shaped association with symptoms of depression. As subjective sleep sufficiency decreased, symptoms of depression increased, indicating a linear inverse-proportional relationship.

Conclusion: The fact that sleep duration and subjective sleep sufficiency exhibited different relationships with symptoms of depression indicates that these 2 sleep parameters each have their own significance with regard to depression. These findings may be useful in the medical management of mental diseases.

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Both depression and sleep disturbances are mental disorders that can be observed often in today's developed countries. Symptoms of various sleep disturbances are often recognized among people suffering from depression and are included in the *International Statistical Classification of Disease and Related Health Problems*, Tenth Revision,[1] and the American Psychiatric Association's *Diagnostic and Statistical Manual of Mental Disorders*, Fourth Edition,[2] as diagnostic criteria for depression. Historically, it has been assumed that depression causes sleep disturbances. However, sleep disturbances may also affect depression, and several studies have provided data suggesting that sleep disturbances are risk factors for the development of depression.[3–7] Treatment for depression usually improves sleep significantly. Conversely, when the sleep disturbance predominates, treatment for it may improve the management of depression.[8] Thus, it can be said that depression and sleep disturbances can mutually become each other's cause and result. Therefore, it is important to clarify the close relationship between these 2 conditions for their prevention and effective management.

From a physiologic perspective, studies on the relationship between depression and sleep disturbances using polysomnography have shown that symptoms such as prolonged sleep latency, increased wakefulness during sleep, and early-morning awakening can be associated with depression.[9] There is also evidence for a relationship between depression and sleep disturbances from an epidemiologic perspective. Ford and Kamerow[3] found that subjects who complained of persistent insomnia were 3 times more likely to develop depression than those without persistent insomnia. Livingston et al.[4] reported that the strongest predictor of future depression among those who were not depressed at baseline was sleep disturbance at baseline, and Breslau et al.[5] reported that the relative risk for the onset of major depression was 4 times greater for subjects with a lifetime history of 2 or more weeks of insomnia than for those without such a complaint.

These studies are significant in that they indicate, through longitudinal observation of the subjects, that sleep disturbances could be a risk factor for the onset of depression. However, these epidemiologic studies have some limitations. First, few studies have analyzed sleep disturbances from diverse perspectives, for example by including items such as sleep duration and subjective sleep sufficiency. Second, few studies have selected subjects that are representative of the general adult population; in most studies, subjects were selected from a particular age group or community. Third, most studies were conducted with small sample sizes; there has been no large-scale survey targeting 10,000 or more individuals.

In our study, an epidemiologic survey targeting over 24,000 subjects representative of the general adult population of Japan was conducted to search for symptoms of depression and to examine the association between those symptoms of depression and sleep disturbances. The purposes of our study were to clarify the prevalence of symptoms of depression and sleep disturbances and to analyze closely the association between symptoms of depression and sleep disturbances (in particular, the associations between symptoms of depression and sleep duration, subjective sleep sufficiency, and insomnia symptoms were to be clarified).

METHOD

Selection of Subjects

The present study was part of a national survey (Active Survey of Health and Welfare) organized by the Statistics and Information Department of the Ministry of Health, Labour and Welfare of Japan. This national survey was planned to collect basic information on health and welfare and included questions concerning symptoms of depression and sleep. The survey was conducted through health centers across Japan.

This survey was conducted in subjects from 300 census precincts in Japan selected randomly from among some 824,000 precincts, which had been apportioned for equal population size. Each census precinct was numbered from north to south, and 300 precincts were selected by choosing precinct numbers at certain intervals. As a result, the sample represented the entire country. A health center with jurisdiction for each precinct was designated. Investigators sent by those health centers visited all the households to distribute the questionnaires and collected them a few days later. The survey targets were all those aged 12 years or older in the 300 sampled precincts. The survey was conducted simultaneously throughout Japan in June 2000. Oral informed consent was obtained from subjects. Participants' privacy was protected in accordance with Declaration of Helsinki guidelines.

Measures

A self-administered questionnaire was devised by 2 of the authors (T.O. and M.U.) with the appropriate official of the Ministry of Health, Labour and Welfare. This questionnaire consisted of 44 items, including items on (1) sociodemographic information such as age, gender, and size of the community; (2) general health status; (3) physical and psychological complaints; (4) information on mental stress; and (5) sleep habits and sleep problems as well as (6) the Japanese version of the Center for Epidemiologic Studies Depression Scale (CES-D).[10]

The CES-D, which is a 20-item inventory designed specifically to assess symptoms of depression in the general population, was used to screen for current depressive states during the period of 1 week leading up to the survey. This questionnaire is adequately reliable and valid for use in a general population. The CES-D yields an item score (range, 0–3) and the sum of the 20-item scores (range, 0–60). Higher scores indicate increasing severity of symptoms of depression. Although this scale is designed to screen, but not diagnose major depression, a score of 16 or higher is highly suggestive of symptoms of depression. In addition, a score of 25 or higher is assumed to be a severer cutoff point in several studies.[11,12] Shima et al.[13] developed the Japanese version of the CES-D, examined its reliability and validity, and recommended the cutoff point to be set at 16, as with the U.S. version of the CES-D.

The following 6 questions about sleep experienced during the previous month (listed here followed by the variables that they targeted) were embedded in the questionnaire:

1. "Do you have difficulty falling asleep?" (yes/no): difficulty initiating sleep (DIS).
2. "Do you wake up during the night after you have gone to sleep?" (yes/no): difficulty maintaining sleep (DMS).

Table 1. Demographic Characteristics of Analyzed Subjects in a Sample of the Japanese Adult General Population (N = 24,686)[a]

Data Set	Percentage in Age Group					
	20–29 y	30–39 y	40–49 y	50–59 y	60–69 y	70+ y
Present study						
Male (N = 11,752)	18	18	19	21	15	9
Female (N = 12,934)	18	18	18	20	14	12
Census						
Male	19	18	17	20	15	12
Female	17	16	16	19	15	17

[a]Data for both the present study and the overall census were obtained in 2000.

3. "Do you wake up too early in the morning?" (yes/no): early morning awakening (EMA).
4. "Do you fall asleep when you must not sleep (for example when you are driving a car)?" (yes/no): excessive daytime sleepiness (EDS).
5. "Do you obtain sufficient rest during sleep?" (very sufficient/sufficient/insufficient/very insufficient): subjective sleep sufficiency.
6. "How many hours do you sleep on average?": sleep duration.

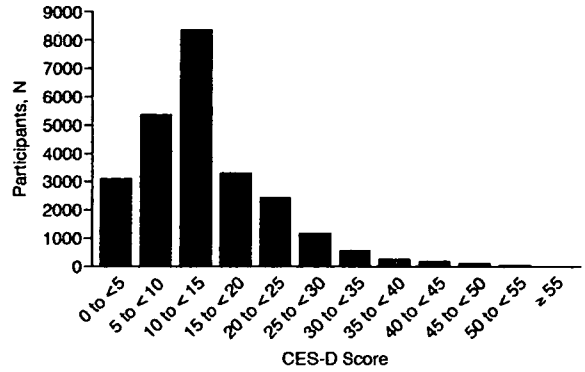
Experience of physical and psychological complaints during the previous month was indicated by an answer of yes or no. These complaints included headache, dizziness, palpitation/dyspnea, epigastric discomfort, constipation/diarrhea, stiff neck/shoulder, backache, easy fatigability, persistent fatigue, irritability, anxiety, and worrying about health.

Statistical Analysis

Questionnaires were returned by 32,729 subjects. The Ministry of Health, Labour and Welfare did not publish the number of residents contacted in the target communities, and so the return rate could not be calculated. However, the collection rates of similar investigations carried out 3, 4, and 6 years prior were 87.1%, 89.6%, and 87.3%, respectively. It can be assumed that since the present study was performed using similar methods, the collection rate is likely to have been similar to those observed previously. The Minister of Health, Labour and Welfare gave us permission to use these survey data. Before analysis, 707 subjects who submitted blank answer forms were excluded from the study. Subjects under 20 years of age (N = 3086) were also excluded, because this study was aimed at adults. In addition, subjects who had not responded to the questions on gender and/or age were also excluded (N = 222). Furthermore, subjects who had omitted 6 or more answers on the CES-D were excluded from the analysis (N = 4028).

For the statistical analysis, the CES-D scores were first calculated. To examine the association between sleep and

Figure 1. Distribution of Center for Epidemiologic Studies Depression Scale (CES-D) Scores in a Sample of the Japanese Adult General Population (N = 24,686)[a]



[a]Mode peaking in the distribution was observed for scores of 10 or higher and below 15.

CES-D scores, we calculated CES-D scores based on responses to the remaining 19 questions after excluding 1 sleep question from the CES-D questionnaire. In addition, because some subjects may have omitted 5 or fewer answers on the CES-D questionnaire, we adjusted for CES-D scores using the following formula, to correct them as a conventional scale of 0 to 60: "CES-D score" = "sum of 19 item scores" × "20/19" × "19/number of answered questions." The prevalence of those having a CES-D score of 16 or higher and of 25 or higher and the mean value and standard deviation of the CES-D scores were then calculated according to gender and age group. Similarly, the associations between symptoms of depression and sleep duration and subjective sleep sufficiency, as well as with sleep disturbances such as DIS, DMS, EMA, and EDS, were examined. The significance of the categorical data, such as the prevalence of symptoms of depression, was analyzed using the χ^2 test, and the significance of the raw data of the CES-D was calculated using the Kruskal-Wallis test. Finally, logistic regression analyses were conducted to investigate factors associated with CES-D scores of 16 or higher and of 25 or higher. The following parameters were used as covariates: gender, age group, community size, physical and psychological complaints, sleep duration, subjective sleep sufficiency, DIS, DMS, EMA, and EDS. An odds ratio was calculated from both the univariate analysis and the multivariate logistic regression analysis with 95% confidence intervals. All analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, Ill.).

RESULTS

The total number of cases analyzed was 24,686 (11,752 men, 12,934 women). The demographic characteristics of the analyzed participants are shown in Table 1.

Table 2. Prevalence of Depressive Symptoms and Mean Center for Epidemiologic Studies Depression Scale (CES-D) Score by Gender and Age Group

Age Group, y	N	Prevalence of Depressive Symptoms, % (95% CI) CES-D Score ≥ 16	CES-D Score ≥ 25	Mean ± SD CES-D Score
Male				
20–29	2,151	28.6 (26.7 to 30.5)	8.9 (7.7 to 10.1)	13.4 ± 8.3
30–39	2,157	23.2 (21.4 to 25.0)	6.2 (5.2 to 7.2)	12.3 ± 7.9
40–49	2,251	26.7 (24.9 to 28.5)	8.2 (7.1 to 9.3)	13.2 ± 8.0
50–59	2,468	24.4 (22.7 to 26.1)	6.6 (5.6 to 7.6)	12.9 ± 7.5
60–69	1,712	23.5 (21.5 to 25.5)	8.1 (6.8 to 9.4)	13.1 ± 8.0
70+	1,013	32.3 (29.4 to 35.2)	14.1 (12.0 to 16.2)	14.6 ± 9.1
Total	11,752	25.9 (25.1 to 26.7)	8.1 (7.6 to 8.6)	13.1 ± 8.0
Female				
20–29	2,329	31.3 (29.4 to 33.2)	11.2 (9.9 to 12.5)	14.0 ± 8.7
30–39	2,362	29.2 (27.4 to 31.0)	9.1 (7.9 to 10.3)	13.2 ± 8.2
40–49	2,368	29.1 (27.3 to 30.9)	9.5 (8.3 to 10.7)	13.7 ± 8.4
50–59	2,592	28.7 (27.0 to 30.4)	8.7 (7.6 to 9.8)	13.6 ± 7.8
60–69	1,766	26.0 (24.0 to 28.0)	7.0 (5.8 to 8.2)	13.2 ± 7.7
70+	1,517	38.6 (36.2 to 41.0)	16.7 (14.8 to 18.6)	16.0 ± 9.8
Total	12,934	30.1 (29.3 to 30.9)	10.1 (9.6 to 10.6)	13.8 ± 8.4
Overall	24,686	28.1 (27.5 to 28.7)	9.1 (8.7 to 9.5)	13.5 ± 8.3

Abbreviation: CI = confidence interval.

Although the percentages of both men and women aged 70 years or older were slightly less than those revealed by the census, the percentages of other age groups were similar.

The distribution of the CES-D scores of the analyzed participants is shown in Figure 1. Mode peaking in the distribution was observed for scores of 10 or higher and below 15.

The prevalence of those having a CES-D score of 16 or higher and of 25 or higher and the mean value and standard deviation of the CES-D scores sorted by gender and by age group are shown in Table 2. The prevalence of depressive symptoms and the mean CES-D scores among women were significantly higher than those among men ($p < .01$ for both). By age group, both the prevalence of depressive symptoms and the mean CES-D scores among those aged 70 years or more showed the highest values.

The associations between (1) CES-D score and (2) sleep duration, subjective sleep sufficiency, and insomnia symptoms are shown in Table 3. As the sleep duration increased in 1-hour increments from 5 to 8 hours, there was a reduction in the prevalence of those having CES-D scores of 16 or higher and 25 or higher and in the mean value of the CES-D scores. However, after sleep duration exceeded 8 hours, the prevalence of those having CES-D scores of 16 or higher and 25 or higher and the mean value of the CES-D scores increased. The association between sleep duration and the mean value of the CES-D scores sorted by age group is shown in Figure 2. For all age groups, a U-shaped association was observed between sleep duration and CES-D score. A U-shaped association was observed in the analysis separated by gender (data not shown). As subjective sleep sufficiency decreased, the prevalence of those having CES-D scores of 16 or higher and 25 or higher and the mean value of the CES-D scores increased in inverse proportion. The association between subjective sleep sufficiency and the mean value of the CES-D scores sorted by age group is shown in Figure 3. For all age groups, a linear and unidirectional association was observed between subjective sleep sufficiency and CES-D score. A linear and unidirectional association was observed in the analysis separated by gender (data not shown). Among those having insomnia symptoms such as DIS, DMS, EMA and EDS, the prevalence of those having CES-D scores of 16 or higher and 25 or higher and the mean value of the CES-D scores were significantly higher than for those not having these insomnia symptoms.

The results of the logistic regression analyses, which were conducted to investigate factors associated with CES-D scores of 16 or higher and of 25 or higher, are shown in Table 4. In the multivariate logistic regression model, sleep duration, subjective sleep sufficiency, DIS, DMS, EMA, and EDS showed significant associations with symptoms of depression. The multivariate logistic regression model also revealed a U-shaped association between sleep duration and symptoms of depression; the odds ratio bottomed out at between 6 and 7 hours of sleep and became higher as sleep duration became either shorter or longer. The multivariate logistic regression model also revealed a unidirectional association between subjective sleep sufficiency and symptoms of depression.

DISCUSSION

The prevalence of those having CES-D scores of 16 or higher and 25 or higher among the general adult population in Japan was 28.1% and 9.1%, respectively. Although a simple comparison should be avoided because the age

Table 3. Prevalence of Depressive Symptoms and Mean Center for Epidemiologic Studies Depression Scale (CES-D) Score by Sleep Duration and Sleep Problems (N = 24,686)

Variable	N (%)	Prevalence of Depressive Symptoms: CES-D Score ≥ 16, % (95% CI)	p Value*	CES-D Score ≥ 25, % (95% CI)	p Value*	Mean ± SD CES-D Score	p Value†
Sleep duration, h[a]			< .01		< .01		< .01
< 5	608 (2.6)	47.9 (43.9 to 51.9)		23.8 (20.4 to 27.2)		18.1 ± 10.8	
5 to < 6	2,672 (11.3)	35.8 (34.0 to 37.6)		12.4 (11.2 to 13.6)		14.8 ± 8.8	
6 to < 7	7,452 (31.5)	27.3 (26.3 to 28.3)		8.1 (7.5 to 8.7)		13.2 ± 8.0	
7 to < 8	7,239 (30.6)	23.5 (22.5 to 24.5)		6.7 (6.1 to 7.3)		12.5 ± 7.5	
8 to < 9	4,442 (18.8)	25.5 (24.2 to 26.8)		7.9 (7.1 to 8.7)		13.1 ± 8.1	
9 to < 10	711 (3.0)	32.1 (28.7 to 35.5)		11.5 (9.2 to 13.8)		14.1 ± 8.6	
≥ 10	562 (2.4)	50.2 (46.1 to 54.3)		27.2 (23.5 to 30.9)		19.4 ± 11.3	
Subjective sleep sufficiency[b]			< .01		< .01		< .01
Very sufficient	4,449 (18.7)	14.5 (13.5 to 15.5)		3.7 (3.1 to 4.3)		10.7 ± 7.1	
Sufficient	11,204 (47.1)	23.2 (22.4 to 24.0)		6.2 (5.8 to 6.6)		12.5 ± 7.3	
Insufficient	6,988 (29.4)	39.5 (38.4 to 40.6)		13.5 (12.7 to 14.3)		15.6 ± 8.7	
Very insufficient	1,145 (4.8)	56.9 (54.0 to 59.8)		29.8 (27.2 to 32.4)		20.3 ± 11.5	
Difficulty initiating sleep			< .01		< .01		< .01
No	20,372 (82.5)	24.0 (23.4 to 24.6)		7.0 (6.6 to 7.4)		12.7 ± 7.7	
Yes	4,314 (17.5)	47.4 (45.9 to 48.9)		19.5 (18.3 to 20.7)		17.5 ± 9.7	
Difficulty maintaining sleep			< .01		< .01		< .01
No	19,574 (79.3)	23.6 (23.0 to 24.2)		6.8 (6.4 to 7.2)		12.6 ± 7.7	
Yes	5,112 (20.7)	45.4 (44.0 to 46.8)		18.1 (17.0 to 19.2)		17.1 ± 9.4	
Early morning awakening			< .01		< .01		< .01
No	19,061 (77.2)	25.6 (25.0 to 26.2)		8.0 (7.6 to 8.4)		12.9 ± 8.0	
Yes	5,625 (22.8)	36.7 (35.4 to 38.0)		13.0 (12.1 to 13.9)		15.4 ± 8.9	
Excessive daytime sleepiness			< .01		< .01		< .01
No	24,011 (97.3)	27.6 (27.0 to 28.2)		8.8 (8.4 to 9.2)		13.4 ± 8.2	
Yes	675 (2.7)	45.3 (41.5 to 49.1)		21.5 (18.4 to 24.6)		17.4 ± 10.0	

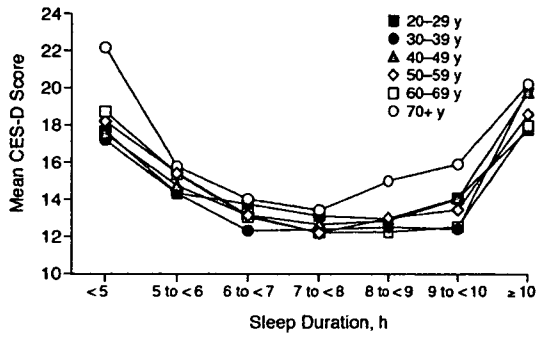
[a]Total N = 23,686. Subjects with missing data were excluded from the analysis.
[b]Total N = 23,786. Subjects with missing data were excluded from the analysis.
*χ^2 test.
†Kruskal-Wallis test.
Abbreviation: CI = confidence interval.

compositions of subject groups are different, the prevalence of those having CES-D scores of 16 or higher reported by most of the studies from Western countries[14–17] is 20% or less, which is lower than that revealed by the present study. A study in Korea[12] targeting subjects aged 20 to 59 years reported a prevalence of 25.3% and 8.7% for those having CES-D scores of 16 or higher and 25 or higher, respectively, which is similar to our results.

The higher prevalence of symptoms of depression rated by the CES-D in our study compared with those reported in other countries may be in part attributable to the fact that subjects aged 70 years or older accounted for about 10% of the subjects in our study, which targeted all adult age groups; that percentage was smaller in the other studies. The prevalence of those having a CES-D score of 16 or higher among the subjects aged 70 years or older in our study was 32.3% for men and 38.6% for women, which was the highest among any of the age groups. These figures pushed up the prevalence of symptoms of depression overall. It has already been reported that the prevalence of symptoms of depression among elderly people is higher,[18,19] and our results support this. Another reason may be the different attitude toward responding to CES-D questions due to differences in race and culture between Japan and Western countries. Iwata et al.[20] pointed out that CES-D scores were likely to be higher among Japanese than among Americans because the Japanese tended to suppress their positive emotional expression.

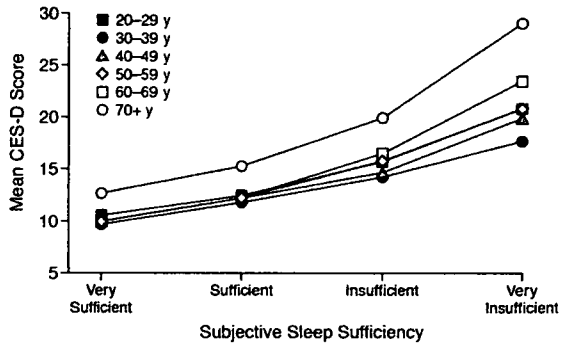
In our study, those getting less than 6 hours of sleep and those getting 8 hours or more were more depressed than those getting 6 to 8 hours of sleep, and a U-shaped association was thus observed between sleep duration and CES-D score. Chang et al.[6] reported, in their long-term prospective study, that those getting 7 hours of sleep or less were at higher risk of becoming depressed than those getting more than 7 hours of sleep. With regard to the association between long sleep duration and depression, Hartmann et al.[21] found in a laboratory study that people who always got more than 9 hours of sleep per night tended to be mildly depressed. Our results reconfirm the results of those previous studies, yet are independently important for the following reasons: (1) our sample size was large (over 24,000) and was representative of the general Japanese population, and (2) our study revealed a U-shaped association between sleep duration and symptoms of depression. This finding was made possible by our breaking down sleep duration into 1-hour increments between less than 5 hours and 10 hours or more. Although a U-shaped association between mortality and sleep dura-

Figure 2. Relationship Between Sleep Duration and Mean Center for Epidemiologic Studies Depression Scale (CES-D) Score by Age Group in a Sample of the Japanese Adult General Population (N = 24,686)[a]



[a]For all age groups, a U-shaped association was observed between sleep duration and CES-D score.

Figure 3. Relationship Between Subjective Sleep Sufficiency and Mean Center for Epidemiologic Studies Depression Scale (CES-D) Score by Age Group in a Sample of the Japanese Adult General Population (N = 24,686)[a]



[a]For all age groups, a linear and unidirectional association was observed between subjective sleep sufficiency and CES-D score.

tion has been reported,[22–26] our report is the first to reveal a U-shaped association between sleep duration and symptoms of depression.

It is easy to assume that people tend to become depressed as a result of short sleep duration or that depression causes people to have less sleep. But why do those who sleep more than 8 hours show a trend toward becoming depressed? This phenomenon may be affected by some confounding factors, such as certain diseases or physical handicaps. To clarify this, we entered 12 physical and psychological complaints included among the survey items as adjustment factors into the logistic model for analysis. However, these factors in themselves do not explain why those who sleep more than 8 hours tend to become depressed. Detre et al.[27] reported that hypersomnia was not an unusual symptom of depression. Therefore, a longer sleep duration may be one of the pathological features of depression. Unfortunately, the mechanism underlying the association between hypersomnia and depression could not be clarified in the present study, and further investigations will be required.

On the contrary, a linear inversely proportional association was observed between subjective sleep sufficiency and CES-D scores, and it was found that as subjective sleep sufficiency decreased, the prevalence of symptoms of depression increased. Although the prevalence of depression among those aged 70 years or older was extremely high, common patterns were observed in all age groups with regard to the relationship between symptoms of depression and both sleep duration and subjective sleep sufficiency. These common patterns were also observed when we performed a gender-stratified analysis. The results suggested a U-shaped association between sleep duration and symptoms of depression and a linear inversely proportional association between subjective sleep sufficiency and symptoms of depression, regardless of gender and age. In addition, it is interesting that different patterns of associations with symptoms of depression were observed for sleep duration and subjective sleep sufficiency. It may be that these 2 sleep parameters each have their own significance.

As to each insomnia symptom, subjects having DIS, DMS, EMA, or EDS were more depressed than subjects who were free of those symptoms. Our study indicated that, among these insomnia symptoms, DIS had the strongest association with symptoms of depression. So far, the associations with DMS and EMA have been more greatly emphasized than that with DIS,[2,28,29] but the present study revealed a contrary result. Sukegawa et al.[30] investigated associations between sleep disturbances and depression in a study targeting elderly Japanese people and reported that an association was indicated between depression and DIS, but that no association was indicated between depression and DMS or EMA. Difficulty initiating sleep may be a symptom of sleep disturbance that appears characteristically among Japanese people suffering from depression. This factor requires future investigation.

There are some limitations to our study. Firstly, as this study was a cross-sectional survey, a causal relationship could not be determined between symptoms of depression and sleep disturbances. However, it is known that symptoms of depression and sleep disturbances can mutually become each other's cause and effect.[31] The investigation of such a causal relationship was outside the scope of this study, and the study's main purpose, which was to clarify which patterns of sleep disturbance are associated with symptoms of depression, was achieved. Secondly, objective data (i.e., physiologic measurement such as electroencephalography) could not be used for the present evaluation of sleep disturbances. Although such measurement

Table 4. Multiple Logistic Regression Results for Prediction of Depression in a Sample of the Japanese Adult General Population (N = 24,686)[a]

	CES-D Score ≥ 16			CES-D Score ≥ 25		
Variable	Adjusted OR	95% CI	p Value	Adjusted OR	95% CI	p Value
Sex			.16			.17
Male	1.00			1.00		
Female	1.05	0.98 to 1.13		1.08	0.97 to 1.20	
Age group, y			< .01			< .01
20–29	1.56	1.40 to 1.74		1.70	1.43 to 2.01	
30–39	1.00			1.00		
40–49	1.20	1.08 to 1.34		1.33	1.12 to 1.58	
50–59	1.24	1.11 to 1.39		1.22	1.02 to 1.46	
60–69	1.11	0.98 to 1.26		1.22	1.00 to 1.50	
70+	1.59	1.39 to 1.83		2.51	2.04 to 3.08	
Size of community			.3			.06
City of ≥ 500,000 people	1.00			1.00		
City of 150,000 to < 500,000 people	0.96	0.87 to 1.06		0.88	0.76 to 1.02	
City of 50,000 to < 150,000 people	0.92	0.83 to 1.02		0.87	0.74 to 1.02	
City of < 50,000 people, suburban district	1.00	0.91 to 1.10		1.01	0.88 to 1.17	
Sleep problem						
Difficulty initiating sleep	1.56	1.44 to 1.70	< .01	1.60	1.43 to 1.79	< .01
Difficulty maintaining sleep	1.49	1.38 to 1.61	< .01	1.44	1.28 to 1.61	< .01
Early morning awakening	1.34	1.23 to 1.44	< .01	1.21	1.08 to 1.36	< .01
Excessive daytime sleepiness	1.22	1.01 to 1.47	.04	1.55	1.23 to 1.95	< .01
Sleep duration, h			< .01			< .01
< 5	1.25	1.02 to 1.54		1.47	1.14 to 1.90	
5 to < 6	1.06	0.95 to 1.19		1.07	0.91 to 1.26	
6 to < 7	1.00			1.00		
7 to < 8	1.08	0.99 to 1.18		1.14	0.99 to 1.32	
8 to < 9	1.36	1.23 to 1.51		1.48	1.26 to 1.75	
9 to < 10	1.98	1.63 to 2.41		2.34	1.75 to 3.13	
≥ 10	4.04	3.25 to 5.01		5.58	4.26 to 7.32	
Subjective sleep sufficiency			< .01			< .01
Very sufficient	0.64	0.57 to 0.71		0.58	0.47 to 0.70	
Sufficient	1.00			1.00		
Insufficient	1.53	1.41 to 1.66		1.64	1.45 to 1.86	
Very insufficient	2.07	1.76 to 2.43		2.96	2.43 to 3.61	

[a]Other adjustment factors included headache, dizziness, palpitation/dyspnea, epigastric discomfort, constipation/diarrhea, stiff neck/shoulder, backache, easy fatigability, persistent fatigue, irritability, anxiety, and worrying about health.
Abbreviations: CES-D = Center for Epidemiologic Studies Depression Scale, CI = confidence interval, OR = odds ratio.

is desirable, it would be very difficult to adopt these in community-based epidemiologic studies, particularly in which data are collected from a large number of randomly selected participants located throughout the country. However, some studies have reported that self-reported data on sleep status do concur, to a certain extent, with physiologic data.[32,33] Thirdly, the present study evaluated sleep habits and sleep disturbance only retrospectively. In future studies, to improve the accuracy of the research results, it would be advisable to perform a prospective evaluation, even on a portion of the subjects, with the aid of sleep diaries, actinography, or polysomnography. Fourthly, as a self-reported survey was adopted, the percentage of respondents aged 70 years or older was less than that of people aged 70 years or older in the general population as revealed by the census. It is assumed that physical difficulties of old age, such as poor eyesight and difficulty in writing, might impede elderly subjects from responding to our questionnaire. Further improvements, such as introducing an interviewing method, would be helpful in the future. Fifthly, adjustments for sampling weight or variance were not conducted in the statistical analyses of this study because necessary data had not been made available to us by the Ministry of Health, Labour and Welfare. However, the influence of not performing adjustments for sampling weight or variance was assumed to be negligible, since the survey precincts in this study were apportioned to be virtually identical in population.

In psychiatric studies, there are 2 methods for case ascertainment of depression: diagnostic methods and nondiagnostic methods.[34] The CES-D is a commonly used nondiagnostic tool. Although there are a few differences in the prevalence of symptoms of depression between that classified by the CES-D and that determined by diagnostic methods used in clinical treatment, the reliability and validity of the CES-D are widely recognized in epidemiologic studies targeting general populations. The relationship between symptoms of depression calculated from the CES-D scores and sleep disturbance can be useful for diagnosis and management of mental disorders. For example, this study suggests that for diagnosis of a patient

who exhibits symptoms of depression it is important to evaluate his or her sleep status multilaterally, from aspects such as sleep duration, subjective sleep sufficiency, and insomnia. Conversely, for diagnosis of a patient who complains of a sleep disorder, it is necessary to examine if he or she has symptoms of depression. Because sleep disturbances may appear in a patient before he or she meets the diagnostic criteria for depression,[35–37] it is always necessary to consider the possibility of depression for management of sleep disturbances. Our present findings do not make it possible to discuss the causal relationship between symptoms of depression and sleep disturbances, but they do suggest that they are closely related. It is suggested that symptoms of depression are milder when sleep duration is between 6 and 8 hours, as subjective sleep sufficiency is higher, or as symptoms of insomnia are milder. We hope that the results of this study will be widely utilized for clinical care in psychiatric medicine.

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Detection of Autoantibodies Against Hypocretin, hcrtr1, and hcrtr2 in Narcolepsy: Anti-Hcrt System Antibody in Narcolepsy

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Study Objectives: The impairment of hypocretin neurotransmission system is considered to play a major role in the pathophysiology of narcolepsy. It has been hypothesized that autoimmune abnormalities underlie the etiology of narcolepsy, based on the tight association with HLA-DRB1*1501/DQB1*0602. It remains unclear if autoantibodies against hypocretin receptors (hcrtr1 and hcrtr2) are involved in narcolepsy.

Design: We have developed a novel radioligand binding assay to address this question. Sera from 181 patients with narcolepsy, 10 patients with other hypersomnias, and 91 control subjects were used. Human [^{35}S]-Hcrt, hcrtr1, and hcrtr2 were synthesized by *in vitro* transcription/translation system. The immune complex of autoantibody and each [^{35}S]-protein were immunoprecipitated and quantified using a radioligand-binding assay.

Results: We detected autoantibodies against hypocretin in 3 patients, hcrtr1 in 1 patient, and hcrtr2 in 5 patients with narcolepsy. Positive reactions were also found against hcrtr1 in 2 and hcrtr2 in 1 control subjects. No relationships were found between these autoantibodies and HLA-DRB1*1501/DQB1*0602 haplotypes, presence of cataplexy, presence of subjective nocturnal sleep disruption, or the score on the Epworth Sleepiness Scale.

Conclusions: Although we have detected autoantibodies against the hypocretin neurotransmission system, our results do not support the hypothesis that autoantibody-mediated dysfunction in the hypocretin system underlies the pathophysiology of narcolepsy.

Keywords: Autoantibody, narcolepsy, hypocretin, orexin, hypocretin receptor, autoimmunity, sleep, radioligand assay

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INTRODUCTION

NARCOLEPSY IS A CHRONIC SLEEP DISORDER THAT AFFECTS BETWEEN 1 IN 1000 AND IN 2000 OF THE POPULATION WORLDWIDE AND APPROXIMATELY 1 in 600 of the Japanese population. This disorder is characterized by excessive daytime sleepiness and cataplexy (loss of muscle tone in response to emotional stimuli with consciousness preserved) and is frequently accompanied by other abnormal manifestations of rapid eye movement (REM) sleep, such as sleep paralysis and hypnagogic hallucinations (dream-like episodes at sleep onset). Based on several models, it has been reported that the hypocretin (Hcrt; also known as orexin) neuropeptidergic system is impaired in narcolepsy. Hcrt consists of 2 peptides (hcrt1 [also called orexin A], and hcrt2 [also called orexin B]) produced from the same precursor (preprohcrt) in neurons whose cell bodies are located in the perifornical lateral hypothalamus.[1] Canine models of autosomal recessive narcolepsy are caused by mutations of the Hcrt receptor 2 (hcrtr2) gene.[2] Murine models with the disruption of preprohcrt or Hcrt receptor gene expression exhibit narcolepsy-like behaviors.[3-5] Hcrt1 is usually undetectable in the cerebrospinal fluid (CSF) of narcoleptic patients with cataplexy.[6-9] Studies using *in situ* hybridization and immunohistochemistry have shown marked reductions in the number of Hcrt neurons in the hypothalamus of postmortem brains from patients with narcolepsy.[10,11]

Narcolepsy is generally nonfamilial and is not linked to mutations of the Hcrt system, except for 1 rare case.[10] Moreover, only 5 pairs out of 19 reported monozygotic twins are concordant for narcolepsy.[12,13] An immunological pathogenesis for narcolepsy has been suggested since the discovery of the tight association between narcolepsy and human leukocyte antigen HLA-DR2.[14] Genetic analysis has now narrowed the HLA susceptibility for narcolepsy to HLA-DQB1*0602.[15] Apart from the tight HLA association, the autoimmune hypothesis for narcolepsy is supported by the peripubertal onset of narcolepsy and the specific degeneration of Hcrt neurons in the lateral hypothalamus. The observation of gliosis-like features around Hcrt and hcrtr2 expressing neurons in the narcoleptic brain has been reported.[11,16] The Hcrt neurons appear to specifically degenerate in narcolepsy without any alteration in adjacent neurons, such as those expressing the peptide melanin-concentrating hormone.[10] Some patients with autoimmune encephalitis have shown symptoms of narcolepsy with cataplexy with reduction of CSF hcrt1 levels.[17] Immunoglobulin G in the CSF of HLA-DQB1*0602 positive narcoleptic subjects with cataplexy binds to rat hypothalamus protein extract.[18] Clinical improvement of cataplexy has been observed in narcoleptic patients after therapeutic intravenous infusion of high-dose normal-human immunoglobulin G.[19] More recently, injection of immunoglobulin obtained from patients with narcolepsy has been found to evoke behavioral arrests in mice,[20] but this remains to be confirmed. It is increasingly apparent that the blood-brain barrier does not always prevent autoantibodies shown in patients with anorexia and bulimia nervosa from reaching their targets in the hypothalamus.[21] Furthermore, hypothalamus, where Hcrt cell bodies are located, is highly vascular. Specific immunologic dysregulation against Hcrt neurotransmission might therefore be involved in the development of narcolepsy.

Recently, the screening of autoantibodies against Hcrt was con-

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ducted using sera and CSF obtained from narcoleptic patients.[22,23] No autoantibodies against Hcrt could be found, suggesting the possibility for the existence of autoantibodies against other components of Hcrt neurotransmission system, such as hcrtr1 and hcrtr2. We have developed a radioligand binding assay system to detect potential autoantibodies against human Hcrt receptors. We produced recombinant human [^{35}S]-Hcrt and Hcrt receptors by *in vitro* transcription/translation and used them as antigens. Using these recombinant proteins, we investigated the presence of autoantibodies against human hcrtr1 and hcrtr2 together with Hcrt autoantibodies in sera obtained from narcoleptic patients.

MATERIALS AND METHODS

Subjects

This research was approved by the ethics committee of all collaborative institutes. Written informed consents were obtained from all participants. All patients were diagnosed clinically, in combination with the Multiple Sleep Latency Test for narcolepsy without cataplexy, at the Neuropsychiatric Research Institute (Tokyo, Japan). Diagnosis was made according to the International Classification of Sleep Disorders, second edition.[24] Blood samples and data related to sleep conditions were collected at the Tokyo Institute of Psychiatry (Tokyo, Japan) and Neuropsychiatric Research Institute (Tokyo, Japan). Control subjects were excluded if they had excessive daytime sleepiness or any signs of immunologic abnormalities based on a questionnaire obtained at the time of blood collection. All participants were Japanese except for 1 Korean and 1 American (Caucasian) narcoleptic patients. Sera from 181 patients with narcolepsy, 10 patients with other hypersomnias, and 91 healthy control subjects were examined. Five milliliters of venous blood were drawn and separated—sera were stored at -80°C until use. The HLA typing for HLA-DRB1 and DQB1 loci were done for all the patients at NPO HLA Laboratory (Kyoto, Japan). Patients with narcolepsy consisted of 171 patients with definite cataplexy (all positive for HLA-DRB1*1501/DQB1*0602) and 10 patients without cataplexy (6 cases were HLA-DRB1*1501/DQB1*0602 positive) and were all unrelated. Patients with other hypersomnias consisted of 6 with idiopathic hypersomnias with long total sleep time and 4 cases of recurrent hypersomnia. We collected the following data to analyze potential relationships with the autoantibody index: age, sex, Epworth Sleepiness Scale[25] (ESS) at the time of blood collection, presence of nocturnal sleep disruption (subjective report), and past history of autoimmune disorders. The mean age and sex distribution are summarized in Table 1. The mean ages of patient groups were not significantly different from that of the healthy control subjects, except for the group of patients with narcolepsy without cataplexy.

Synthesis of Recombinant [^{35}S]-Hcrt, hcrtr1, and hcrtr2

The open-reading frames of Hcrt, hcrtr1, and hcrtr2 were obtained by reverse transcript-polymerase chain reaction (PCR) amplification using poly-A RNA obtained from the human hypothalamus and hippocampus (CLONTECH Laboratories, Inc., Palo Alto, CA). The first-strand cDNA was synthesized using ReverTraAce (TOYOBO, Tokyo, Japan) with random hexamers according to the manufacturer's instructions. The following primer pairs for HCRT with either an EcoR I or a Xho I site, 5'-GGAATTCatgaaccttccttccacaaaggt-3' and 5'-GGCGAGCTCtcagatcccggactgtcctcc-3' (the EcoR I and a Xho I sites are capitalized), was used for HCRT amplification. PCR was carried out using a high fidelity DNA polymerase, KOD-plus (TOYOBO, OSAKA, Japan) and Hcrt PCR product was ligated into the pET28a (+) expression vector (Novagen, Madison, WI) (HCRT/pET28a). [^{35}S]-methionine labeled Hcrt was produced using HCRT/pET28a, TNT Quick coupled Transcription/Translation System (Promega, Madison, Wisc), and [^{35}S]-methionine (Amersham Biotech, Arlington Heights, IL) according to the manufacturer's instructions. For hcrtr1 and hcrtr2, first PCR were performed using the following primer pairs, hcrtr1: 5'-atggagccctcagccaccccagg-3' and 5'-tcagggcagcactgtggtgac-3'; hcrtr2: 5'-atgtccggcaccaaattggagg-3' and 5'-ctaccagttttgaagtggtcc-3'. After the addition of 3' adenine overhangs by ExTaq (TAKARA, TOKYO, Japan), these PCR products were cloned into pGEM-T Easy vector (Promega). To add the T7 promotor, a second PCR was performed using KOD-plus DNA polymerase, cloned-vectors as templates, and the following primer pairs, hcrtr1:
5'-GGATCCTAATACGACTCACTATAGGGAGCCACCatggagccctcagccaccccagg-3' and
5'-tcagggcagcactgtggtgac-3'; hcrtr2:
5'-GGATCCTAATACGACTCACTATAGGGAGCCACCatgtccggcaccaaattggagg-3' and
5'-ctaccagttttgaagtggtcc-3' (The sequences of T7 promotor, spacer, and KOZAK translation initiation sequence are capitalized). [^{35}S]-methionine-labeled hcrtr1 and hcrtr2 were produced using second PCR products, TNT T7 Quick for PCR DNA (Promega), and [^{35}S]-methionine according to the manufacturer's instructions. Each mixture, including [^{35}S]-methionine-labeled protein, was applied to the NICK column (Amersham Biotech) to remove free [^{35}S]-methionine. SDS-PAGE analysis was carried out, and a single band corresponding to each protein (open reading frames) was found in the BAS-imaging system (data not shown). Each [^{35}S]-labeled human protein was adjusted to a 20,000 counts per minute (cpm) per 20 µL concentration by the reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% BSA, 0.1% Tween-20, and 0.1% NaN3, pH 7.4) and stored at -80°C until use.

Table 1—Age, Sex and Autoantibodies to Hcrt Neurotransmission Systems in Hypersomnia Patients and Healthy Control Subjects.

Subjects	Number examined	Age (years (mean ± SD)	Male/Female		Number of positive antibodies		
			raw number	Sex ratio	HCRT	HCRTR1	HCRTR2
Narcolepsy	181	45.2 ± 17.8	110/71	1.55	3	1	5
(With cataplexy)	(171)	(46.1 ± 18.0)	(104/67)	(1.63)	(3)	(1)	(4)
(Without cataplexy)	(10)	(27.8 ± 4.2)*+	(6/4)	(1.50)	(0)	(0)	(1)
Other hypersomnias	10	38.9 ± 18.1	2/8	0.25	0	0	0
Healthy control subject	91	42.9 ± 11.1	40/51	0.78	0	2	1

*The mean age was significantly different from healthy control subjects at $p < 0.05$. + The mean age was significantly different from narcoleptic patients with cataplexy at $p < 0.05$. HCRT: hypocretin; HCRTR1: HCRT receptor 1; HCRTR2: HCRT receptor 2

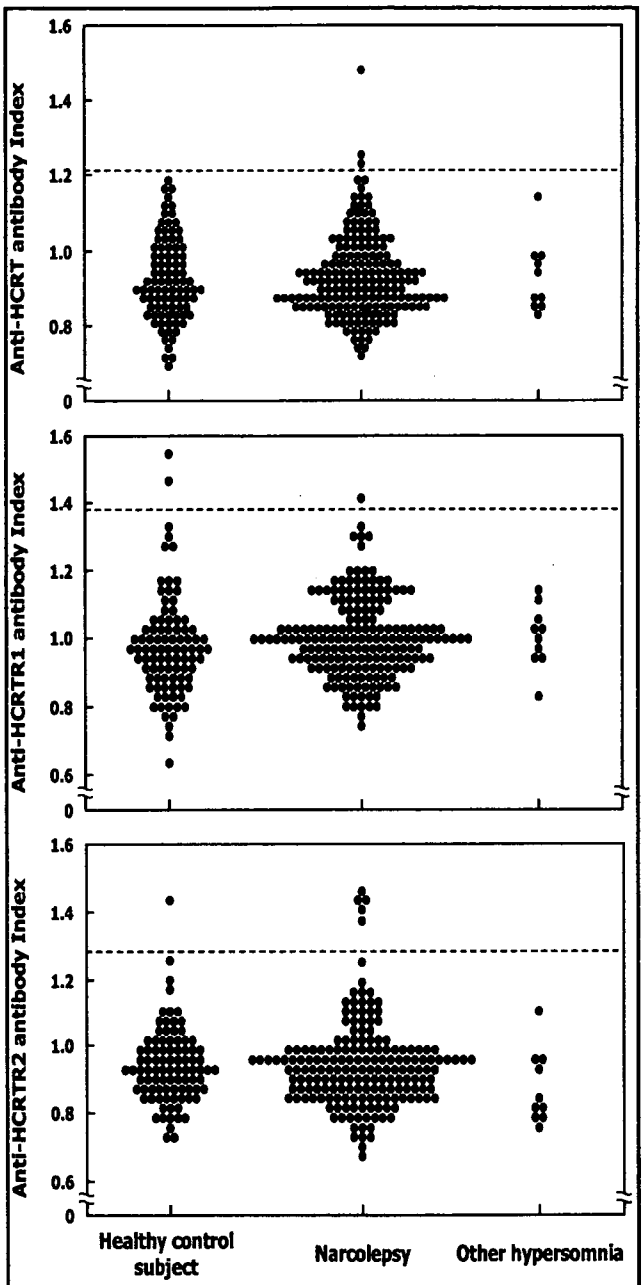

Figure 1—Index of antibodies to human hypocretin, hypocretin receptor 1, and hypocretin receptor 2 in patients with narcolepsy and other hypersomnias and healthy control subjects. Each dot corresponds to each person. The dotted line denotes the mean + 3 SD levels of healthy control subjects. Indexes above this are considered positive for autoantibody.

Radioligand Binding Assay

The detailed method for the radioligand binding assay was described previously.[26] The mixture containing 1 μL of patient serum and 20,000 cpm of each [^{35}S]-labeled human protein was incubated overnight at 4°C (50 μL in total). The reaction mixtures containing immune complex were transferred to a 96-well filtration plate (Millipore Corp., Benford, MA). Ten microliters of 50% Protein-G Sepharose 4FF (Amersham Bioscience) were added to each well. The plate was incubated for 45 minutes at room temperature for binding of protein-G to the immune complex and then washed 10 times with 200 μL of washing buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, and 1% Tween-20, pH 7.4) using MultiScreen vacuum manifold (Millipore). The filter was dried, and liquid scintillation counter, MicroScint 0 (PerkinElmer Life Science, Boston, MA), was added to each well. The precipitated and labeled proteins were quantified in duplicate using a Top-Count NXT apparatus (PerkinElmer Life Science). The intraassay coefficient of variation with these radioligand assays ranged from 4.5% to 5.1%, while the interassay coefficient variation ranged from 6.1% to 9.5%. In order to avoid interassay variation, the results were expressed by autoantibody index calculated as follows; (cpm of the sample serum/cpm of the pooled 91 healthy control sera). The cut-off value was calculated as the mean + 3 SD in healthy control subjects. The cut-off value with mean + 3SD is arbitrary. We selected this cut-off value based on the absorption tests in our previous experiment.[27,28] The mouse anti-Hcrt monoclonal antibodies and the rabbit polyclonal anti-Hcrt serum[29] were used as the positive standard for anti-Hcrt antibody.

Statistical Analysis

The distribution pattern was tested for 3 antibody indexes using normal probability paper. A normal distribution of antibody indexes was confirmed in hypersomnia patients and healthy control subjects. Distributions of antibody indexes among hypersomnia groups and the healthy control group were compared using the Mann-Whitney U test. The Mann-Whitney U test was also used to compare the distribution of antibody indexes among the divided groups according to the association to the following features; sex, HLA-DRB1*1501/DQB1*0602 (data available only for patients), cataplexy, and nocturnal sleep disruption. The correlations between antibody indexes and age or ESS at the time of blood sampling were examined using the Spearman correlation coefficient test. A p value less than .05 was considered as statistically significant.

RESULTS

Using a radioligand-binding assay, we examined autoantibodies against Hcrt neurotransmission systems in sera obtained from patients with narcolepsy and compared them with the sera of patients with other hypersomnias and healthy control subjects (Figure 1, Table 1). As a positive control, we used mouse monoclonal antibody and rabbit polyclonal antiserum. They showed positive reactions, and anti-Hcrt antibody indexes were 1.929 on 1:5000 dilutions and 2.155 on 1:1000 dilutions, respectively, showing the validity of our radioligand-binding assay.

There were no differences regarding the average indexes of anti-Hcrt, hcrtr1, and hcrtr2 antibody between patients with narcolepsy and healthy control subjects, patients with narcolepsy and those other hypersomnias, or patients with other hypersomnias and healthy control subjects (Figure 1, Table 1). With a cut-off values above the mean of + 3 SD of healthy control subjects (Hcrt: 1.219, hcrtr1: 1.388, hcrtr2: 1.274), positive reactions against recombinant Hcrt, hcrtr1, and hcrtr2 were found in 3, 1, and 5 patients with narcolepsy, respectively (Figure 1). As a whole, 8 patients with narcolepsy had anti-Hcrt neurotransmission system antibodies, with 1 having positive reactions against both hcrtr1 and 2. No positive reactions were detected in patients

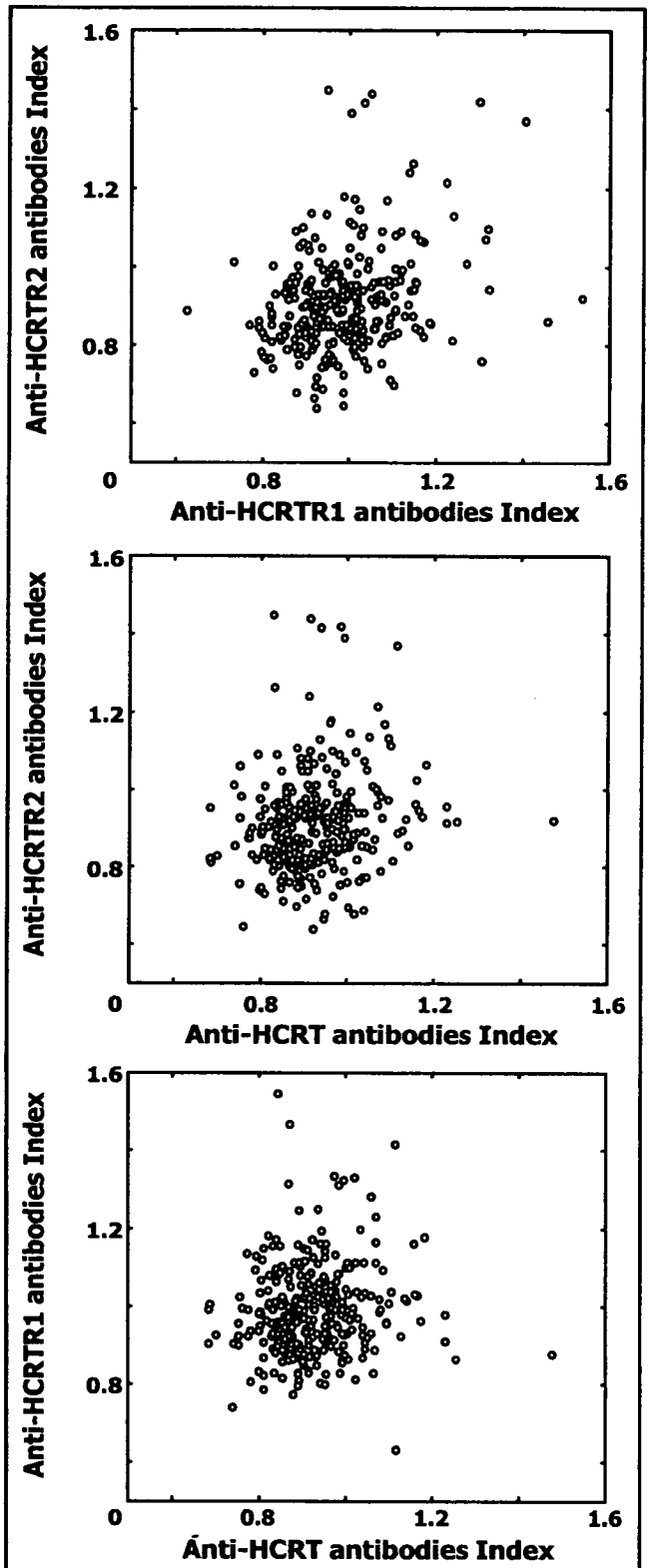

Figure 2—Comparison among 3 antibodies to human hypocretin, hypocretin receptor 1, and hypocretin receptor 2 in all subjects examined (n = 282). The Spearman correlation coefficient test was used to analyze the relationship among 3 different autoantibodies. There are no correlations among 3 antibody indexes.

with other hypersomnias, while healthy control subjects showed positive reactions against hcrtr1 (in 2 subjects) and hcrtr2 (in 1 subject).

Antibody indexes were compared with each other in order to clarify the relations among 3 different autoantibodies. No correlations were found among all subjects examined (Figure 2) or among patients with narcolepsy (data not shown).

All patients having autoantibodies against Hcrt neurotransmission systems were positive for HLA-DRB1*1501/DQB1*0602 except for 1 patient with "narcolepsy without cataplexy" who had an autoantibody against hcrtr2. The relationship between autoantibody indexes and collected clinical and demographic data were analyzed. We could not find any significant differences except for a sex difference in the mean antibody indexes. No differences in the mean antibody indexes were observed among the divided groups according to the association with HLA-DRB1*1501/DQB1*0602 (data available only for patients), presence of cataplexy, or presence of nocturnal sleep disruption, using the Mann-Whitney U test. No correlations between antibody indexes and age, or ESS, at the time of blood sampling were found using Spearman correlation coefficient test. The mean anti-Hcrt antibody index was significantly higher in women (n = 133, mean ± SD; 0.941 ± 0.113) than that in men (n = 156, mean ± SD; 0.909 ± 0.083) (p = .018). The sex differences were also noted in the subgroup of narcoleptic patients (women: n = 71, mean ± SD; 0.957 ± 0.114) (men: n = 110, mean ± SD; 0.911 ± 0.085) (p = .007).

DISCUSSION

The autoimmune hypothesis for narcolepsy has remained attractive because of the tight HLA association, peripubertal onset, specific degeneration of Hcrt neurons, and the existence of functional antibodies involving the cholinergic system in sera. In this study, we have conducted the first screening for autoantibodies against Hcrt receptors in human serum and found the existence of potential autoantibodies in some patients against Hcrt, hcrtr1, and hcrtr2 using radioligand-binding assay.

The radioligand binding assay provides several methodologic advantages compared with conventional methods used previously to investigate the autoimmune basis of narcolepsy. The recombinant proteins translated from full open reading frames are easily available as antigens in our system, compared with the conventional protein overexpression systems. It is not necessary to extract and denature the antigen for *in vitro* transcription/translation system. The Millipore MultiScreen system enables us to apply this system to high-throughput use. Repetitive washing (10 times) with high stringency detergent (1% Tween-20) can reduce background to negligible levels without any intricate operations. The radioligand binding assay also has an advantage with its ability to detect autoantibodies against conformational antigens, as compared to other methods.[30,31] It should, however, be noted that tissue-specific posttranslational modifications of antigens are still not completely the same as natural ones.

We have shown the presence of autoantibodies in sera from both narcoleptic patients and healthy control subjects. Our observations also suggest that these autoantibodies are not likely to contribute to the pathophysiology in the majority of narcoleptic patients. They might be naturally occurring autoantibodies without pathologic function or might not cross the blood-brain barrier to cause narcolepsy. Considering the physiologic importance

of Hcrt neurotransmission system in sleep-wake regulation, it is worth discussing possible roles for these autoantibodies.

Recently, potential autoantibodies against rat hypothalamic protein using enzyme-linked immunosorbent assay have been reported in CSF obtained from HLA-DQB1*0602 positive narcoleptic patients with cataplexy.[18] On the other hand, these researchers have previously reported negative results in detecting anti-Hcrt antibodies in sera and CSF obtained from 41 narcoleptic patients, employing immunoblotting, and immunoprecipitation for Hcrt-expressing cell lines.[22] Their data have shown lower mean antibody reaction against C-terminal peptide of Hcrt in CSF from narcoleptic patients compared with that of healthy control subjects. All techniques quoted might fail to detect antibodies against various conformational antigens.

In our study, 3 patients with narcolepsy had positive reactions against [^{35}S]-recombinant Hcrt protein. These might be autoantibodies against conformational antigens, which could not be detected by other systems using linearized , partial or denatured antigens.[32] Since we used high-stringency detergent and washed the plates frequently, autoantibodies detected in the present study might have high affinities with conformational antigens. Three narcolepsy patients with positive reactions against Hcrt were all women with HLA-DRB1*1501/DQB1*0602 and cataplexy. The sex difference on the reaction of autoantibodies against Hcrt might reflect the difference in immunologic background between men and women. We need to consider the significant differences in sex ratio between the narcoleptic group and controls (110/71 = 1.55 versus 40/51= 0.78, Table 1). It is one limitation in this study and the possible reason why only anti-Hcrt antibodies did not appear in healthy control subjects.

Five patients with narcolepsy showed positive reactions against hcrtr2. One patient had autoantibodies against both hcrtr1 and hcrtr2, although no correlations among the 3 autoantibody indexes were found. The coexistence of autoantibodies against hcrtr1 and hcrtr2 suggests that this patient might produce a high level of some sorts of different autoantibodies. However, this patient (25-year-old woman with a disease duration of 13 years) showed a typical clinical course of narcolepsy without any specific complications. Therefore, these autoantibodies might not have a pathogenic role. Regarding this patient, it is worthwhile measuring the concentrations of CSF Hcrt and total immunoglobulin G in the future. We can also assert that cross-reacting antigenicity does not occur among these autoantibodies, based on the lack of correlation among the 3 autoantibodies (see Figure 2). Autoantibodies against these 3 proteins might be produced independently. One "narcolepsy without cataplexy" patient and 1 healthy control subject showed positive reactions against hcrtr2. This patient with narcolepsy without cataplexy was negative for HLA-DRB1*1501/DQB1*0602. The excessive daytime sleepiness seen in this patient could have an autoimmune etiology, but it might be driven more by environmental factors rather than HLA haplotype. Total of eight patients and 3 healthy control subjects with positive reactions to Hcrt, hcrtr1, and hcrtr2 denied the present or past history of complication from autoimmune diseases. Patients with positive reactions have narcolepsy symptoms and clinical courses similar to the majority of narcoleptic patients.

Interestingly, further inquiry revealed that 2 healthy control subjects with positive reactions against hcrtr1 had a past history of excessive daytime sleepiness in their school days. It has been speculated that traumatic events and strong stress induces disruption of the blood-brain barrier.[33,34] It has been reported that 1 type of naturally occurring antibody in the blood of control subjects has an agonist-like activity against mu-opioid receptor.[35] If anti-hcrtr1 antibodies have antagonist-like functions, the transient impairment of blood-brain barrier might have occurred in these healthy control subjects. Anti-hcrtr1 antibodies detected in 2 healthy control subjects might have some functions in sleep-wake regulation. On the other hand, Hcrt receptors are reported to be expressed in peripheral tissues.[36,37] Anti-receptor antibodies may therefore be unrelated to sleep-wake regulation.

Smith et al[20] studied immunoglobulins purified with Protein-A Sepharose in 9 narcoleptic patients and showed that all narcoleptic patients examined had functional antibodies involving the cholinergic system. In this study, we did not examine antibodies specific to the cholinergic system. Our system, however, could easily be adapted for confirmation of these and other potential antigen targets.

In conclusion, serum autoantibodies against the Hcrt and the 2 known Hcrt receptors were detected in a few narcoleptic patients. Our results showed, however, no differences in the incidence of positive numbers between narcoleptic patients and healthy control subjects and the lack of relationship between narcolepsy and these autoimmunities against Hcrt neurotransmission systems. These autoantibodies in the serum might be unrelated to the development of narcolepsy. Future analysis of CSF from patients and controls may provide further information regarding potential autoantibodies specific to the hypocretin neurotransmitter system.

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健康日本２１こころの健康づくりの目標達成のための休養・睡眠のあり方に関する根拠に基づく研究

平成１８年度　総括・分担研究報告書

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