J. Physiol. (1984), **353**, *pp.* 305–315
With 4 text-figures
Printed in Great Britain

ULTRADIAN RHYTHM OF GROWTH HORMONE SECRETION AND SLEEP IN THE ADULT MALE RAT

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(*Received 22 November 1983*)

SUMMARY

1. The correlation between growth hormone (GH) secretion and the sleep–wakefulness cycle in the adult male rat was studied by serial blood sampling at 10 min intervals. Electroencephalograms (e.e.g.s) recorded continuously during the blood sampling were scored into wakefulness and sleep, and the amount of sleep for every 10 min was plotted against the GH value as assessed by radioimmunoassay.

2. The power spectrum analysis and the least-squares method applied to the time series of GH concentrations in the control rat revealed that the mean (±S.E. of the mean) periodicity was 2·93±0·10 h for the period from 00.00 to 12.00 h in five rats and 2·85±0·06 h for the period from 12.00 to 24.00 h in eight rats.

3. For the animals in which the e.e.g. was recorded simultaneously with blood samplings from 12.00 to 24.00 h, the cross-correlation analysis performed between the time series of the amount of sleep and the GH value revealed a significant positive correlation at time lags of 0–10 min in six and at time lags of 20–50 min in four out of fourteen rats. The number of animals having a positive correlation at short time lags seemed relatively less, but in most animals, there was observed a definite relationship that each GH peak occurred with a consistent time lag ranging from 40 to 70 min following the onset of sleep cycle.

4. Sleep deprivation performed from 13.00 to 16.00 h during the sampling period from 11.00 to 19.00 h was effective in preventing high-level GH pulses which otherwise should appear during this time of the day.

5. Concurrent measurements of corticosterone concentrations in the sleep-deprivation experiment revealed that peak values of corticosterone secretory episodes were not influenced by sleep deprivation.

6. These findings indicate that the GH secretion in the adult rat is also correlated with the sleep–wakefulness cycle, although somewhat differently from the immature rat.

INTRODUCTION

Since the striking demonstration made by Tannenbaum & Martin (1976), it has been established that the secretion of growth hormone (GH) in the rat occurs episodically, expressing an ultradian rhythm. Investigations which have been undertaken to determine the basis of this GH secretory pattern, however, have failed

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to indicate any specific factor responsible for it (for review, see Martin, 1976). Sleep, which has been recognized for humans as the most likely factor, has been ruled out for the adult male rat (Willoughby, Martin, Renaud & Brazeau, 1976). However, our most recent experiment demonstrated that in the immature rat, GH secretion occurred in association with the sleep cycle (Kawakami, Kimura & Tsai, 1983). The study suggested that a relationship similar to that in humans exists in the rat between the mechanisms for GH secretion and sleep. However, the former experiment was done on the immature rat, and thus the possibility could not be denied that certain developmental changes with respect to the relationship between both mechanisms occurred. The present study was therefore undertaken on the adult rat in order to determine the effect of sleep on GH secretion in this species.

METHODS

Animals and surgery

Adult male Wistar rats were placed four or five animals to a cage under controlled environmental conditions. The room was lit from 05.00 h to 19.00 h daily, and food and water were available *ad libitum*. The animals which were to be used for recording the electroencephalogram (e.e.g.) concurrently with blood samplings were implanted with stainless-steel electrodes over the cerebral cortex under anaesthesia with ether at least 1 week before the sampling experiment. Intracardiac cannulae were placed in rats anaesthetized with ether on the day before the sampling experiment. After surgery, the animals were kept in individual cages which were used during samplings.

Blood sampling

All animals, not only those with e.e.g. recording but also those without it, were habituated to the experimental room for at least 3 h periods. Unanaesthetized and unrestrained rats were bled for the determination of GH at 10 min intervals during a 12 h period beginning at 12.00 or 00.00 h. Sequential 30 μl blood samples for the determination of GH were collected with a 100 μl microsyringe and replaced by an equal volume of heparinized (10 u./ml) 0·9 % saline. Blood was added directly to assay tubes containing 300 μl of the buffer solution. For the determination of corticosterone, simultaneously with that of GH, blood samples were taken during an 8 h period from 11.00 to 19.00 h. An additional 5 μl blood was collected and added to tubes containing 50 μl heparinized (10 u./ml) 0·9 % saline. The total amount of blood withdrawn during the 8 or 12 h period was approximately 1·6 ml, considering a 8·0 μl dead volume in a single sampling by our cannula system, and thus the loss was less than 10 % of the total blood volume in an animal weighing approximately 300–350 g. This was regarded as negligible, on the basis of previous data on the immature rat (Kawakami *et al.* 1983). The exact time of blood withdrawal was marked on the e.e.g. record.

E.e.g. recording and scoring

E.e.g.s were recorded continuously during the blood sampling in all animals except a group of animals which served in the experiment to obtain GH secretory profiles. Recordings were performed through suspended recording cables coupled to screw electrodes via stainless-steel clips on a twelve-channel electroencephalograph (Nihon Kohden Co.) at a slow paper speed of 1 mm/s.

The e.e.g. records were scored in 1 min epochs into two stages, wakefulness and sleep, the latter including slow waves, spindles and paradoxical sleep, taking behaviour into account (Kimura & Kawakami, 1981). The amount of sleep in every 10 min period was calculated for each animal, and was plotted against the GH level at the end of the 10 min period, as described previously (Kawakami *et al.* 1983).

Sleep deprivation

A 3 h sleep deprivation was performed from 13.00 to 16.00 h, following the initial 2 h undisturbed condition. It was done by means of mild stimuli such as the clapping of hands and patting the cage, and, when the animal was not wakened by these sounds, by touching the tail and body gently.

One observer was constantly reading the e.e.g. recording as it appeared on the paper and made such sounds at the onset of the sleep stage.

Hormone assay

Whole blood samples were analysed for GH by the double-antibody radioimmunoassay using materials supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). The GH values (ng/ml whole blood) are expressed in terms of NIAMDD rat GH-RP-1. The mean of minimally detectable amounts of GH (95% confidence limits of buffer controls) on nine assays was $13{\cdot}1 \pm 8{\cdot}1$ (S.D.) ng/ml. The upper limit for the assay system was 256 ng/ml. The within- and between-assay variances on the nine assays, which were calculated from the triplicate determinations in each assay for a pool of rat serum containing $73{\cdot}7 \pm 3{\cdot}4$ ng/ml ($n = 27$), were 10·4 and 11·9%, respectively.

Corticosterone was determined by the protein-binding methods of Murphy (1967) and Takahashi, Honda, Kobayashi, Hayafuji, Otani & Takahashi (1979).

Data analysis

Characteristics of the time series data of GH concentrations and of the amount of sleep were clarified by statistical analysis of the power spectrum followed by the least-squares spectrum method, as described previously (Kawakami *et al.* 1983). Through these procedures, the period of the dominant component, as well as the acrophase (time of peak), can be found. Cross-correlation analysis was performed between the time series of GH concentrations and the amount of sleep to analyse the influence of sleep on GH. The analysis was also performed to analyse the influence of sleep on corticosterone concentration. The correlation was calculated for each of the nineteen time lags, i.e. 0–18 (= 0 to 180 min), considering that approximately 3·0 h periodicity is dominant in the GH and corticosterone series (Kawakami *et al.* 1983). This was to correlate an amount of sleep with GH values, or the GH value with corticosterone values obtained between that time and a time up to 180 min later.

RESULTS

GH secretory profile in the control adult male rat

The individual GH secretory profiles of two representative adult male rats during 12 h sampling periods from 00.00 to 12.00 h and from 12.00 to 24.00 h are shown in Fig. 1. In each animal, GH was secreted in an episodic manner and exhibited an ultradian rhythm, which confirmed previous findings (Tannenbaum & Martin, 1976). The GH secretory periodicity as determined by the power spectrum and the least-squares spectrum method applied to the time series of GH concentrations was in the range 2·5–3·2 h during the 24 h period sampled. The mean ($\pm$S.E. of the mean) period was $2{\cdot}93 \pm 0{\cdot}10$ h for the period from 00.00 to 12.00 h ($n = 5$) and $2{\cdot}85 \pm 0{\cdot}06$ h for the period from 12.00 to 24.00 h ($n = 8$), showing no significant difference from each other.

Relationship of GH secretory profile with the profile of the sleep–wakefulness cycle

In this series of experiments, blood samples were taken during a 12 h period from 12.00 to 24.00 h, concurrently with recording of e.e.g.s. The mean ($\pm$S.E. of the mean) periodicity estimated for the time series of GH concentration obtained in this series of experiments was $3{\cdot}04 \pm 0{\cdot}11$ h ($n = 14$), which was not significantly different from that obtained for the control male rat described above. The mean ($\pm$S.E. of the mean) periodicity for the time series of the amount of sleep was $3{\cdot}07 \pm 0{\cdot}19$ h, suggesting the existence of a similar ultradian rhythm to that in the GH time series. It was noted further that the periodicities of GH and sleep were identical in ten (71%) of fourteen rats, whereas in the others they were different from each other, by 30–70 min.

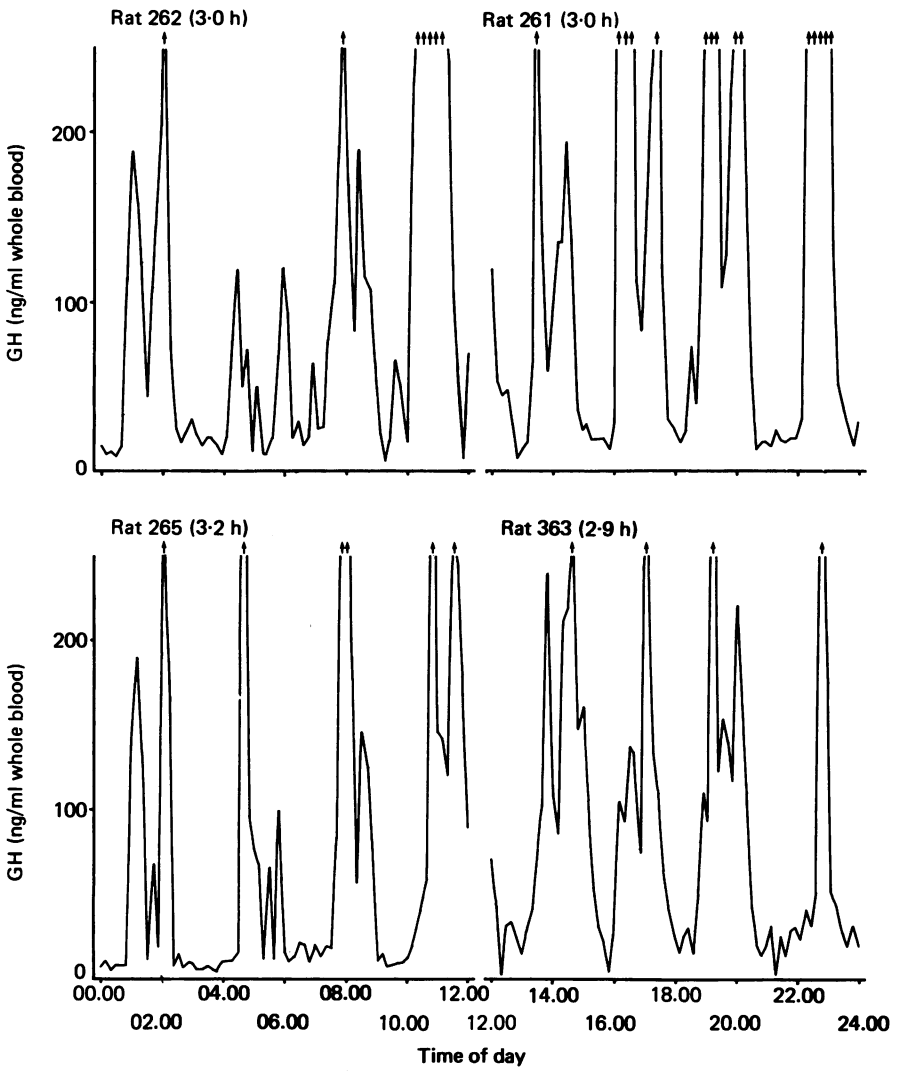

Fig. 1. GH profiles determined by serial blood collection at 10 min intervals through the intracardiac cannula during 12 h periods from 00.00 to 12.00 h and from 12.00 to 24.00 h in the adult male rat. Individual profiles of four representative animals are shown. Arrows indicate values exceeding 256 ng/ml whole blood, the upper limit of the assay curve. Numbers in parentheses indicate periodicity.

Cross-correlation analysis performed between the time series of GH concentrations and the amount of sleep revealed the highest significant ($P < 0{\cdot}001$ or $0{\cdot}005$) and positive cross-correlation coefficients at time lags of 0–10 min in six and at 20–50 min in four out of fourteen rats examined. In the remaining animals, there were two with significant positive coefficients at time lags of 100 and 140 min. The proportion of positive correlations at shorter time lags (0–10 min; 42 %) was apparently less than in the immature rat: 96 % in the male (Kawakami *et al.* 1983) and 79 % in the female

(Kawakami, Kimura & Tsai, 1984). Instead, the proportion of positive correlations at longer time lags (20–50 min; 29%) increased. Because whether or not the periodicities of GH and sleep rhythms were identical was not related to the presence of a significant cross-correlation coefficient, this seemed mainly due to the phase difference between two ultradian rhythms which was frequently observed (see later).

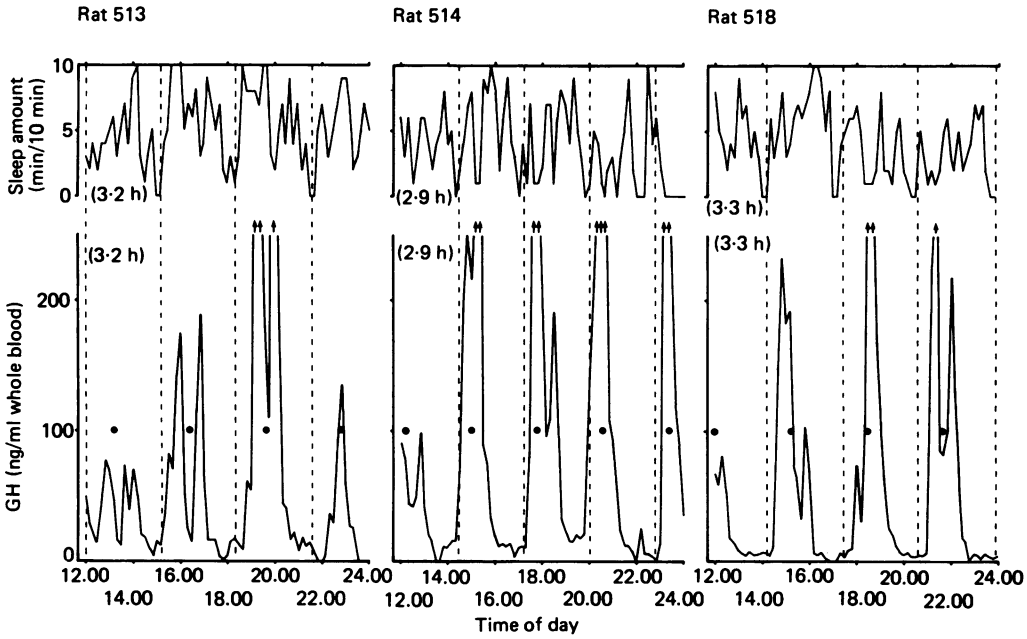

Fig. 2. Sleep–wakefulness cycle profiles plotted against GH profiles. Individual profiles for a sleep–wakefulness cycle and for the GH concentration in three representative animals are shown. The numbers in parentheses indicate the periodicity. In order to show the phase difference between the time series of GH concentrations and the amount of sleep clearly, vertical dotted lines defining each sleep cycle are drawn across both time series, on the basis of periodicity and acrophase estimated for the time series of sleep amount. Acrophases for the GH secretory rhythms are indicated by (●) on the time series of GH.

In Figs. 2 and 3 are shown some examples of individual time series of GH concentrations against which time series of the amount of sleep are plotted, as described previously (Kawakami *et al.* 1983). In order to show the phase difference between them clearly, vertical lines defining each sleep cycle at the trough time are drawn. The trough time was calculated on the basis of the periodicity and acrophase that were estimated for the time series of the amount of sleep. Acrophases, estimated also for the time series of GH concentration, are indicated by filled circles on the time series of GH concentrations.

A set of time series of GH concentrations and the amount of sleep from rat 513 shows a typical example in which ultradian rhythms of both time series have identical periodicities, with a small phase difference. In this case, each GH secretory episode almost exactly coincided with a sleep cycle without a marked time difference in their

peaks, i.e. each GH peak preceded the sleep peak by less than 20 min. The cross-correlation coefficient was significant at a time lag of 10 min ($r_1 = 0{\cdot}454$, $P < 0{\cdot}001$) and the highest coefficient was at a time lag of 20 min ($r_2 = 0{\cdot}551$, $P < 0{\cdot}001$). Other sets of time series from rats 514 and 518 are typical examples that

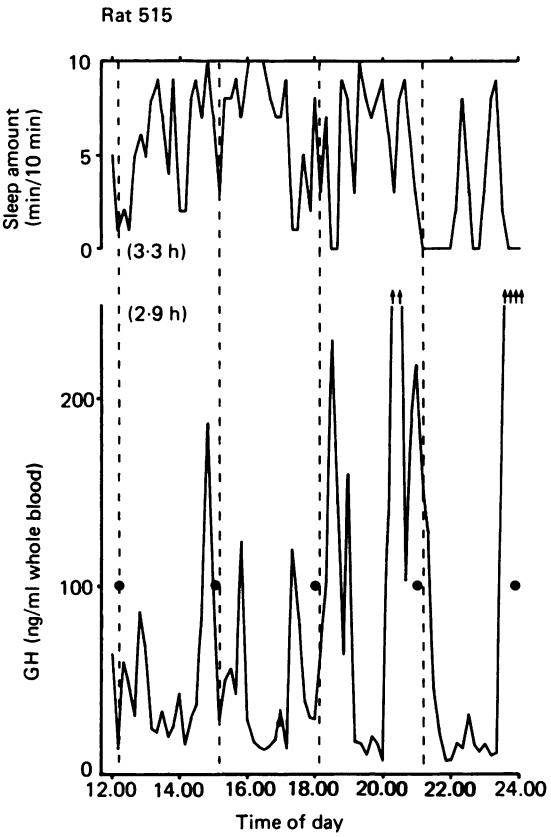

Fig. 3. Sleep–wakefulness cycle profiles plotted against GH profiles. For further details see Fig. 2 and also the text.

have considerable phase differences between both GH and amount of sleep ultradian rhythms. In those cases, the periodicities of the time series of amount of sleep and of GH concentrations were also similar. However, the GH peaks occurred considerably earlier, 49 and 54 min respectively, than the peaks of amount of sleep. In other words, in these cases GH peaks occurred with a consistent time lag of approximately 50 min after the onset of a sleep cycle. Together with all ten cases in which the GH and sleep rhythms had identical periodicities, there was a relationship such that the GH peak preceded the sleep peak by 20–50 min. Then, the time between the onset of a sleep cycle and a GH peak was in the range 40–70 min, and thus a GH peak generally coincided with the early phase of a sleep cycle, considering that the periodicity of sleep cycle was approximately 3·0 h. Even so, the cross-correlation coefficient in rat 514 was not significant at any time lag, whereas that in rat 518 was highest at time lag of 20 min.

The relationship between the GH and sleep rhythms was quite peculiar in rat 515 (Fig. 3), which was an example of four (28 %) among fourteen rats examined. In those animals, periodicities of the time series of the GH concentrations and the amount of sleep differed considerably from each other, and thus no consistent relationship of the GH peak time with the onset of the sleep cycle was obtained. However, it was noticeable that each GH secretory pulse occurred corresponding to or following the increase in the amount of sleep. This was supported by the significant ($P < 0{\cdot}05$) positive cross-correlation coefficient obtained at a time lag of 30 min.

TABLE 1. Over-all mean (±S.E. of the mean) hormone concentrations and mean (±S.E. of the mean) amounts of sleep in the sleep-deprivation experiments

	Undisturbed ($n = 248$)	Sleep deprived (n = 144)	Significance (P)
GH (ng/ml whole blood)	68·6±6·0	47·6±1·6	< 0·001
Corticosterone (μg/100 ml whole blood)	33·0±0·7	38·3±1·0	N.s.
Amount of sleep (min/10 min period)	4·44±0·32	1·92±0·13	< 0·01

The numbers in parentheses indicate the total number of samples obtained from the eight rats during each undisturbed and sleep-deprived condition. N.s., not significant.

Effects of sleep deprivation on GH secretory profile

An involvement of sleep in the induction of GH secretion was studied in eight animals by the sleep-deprivation experiment, in which wakefulness and sleep were determined by e.e.g. recordings. In Fig. 4 are shown three typical individual time series of GH concentrations during the 8 h (11.00 to 19.00 h) period in which a 3 h (13.00 to 16.00 h) sleep-deprivation episode was inserted. The time series of the amount of sleep was plotted against the GH time series, as described before.

As seen in Fig. 4, a small amount of sleep was taken by the animals despite all of our attempts to keep them in a state of complete wakefulness for 3 h. However, no high-level GH pulses were observed during this 3 h sleep deprivation. In almost all animals examined, the GH pulses which occurred during the sleep deprivation were of small amplitude, and high-amplitude GH pulses always appeared with the cessation of sleep deprivation. In support of this, the over-all mean (±S.E. of the mean) amount of sleep during the sleep-deprived period (Table 1) was quite small compared with that during the undisturbed period, and the over-all mean GH concentration obtained in the sleep-deprived period was significantly lower than that for the undisturbed period.

It was thus highly conceivable that a decrease in the amount of sleep was associated with an attenuation of GH secretion. The secretory rhythm of GH, however, seemed to be kept normal even under the sleep-deprived conditions. When the periodicity was calculated for the 8 h GH time series obtained in the experiment on sleep deprivation, similar values, 2·93±0·15 h as the mean (±S.E. of the mean) for eight animals, to those for the undisturbed animals were obtained. Therefore, it was most likely that a decrease in the amount of sleep was responsible for the attenuation of GH secretion, but was not responsible for an occurrence of GH secretion which would have undergone the 3·0 h ultradian rhythm.

By analysing the cross-correlation coefficients between the amount of sleep and the

GH secretion in all 8 h (11.00 to 19.00 h) series, the highest positive and significant cross-correlation coefficients were obtained at time lags of 0–10 min for five and time lags of 30–40 min for two of eight rats. The number of animals showing a positive correlation was similar to that in the undisturbed animals.

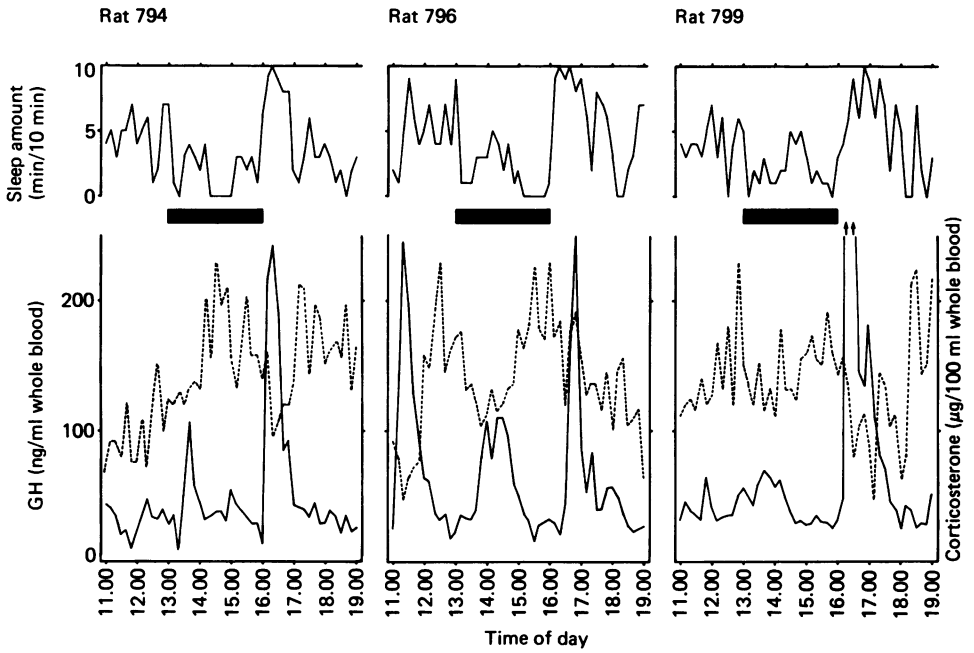

Fig. 4. Effect of a 3 h sleep deprivation on GH (filled line) and corticosterone (dotted line) profiles. Individual GH and corticosterone profiles against the sleep–wakefulness cycle profiles in three representative animals are shown. The dark areas are periods of sleep deprivation.

Effects of sleep deprivation on the corticosterone secretory profile

In the sleep-deprivation experiment, concurrent measurements of corticosterone concentrations were performed in the same samples as those in which GH concentrations were measured, in order to ascertain whether the sleep-deprivation procedure in the present experiment acted as a stressful stimulus. The time series of the corticosterone concentrations are plotted against the time series of the amount of sleep with those of GH concentrations in Fig. 4.

As apparently seen in those individual profiles, corticosterone levels fluctuated significantly over an 8 h period. Although not as distinct as GH, two or three secretory episodes of corticosterone seemed to occur during the 8 h period. These corticosterone secretory episodes showed an inverse relationship with those of GH secretion, i.e. at the time when GH secretion occurred, corticosterone secretion did not occur, and at the time when GH secretion ceased to occur, corticosterone secretion occurred. In accord with this, cross-correlation coefficients calculated between the GH and corticosterone concentrations were significantly ($P < 0{\cdot}01$ or $0{\cdot}05$) negative in four of eight rats. Further, an ultradian rhythm with a mean (±S.E. of the mean)

periodicity of $3{\cdot}39 \pm 0{\cdot}23$ ($n = 8$) was also found in the time series of corticosterone concentrations, corresponding to the ultradian rhythm of GH with approximately 3·0 h periodicity in this series of experiments.

It did not appear that peak values of those corticosterone secretory episodes were influenced by sleep deprivation. The peak value ranged from 55 to 65 μg/100 ml for either the sleep-deprived or the undisturbed period. Further, there was no significant difference between the over-all mean concentration of corticosterone calculated for each of the sleep-deprived and undisturbed periods (Table 1).

Relationship of ultradian rhythm of GH secretion with the time of day

In order to estimate the relationship of the ultradian rhythm of GH to the time of day, the time series of the number of GH peaks, which appeared in the twenty-seven GH time series obtained from the experiments on the control and on the relationship with the sleep–wakefulness cycle, was analysed for the periodicity, as described previously (Kawakami *et al.* 1984). It was considered that the time at which most animals showed GH secretory peaks would be estimated through this procedure.

By analysing the time series so obtained over a 24 h period from a total of twenty-seven animals, it was estimated that the time series of the number of GH peaks had a periodicity of 3·03 h, almost exactly 3·0 h, and peak times were estimated to be 01.47 (around 02.00), 04.49 (05.00), 07.51 (08.00), 10.53 (11.00), 13.55 (14.00), 16.57 (17.00), 19.59 (20.00) and 23.01 (23.00) h.

DISCUSSION

The secretory rhythm of GH secretion in the adult male rat had almost exactly 3·0 h periodicity across the light–dark cycle, in agreement with an approximately 3·3 h periodicity described previously by Tannenbaum & Martin (1976). This ultradian rhythm has been suggested to be endogenous, and to be entrained by the light–dark cycle as a cue (Tannenbaum & Martin, 1976). The results of the present study could fix definite times specific for the GH secretory peak as 02.00, 05.00, 08.00, 11.00, 14.00, 17.00, 20.00 and 23.00 h. This timetable was very similar to that obtained for the immature rat: 02.00–03.00, 05.00–06.00, 08.00–09.00, 11.00–12.00, 14.00–15.00, 17.00–18.00, 20.00–21.00 and 23.00–24.00 h (Kawakami *et al.* 1984).

It was revealed that 71 % of animals examined had a significant positive correlation at time lags of 0–50 min between the amount of sleep and GH concentration. However, the number of animals having the highest positive correlation at time lags of 0–10 min was less than that in the immature rat (Kawakami *et al.* 1983, 1984), and instead the number having the correlation at time lags of longer period (20–50 min) increased. This seemed mainly due to the significant phase difference between the two ultradian rhythms. Although the periodicities of GH and sleep rhythm were almost identical in most animals, there was an apparent difference in their peak times. That is, the peak of each GH episode in individual animals occurred before the peak of a sleep to which the GH episode related. The phase difference ranged from approximately 20 to 50 min, being different between animals. This 20–50 min difference coincided well with the time lags where the amounts of sleep and GH values showed the highest positive cross-correlation coefficients in 29 % of animals.

The above also seems to indicate a definite relationship between the GH secretion and sleep–wakefulness cycle such that each secretory episode takes place during the early phase of the sleep cycle, recurring with a 3·0 h period ultradian rhythm. Consistent time lags of 40–70 min were estimated between the onset of the sleep cycle and the GH peak. It is possible therefore that the GH secretion in the adult rat is also somehow correlated with the sleep–wakefulness cycle, as first demonstrated in the immature rat (Kimura & Kawakami, 1981; Kawakami *et al.* 1983, 1984).

The possible correlation of GH secretion with sleep was further supported by the results of sleep-deprivation experiments. It was found that when the amount of sleep was decreased by the deprivation procedure the amplitude of GH pulses apparently became smaller, the pulses sometimes disappearing. Through the corticosterone measurement, it was confirmed that the procedure of sleep deprivation did not act as a stress, which has been known to inhibit GH secretion in the rat (Kokka, Garcia, George & Elliot, 1972; Martin, 1976; Terry, Saunders, Audet, Willoughby, Brazeau & Martin, 1977). Peak values of corticosterone pulses, which were also demonstrated to appear episodically, were not different between the sleep-deprived and undisturbed periods. The over-all mean corticosterone concentrations for each of the periods were not different from each other, either. Considering the fact that, even under the sleep-deprived conditions, the GH secretory rhythm was kept normally but the pulse amplitudes were smaller than those under the undisturbed conditions, it seems relevant to say that the GH releasing mechanism operates on the basis of its own 3·0 h period ultradian mechanism, but its activity would be potentiated under sleep, which also recurs on the basis of a separate 3·0 h period ultradian rhythm. That means that the mechanisms for both the sleep and GH secretion would function on the basis of their own 3·0 h period ultradian mechanisms, independently of each other, but normally both would be synchronized under the influence of a higher oscillator(s). Rat 515 may provide strong evidence supporting this concept; in this case, the mechanisms for both rhythms would have been desynchronized and thus the GH secretion would have occurred due to its own rhythm. It may be said that for the releasing mechanism of GH to operate, sleep itself is necessary but that sleep recurring with a 3·0 h periodicity is unnecessary, since the mechanism of GH secretion itself is able to function with a 3·0 h periodicity, if in a sleep state.

The above consideration, at the same time, points to the interesting possibility of a multiplicity of pace-makers. Although the timely activation of the GH secretory mechanism under the continuous sleep state supports this, the most decisive evidence is present in rat 515, in which two ultradian rhythms were apparently showing different periodicities. Such dissociation could have resulted from some lack of entrainment of the two rhythms to the environmental cues. The possibility has been reported for the human circadian system (Aschoff, 1969). The circadian rhythms of activity–rest and body temperature free-ran at different frequencies under conditions lacking cues in about 16% of subjects. Although the frequency of occurrence of such dissociation is low in either the rat or the human, it indicates directly the existence of multiple pace-makers for the ultradian and circadian rhythms. A study is under way to obtain more evidence in our laboratory.

The association between sleep and GH secretion in the adult rat was considerably different from that in the immature rat (Kawakami *et al.* 1983, 1984). In most

immature rats, no considerable time difference between the peaks of GH episode and sleep cycle was observed. For instance, the peaks of GH and sleep were at 14.03 h and 17.07 h, and at 13.57 h and 17.05 h, respectively (Kawakami *et al.* 1984). Further, the sleep during either the early or late phase of a sleep cycle coincided with GH secretion, probably forming a basis for the highest cross-correlation coefficients at time lags of 0–10 min in most immature animals. All of these findings, together with the findings in the adult rat, point to a difference between regulatory mechanisms in immature and mature animals.

We wish to thank the National Institute of Arthritis, Metabolism and Digestive Diseases for supplying the radioimmunoassay materials. The authors also thank Dr K. Wakabayashi, Institute of Endocrinology, Gunma University, for his kind help with the measurement of GH. The present study was supported by the Grant-in-Aid for Scientific Research, from the Ministry of Education, Japan.

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