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CORRELATION OF GROWTH HORMONE SECRETION TO SLEEP IN THE IMMATURE RAT

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SUMMARY

1. The correlation between growth hormone (GH) secretion and the sleep–wakefulness cycle in the 29–31 day-old male rat was studied by serial blood sampling through an intracardiac cannula at 10 min intervals from 12.00 to 18.00 h. E.e.g.s recorded continuously during the blood sampling period 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 periodicity was $3{\cdot}12 \pm 0{\cdot}09$ h (mean $\pm$ S.E. of the mean) in fourteen rats examined.

3. For the animals in which the e.e.g. was recorded simultaneously with blood samplings, the cross-correlation analysis performed between the time series of the amount of sleep and the GH value revealed a significant positive correlation between the GH level and the amount of sleep during the preceding 10 min.

4. The time series of the sleep amount and the GH value had mean periodicities of $3{\cdot}09 \pm 0{\cdot}17$ and $3{\cdot}05 \pm 0{\cdot}13$ h (mean $\pm$ S.E. of the mean), respectively, in twenty-seven rats examined.

5. Sleep deprivation performed between 13.00 and 15.00 h was effective in preventing GH secretory bursts which otherwise should appear during this time period of the day.

6. Corticosterone measurement by protein-binding assay revealed that the procedure of sleep deprivation did not act as the 'stress' which has been known to inhibit GH secretion in the rat.

7. These findings indicate that GH secretion in the rat is sleep-related, as has been proved in the human.

INTRODUCTION

It has been proved that the secretion of growth hormone (GH) in the rat is of an episodic nature (Tannenbaum & Martin, 1976) as in other experimental animals and humans (Martin, 1976). In marked contrast to the close relationship of GH secretory

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episode to the sleep cycle which has been observed in humans, an episodic GH secretion in the rat that occurred regularly every 3–3·5 h has been proposed to be independent of sleep (Willoughby, Martin, Renaud & Brazeau, 1976). However, it was observed in our last experiment using immature rats that the GH level in animals decapitated during sleep was higher than during the arousal state (Kimura & Kawakami, 1981). The studies reported here were designed to determine the correlation between GH secretion and the sleep–wakefulness cycle in more detail using a repeated sampling method.

METHODS

Animals and surgery

Male Wistar rats, which were received at 22 days of age, were placed four to five animals to a cage under controlled environmental conditions. The room was lighted from 05.00 to 19.00 h daily, and food and water were available *ad libitum*. At 23–24 days of age, approximately 7 days before the sampling experiment, the animals which were to be used for recording the e.e.g. concurrently with blood samplings were implanted with stainless-steel screw electrodes over the cerebral cortex under anaesthesia with ether following the method described previously (Kimura & Kawakami, 1981). Intracardiac cannulae were placed in rats anaesthetized with ether on the day before the sampling experiment. The cannulae were brought subcutaneously to the back of the neck. After surgery, the animals were kept in transparent plastic cages which were used during samplings.

E.e.g. recording

E.e.g.s were recorded continuously during the blood sampling period. Recordings were performed through suspended recording cables coupled to screw electrodes via stainless-steel clips on a 12-channel electroencephalograph (Nihon Kohden Co.) at a low paper speed (1 mm/s). The e.e.g. records were scored in 1 min epochs into two stages; wakefulness and sleep (slow waves, spindles and paradoxical sleep) taking the behaviour into account. The behaviour of animals was watched carefully during the recording of the e.e.g.s and noted on the e.e.g. recording paper. Further details of the scoring method were reported in an earlier paper (Kimura & Kawakami, 1981). In addition, since it was observed in the same previous study that the GH level in the animals decapitated during paradoxical sleep was not significantly different from that during slow waves and spindles, the state of paradoxical sleep was included in the sleep stage. The amount of sleep in every 10 min period was calculated for each animal.

Blood sampling

Before starting the 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 24 h. Sequential 30 μl blood samples were collected with a 100 μl microsyringe and replaced with an equal volume of heparinized (10 i.u./ml) 0·9% saline.

Unanaesthetized and unrestrained rats were bled at 10 min intervals for a 6 h period beginning at 12.00 h. Blood was added directly to assay tubes for determination of GH content. The exact time of blood withdrawal was marked on the e.e.g. record by switching a marker. Blood samplings for corticosterone assay were done similarly to those for GH, but only 10 μl blood was added to assay tubes containing 50 μl heparinized (100 i.u./ml) 0·9% saline, discarding the remaining 20 μl blood.

All blood samplings were performed in the rat at 29–31 days of age. The total amount of blood withdrawn during this 6 h period was approximately 0·8 ml considering a 7·5 μl dead volume in a single sampling by our cannula system, which was unavoidable in order to minimize the stressful stimuli and loss of blood, and thus altogether about a 20% loss of total blood volume resulted. Haematocrits measured before and after the sampling period in a group of animals ($n = 6$) were $37·3 \pm 0·5$ and $28·5 \pm 0·9$ (mean $\pm$ S.E. of the mean), respectively, revealing a decrease of 23·6%. It had been confirmed in the rat at 24 days of age that an 18% loss of total blood volume and thus a 20% decrease in haematocrits did not affect significantly the weight gain afterwards (Kimura & Kawakami, 1982). Details of this method were reported previously (Kimura & Kawakami, 1982).

Sleep deprivation

Two-hour sleep deprivation was done from 13.00 to 15.00 h, after the first 1 h of free and undisturbed conditions. It was done by means of mild stimuli such as the clapping of hands and patting of the cage, and when the animal was not awakened 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 method using materials supplied by the N.I.A.M.D.D. The GH values (ng/ml whole blood), calculated considering the dilution in 7·5 μl saline of each sample which occurred due to the sampling procedure described above, are expressed in terms of N.I.A.M.D.D. rat GH-RP-1. The mean minimally detectable amount of GH (95% confidence limits of buffer controls) on five assays was 7·2 $\pm$ 2·8 (S.D.) ng/ml. The upper limit of the assay system was 128 ng/ml, and thus all values above this point are expressed as 128 ng/ml in the Figures. The cumulative within- and between-assay variance for the five assays, which were calculated from the triplicate determinations in each assay for pool of rat serum containing 93 $\pm$ 9 ng/ml (n = 15), were 10·0 and 7·4%, respectively. Corticosterone was determined by the protein-binding methods of Murphy (1967) and Takahashi, Honda, Kobayashi, Hayafuji, Otani & Takahashi (1979).

Data analysis

All data were applied to the statistical analysis of the power spectrum to find out periodical characteristics of the time series data of GH concentrations and of sleep amounts during the undisturbed condition. The spectrum was computed by means of a Fourier transform of the autocovariance function of the series (Sasaki, 1978). The precise periodicity was identified by using the least-squares spectrum method (Halberg, Engeli, Hamburger & Hillman, 1964). Cross-correlation analysis was performed between the GH time series and the sleep time series to analyse the influence of sleep on GH. Calculation was done for each of the 7 time lags, 0–6 (= 0 to 60 min), in the GH series, to correlate any amount of sleep with GH values obtained between the same time and up to 60 min afterwards. Student's t test was used for testing the significance of differences between over-all means of hormone concentration and of sleep and wakefulness amount.

RESULTS

GH secretory profiles in the control immature male rat

Power spectrum analysis revealed the existence of an ultradian rhythm with a periodicity of 2 or 3 h in the time series of GH concentrations of fourteen control rats. The precise periodicity as determined by the least-squares spectrum method was in the 2·6–3·8 h range, and the mean for all fourteen rats was 3·12 $\pm$ 0·90 h (mean $\pm$ S.E. of the mean). Peak times of the GH burst as determined by the acrophase calculated were almost the same for all the animals. The mean time for the earlier burst was 13.46 h and 16.51 h for the later burst.

In Fig. 1 are shown the individual GH secretory profiles for the representative five rats with the typical two GH episodes, whose peak levels were higher than 128 ng/ml whole blood.

Correlation between GH secretory profile and sleep–wakefulness cycle

In the twenty-seven animals in which the e.e.g. recording was performed concomitantly with the blood sampling from 12.00 to 18.00 h, power spectrum analysis and the least-squares spectrum method revealed that they had an ultradian rhythm of 2·1–4·5 h periodicity in the time series of GH concentrations, the mean periodicity

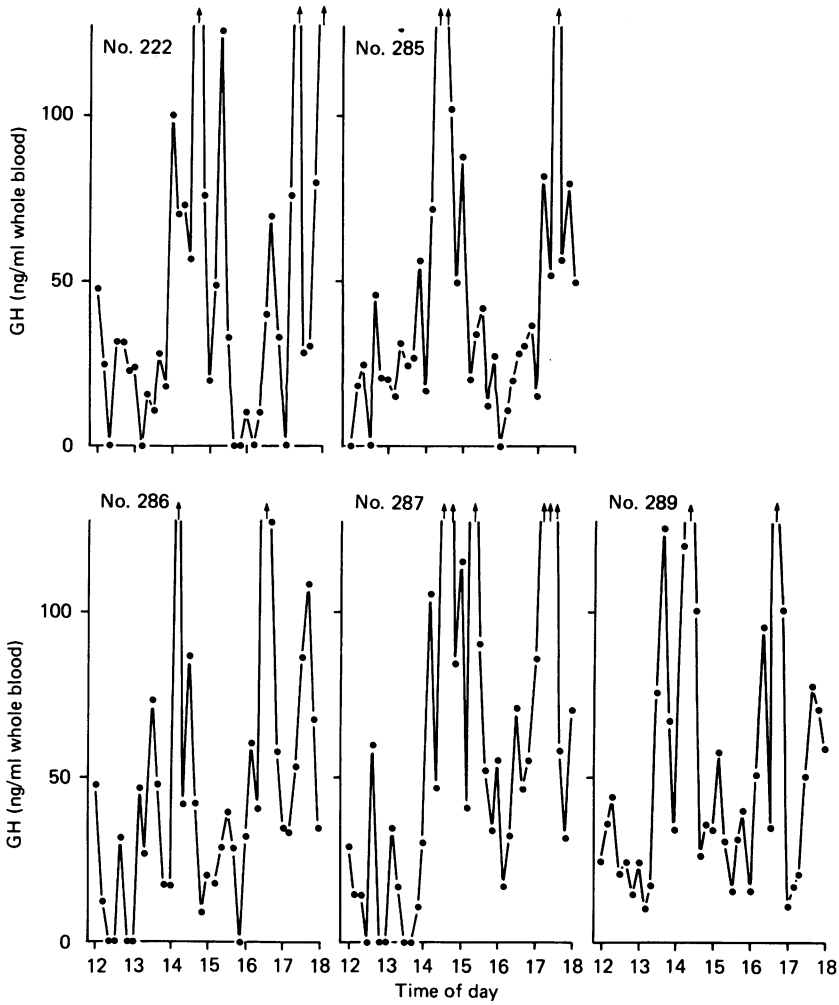

Fig. 1. GH profiles determined by serial blood collection at 10 min intervals through the intracardiac cannula during a 6 h period in male rats 29–31 days of age. Individual profiles in five representative animals are shown. Arrows indicate values exceeding 128 ng/ml whole blood, the upper limit of the assay curve.

being 3·05±0·13 h (mean±S.E. of the mean, $n = 27$). In those rats, the mean peak time for the earlier burst was 13.36 h and for the later one 16.39 h.

The profile of the sleep–wakefulness cycle was expressed with the time series of sleep amount at 10 min intervals and a give sleep amount represented the total duration (min) of sleep which occurred in the preceding 10 min. The sleep amounts were plotted against the GH levels found at the end of the 10 min period. Fig. 2 illustrates the individual sleep–wakefulness cycle profiles plotted against GH profiles in the representative five rats.

The power spectrum analysis and the least-squares spectrum method applied to the time series of sleep amount revealed a 2·2–4·5 h periodicity. The mean periodicity was 3·09±0·17 h (mean±S.E. of the mean, $n = 27$), being almost completely analogous

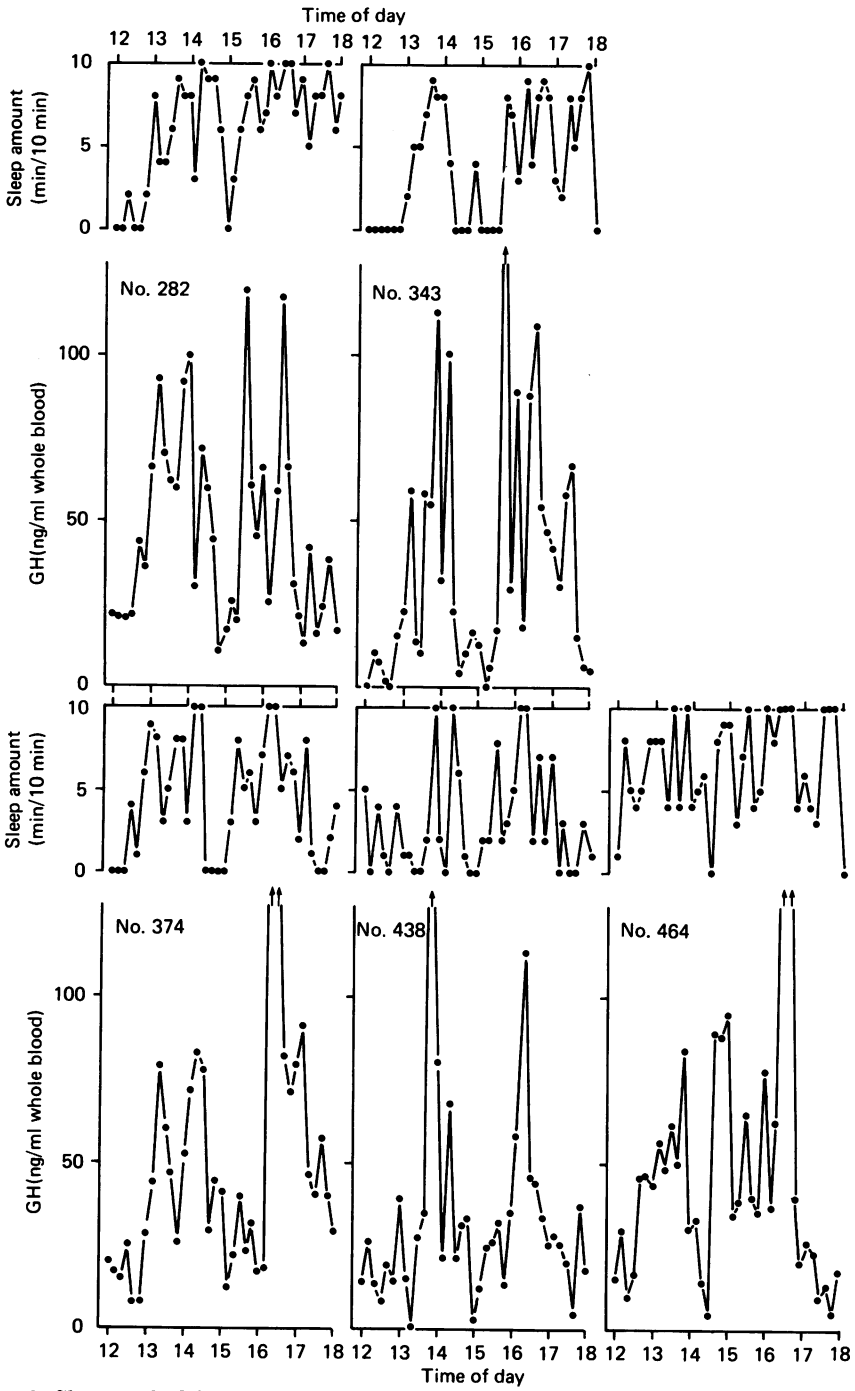

Fig. 2. Sleep–wakefulness cycle profiles plotted against GH profiles. The profiles of the sleep–wakefulness cycle were expressed with the time series of sleep amount at 10 min intervals which were obtained from the e.e.g. recording performed concomitantly with the blood sampling, and a given sleep amount represented a total duration (min) of sleep which occurred in the preceding 10 min. Individual profiles for sleep–wakefulness cycle and for GH in five representative animals are shown. For further details see Fig. 1.

to that for GH. The mean peak time was 13.20 h for the earlier sleep phase and 16.25 h for the later one. These peak times were also analogous to the peak times for GH bursts. In agreement with those, it was easily discovered, simply by comparing the time series of amount of sleep and GH concentration, that when the amount of sleep was small, i.e. during the wakefulness cycle, the GH concentration was low, while when the amount of sleep was great, i.e. during the sleep cycle, the concentration was high. Evidently, GH peaks occurred corresponding with high amounts of sleep. Cross-correlation analysis performed between both time series revealed a consistent and significant relationship between GH secretion and the amount of sleep in twenty-six of twenty-seven animals. The majority (69 % of the twenty-six rats had the highest, significant ($P < 0{\cdot}01$ or $0{\cdot}05$) and positive cross-correlation coefficients at time lag 0 min. In 31 % of the rats, the highest and most significant positive coefficient was obtained at the time lag 10 min. These findings were highly indicative of a relationship between sleep and GH secretion in the rat. Further, it was indicated that the influence of sleep on GH secretion appeared rapidly within 10 or 20 min, which was estimated from the time lag of 0 or 10 min where the most significant correlation was obtained. As described above, each amount of sleep reflected the sleep which occurred in the preceding 10 min.

Effect of sleep deprivation on GH secretory profile

A possible involvement of sleep in the induction of GH secretion was studied further by the sleep deprivation experiment. In Fig. 3 are shown individual profiles of GH concentrations and the amount of sleep for five rats during the 6 h period in which a 2 h sleep deprivation episode was inserted. Despite our attempt to keep the animals in a state of complete wakefulness for the 2 h, from 13.00 to 15.00 h, it was found at the e.e.g. scoring that they lapsed into several sleep periods of short duration. However, it was apparent that no rats had a high GH pulse as observed in the undisturbed animals during this time period of a day, but they showed only small GH pulses. High level GH secretory bursts appeared with the cessation of sleep deprivation. Seven (64 %) of eleven rats subjected to the 2 h sleep deprivation showed these typical GH profiles, indicating that the lack of sleep during the time from 13.00 to 15.00 h corresponded with the lack of GH secretory episodes which should otherwise have occurred during this time period. The other four rats did not show apparent GH peak occurrence after the cessation of sleep deprivation.

The over-all mean GH concentrations obtained in each of the undisturbed and sleep-deprived periods for the eleven rats are shown in Table 1. The over-all mean amounts of sleep and wakefulness, the latter of which was calculated as 10 min minus the amount of sleep, are shown, too. The mean amount of sleep during the sleep-deprived period was clearly and significantly low compared with that during the undisturbed period. During such sleep-deprived period, the mean GH concentration was significantly low compared with that during the undisturbed period.

By analysing the cross-correlation coefficients between the amount of sleep and the GH concentration in all 6 h (12.00–18.00 h) series, the highest positive cross-correlation coefficients were obtained at time lag 0 min in six and time lag 10 min in four of all eleven rats, although in these latter cases coefficients at time lag 0 min were significant, too. Other two rats revealed no significant positive correlation at

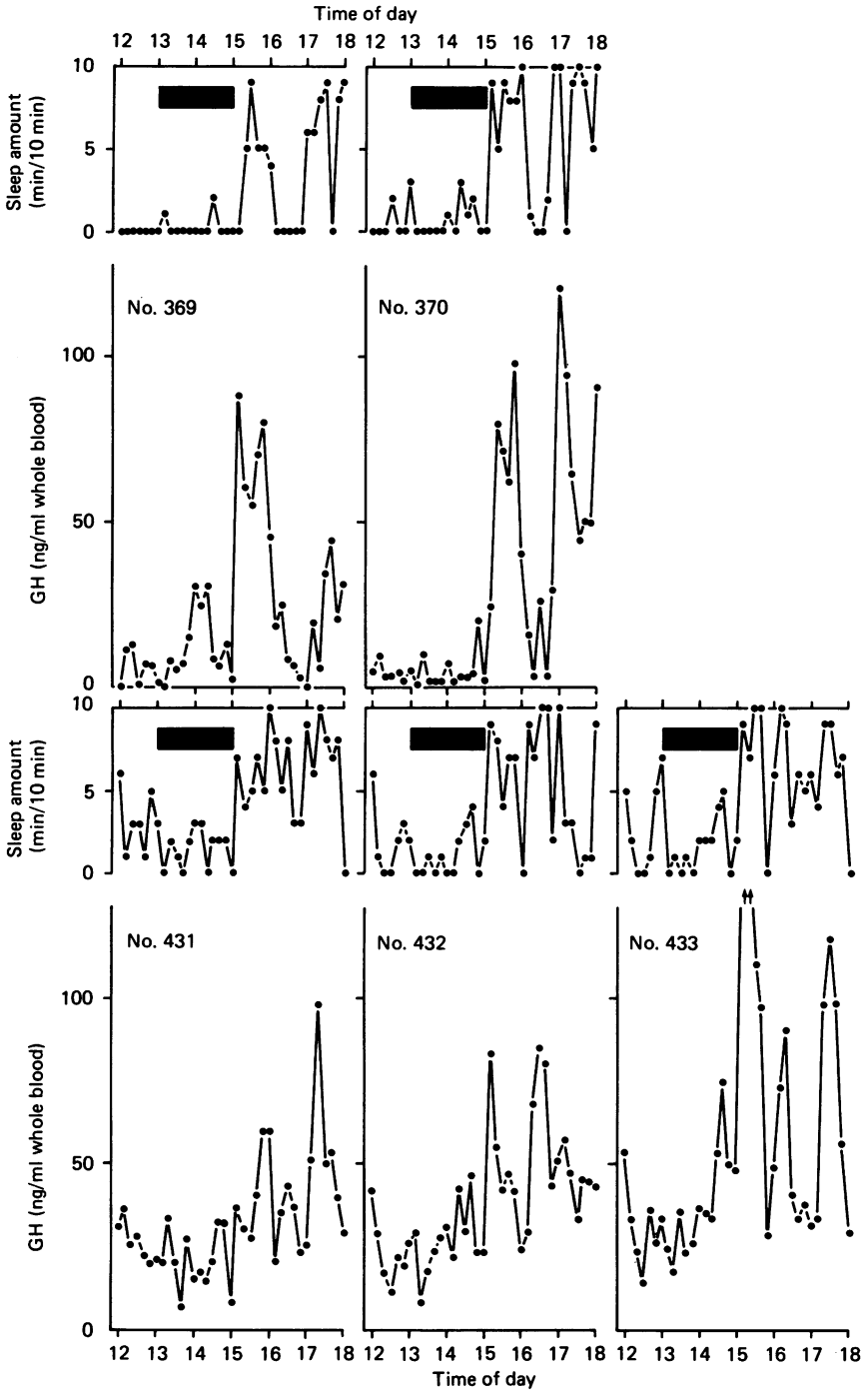

Fig. 3. Effect of a 2 h sleep deprivation on GH profiles. Individual GH profiles plotted against the sleep–wakefulness cycle profiles are shown. The dark areas are periods of sleep deprivation. For further details see Fig. 1.

time lags of 0–60 min. Even when the correlation coefficient was calculated for the 2 h (13.00–15.00 h) of sleep deprivation, eight rats showed significant positive correlation coefficients at time lags of 0–50 min, mostly 0–10 min. Thus, a significant relationship between the amount of sleep and the GH concentration was found in the sleep-deprived animals as in the undisturbed ones.

Effect of sleep deprivation on corticosterone profiles

In order to ascertain whether the sleep deprivation procedure employed in the present experiment acted as a stressful stimulus 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), corticosterone concentrations were measured in nine rats over the same time course of sleep deprivation experiments.

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

	Undisturbed	Sleep-deprived	Significance (P)
Experiment on GH measurement in eleven rats	(n = 275)*	(n = 132)	
GH (ng/ml whole blood)	34·9±1·5	21·3±1·1	<0·001
Amount of sleep (min/10 min period)	3·6±0·2	0·9±0·1	<0·001
Amount of wakefulness (min/10 min period)	6·4±0·2	9·1±0·1	<0·001
Experiment on corticosterone measurement in nine rats	(n = 225)	(n = 108)	
Corticosterone (μg/100 ml whole blood)	14·1±0·5	18·9±0·8	<0·001
Amount of sleep (min/10 min period)	5·2±0·2	1·0±0·1	<0·001
Amount of wakefulness (min/10 min period)	4·8±0·2	9·0±0·1	<0·001

* n indicates the total number of samples obtained from the eleven or nine rats during each undisturbed and sleep-deprived conditions. Amount of wakefulness was calculated as 10 min minus the amount of sleep.

Five individual profiles of corticosterone concentrations and the amount of sleep during the 6 h period in which a 2 h sleep deprivation episode was inserted are shown in Fig. 4. Apparently, corticosterone levels fluctuated significantly over the 6 h period. A secretory episode was evident during the 2 h sleep-deprivation period and other one or two episodes were observed during the remaining undisturbed period. There was no significant difference between both peak values that occurred during the sleep deprivation and undisturbed period; the peak ranged from 25–39 μg/100 ml for either period.

When the over-all mean concentration of corticosterone was calculated in each of the undisturbed and sleep-deprived periods (Table 1), the level during the sleep-deprived period was significantly higher than that during the undisturbed period.

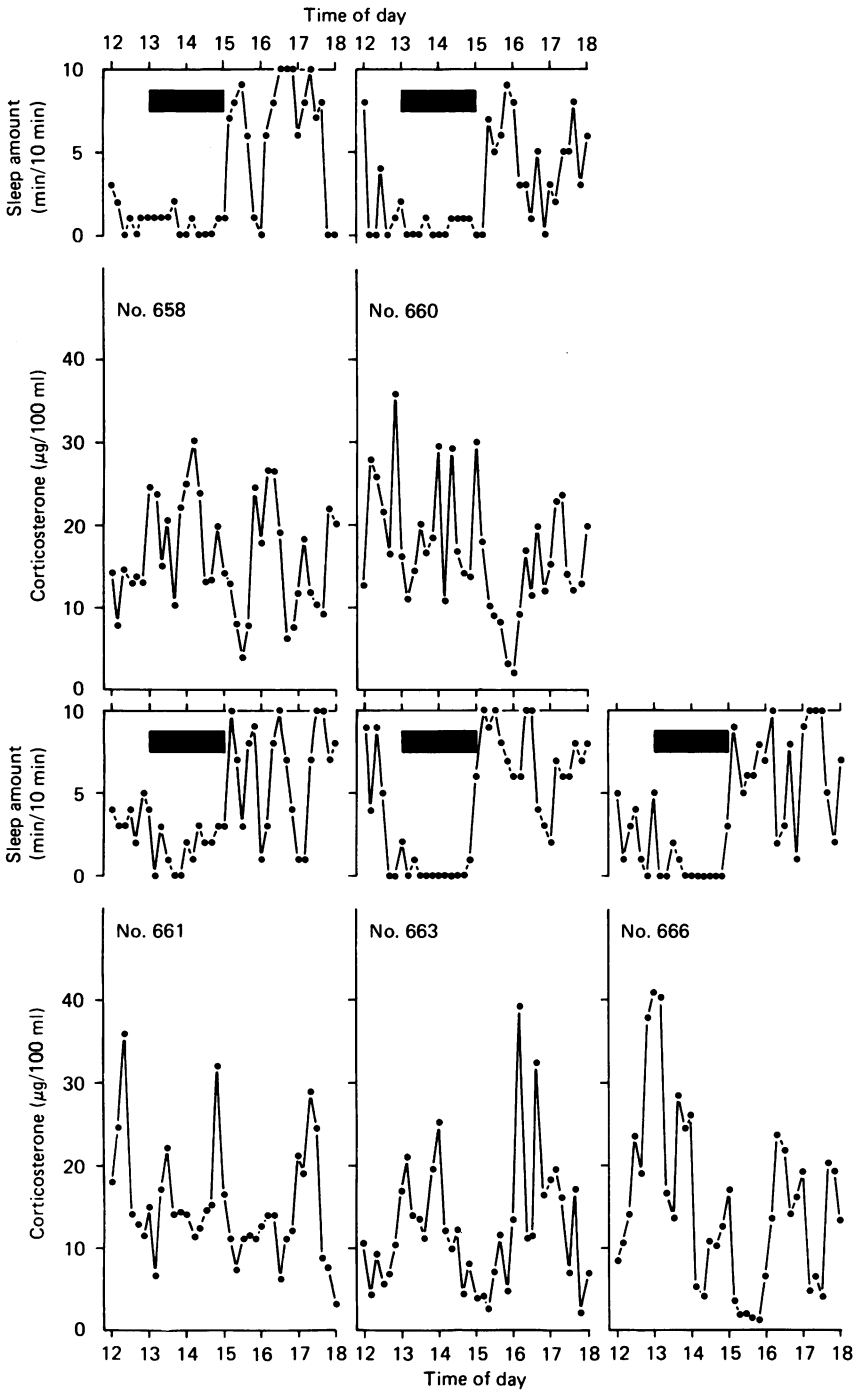

Fig. 4. Effect of a 2 h sleep-deprivation on corticosterone profiles. Individual corticosterone profiles plotted against the sleep–wakefulness cycle profiles in five representative animals are shown. The dark areas are periods of sleep deprivation. For further details see Figs 1 and 2.

From this high level corticosterone during the sleep-deprived period, it was not simply inferred that sleep deprivation acted as stressful stimuli. The analysis of the cross-correlation coefficients revealed significant negative coefficients at the time lags of 0 and 10 min between the amount of sleep and the corticosterone concentration, whereas it revealed significant positive coefficients at the time lags of 0 and 10 min between the amount of wakefulness and the corticosterone concentration. In addition, during the sleep-deprived period, the amount of wakefulness was significantly high compared with that during the undisturbed period. Therefore, it was very reasonable that a higher concentation of corticosterone was observed during the sleep-deprived period corresponding with a higher amount of wakefulness. Comparing the corticosterone value per 1 min wakefulness during the sleep-deprived period with that during the undisturbed period, it would apparently be said that the wakefulness during the sleep-deprived period never exerted a stronger effect on the secretion of corticosterone than the wakefulness did during the undisturbed period. This could be a strong evidence that the sleep deprivation procedure taken in the present experiment was not 'stress', if it was accepted that the 'spontaneous wakefulness' itself was not 'stress'.

DISCUSSION

The episodic secretion of GH, which appeared to be governed by an ultradian rhythm, was first demonstrated in the results of a sequential blood sampling experiment using cannulated adult male rats (Tannenbaum & Martin, 1976), and was found to have been developed by 22 days of age in an experiment using a similar cannulation method (Edén, 1979; Edén, Albertsson-Wikland & Isaksson, 1978). In the latter report, there was noted two distinct GH secretory episodes during a 6 h period from 09.00 to 15.00 h in the 30 day-old male rat sampled at 30 min intervals, which we confirmed first in the present experiment with 10 min sampling intervals. Further, the timing of a secretory episode, about 14·00 h, as observed in the present study, was consistent with that observed in the earlier studies. Although it was argued in the earlier reports (Tannenbaum & Martin, 1976; Edén, 1979) that it took approximately 7 days for animals to recover from the stress of surgery and anaesthesia (pentobarbitone sodium), our surgery and anaesthesia (ether) seemed not to affect the health of animals very much, based on the GH secretory patterns our animals showed. It was therefore considered acceptable to study the correlation between the GH secretion and sleep using this frequent sampling method in the immature rat.

The results of the present experiment indicate that the GH secretion in the rat is sleep-related, as has been proved in humans (Takahashi, Kipnis & Daughaday, 1968). The main items of evidence of this include the following: (1) GH concentrations in the blood demonstrated a significant positive correlation with the amount of sleep in the preceding 10–20 min; (2) the sleep deprivation performed during a period in which the majority of rats exhibit GH secretory bursts prevented them from occurring, and (3) the periodicity of GH secretory episodes was almost exactly the same as that for the sleep–wakefulness cycle. The attempt to correlate GH secretion with sleep has failed in the study using adult male rats (Willoughby *et al.* 1976*a*).

Despite the fact that they employed a quite similar method to ours, no correlation could be obtained between the GH level and sleep either in the visual inspection of hormone profiles and concurrent sleep phase patterns or on the scattergrams in which the duration of sleep in 5, 10 or 15 min intervals was plotted against the GH level. The reasons for their failure are not clear, but the major one seems to be that they scored the e.e.g. records into five stages adopting the method frequently used for humans. This would lead to the complication of sleep–wakefulness profiles, and thus the difficulty to correlate hormone levels, considering the fact that rats repeat much shorter sleep–wakefulness cycles than humans. Further, it has been reported that the structural component of the rat had only two stages: a slow-wave stage and a paradoxical sleep stage (Webb & Duke, 1981). Our treatment of e.e.g. data was rather simple and clear cut but seemed adequate for this type of pilot study.

The deprivation of sleep was drastically effective in preventing the occurrence of GH secretory bursts, while an acute increase in the amount of sleep after the cessation of sleep deprivation was accompanied by them. These results appear to clearly indicate the relationship of sleep to GH secretion, too. The possibility that the procedure for sleep deprivation acted as the 'stress' would be denied by the findings that the corticosterone secretory episode which occurred during the sleep deprivation was comparable in the amplitude to that during the undisturbed period and that the corticosterone value per 1 min wakefulness was not higher during the sleep deprivation than during the undisturbed period. That the secretion of corticosterone occurred episodically in the rat has been described by Carillo, Duke & Dunn (1980) and the results of the present experiment are in good agreement with that. In addition, the results of the present experiment indicate that the episodic secretion of corticosterone occurs in association with the spontaneous wakefulness in the rat, showing an inverse relationship to the GH secretion which occurs in association with the sleep.

That the sleep–wakefulness rhythm in the rat has an ultradian component of 2–4 h periodicity has been reported previously (Ibuka, Inouye & Kawamura, 1977). The periodicity in our rats determined by the analysis of the time series of sleep amounts during the 6 h period was 2·2–4·5 h, which was in agreeement with that of the latter report. Taking into consideration the close correlation between sleep amounts and GH levels clarified in the present experiment, it is possible that the sleep–wakefulness cycle is, in some manner, related to the ultradian rhythm of GH secretion and this idea finds support in the fact that the periodicity of GH secretion as determined in the present experiment was 2·1–4·5 h, being analogous to that for the sleep–wakefulness cycle.

The present study revealed that in rats the secretion of GH was linked to sleep as in humans. A similar correlation was indicated in dogs, but only under unphysiological condition, as after sleep deprivation (Takahashi, Ebihara, Nakamura & Takahashi, 1981). Further, in non-human primates (baboon and rhesus monkey), conflicting results have been reported on either the spontaneous correlation or the effect of sleep deprivation (Parker, Morishima, Koerker, Gale & Goodner, 1972; Jacoby, Sassin, Greenstein & Weitzman, 1974; Jacoby, Smith, Sassin, Greenstein & Weitzman, 1975; Quabbe, Gregor, Bumke-Vogt, Eckhof & Witt, 1981). Considering the fact that extensive findings have been accumulated for the rat on the brain control sites of GH secretion including the functional relationship of these neural sites with

the sleep mechanism (Frohman, Bernardis & Kant, 1968; Martin, 1972; Willoughby, Terry, Renaud & Brazeau, 1976*b*; Terry & Martin, 1981; Martin, Kontor & Mead, 1973), the present result may offer a great advantage in further investigation in the rat. This may mean that the rat can provide a good model of human sleep-related GH secretion.

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