Diurnal variations and sleep deprivation-induced changes in rat hypothalamic GHRH and somatostatin contents

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Gardi, J., O. Obá, J., J. Fang, J. Zhang, and J. M. Krueger. Diurnal variations and sleep deprivation-induced changes in rat hypothalamic GHRH and somatostatin contents. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1339–R1344, 1999.—Previous reports indicate that hypothalamic growth hormone-releasing hormone (GHRH) promotes sleep and is involved in sleep regulation. The aim of our experiments was to determine whether the GHRH and somatostatin contents of the rat hypothalamus have diurnal variations and whether they are altered by sleep deprivation (SD). Hypothalamic samples were collected at 10 time points during the 24-h light-dark cycle. SD started at light onset. Hypothalamic samples were obtained after 4 and 8 h of SD and after 1 and 2 h of recovery following 8 h of SD. The peptides were determined by means of radioimmunoassay. GHRH displayed significant diurnal variations with low levels in the morning (a transient rise occurred at 1 h after light onset), gradual increases in the afternoon (peak at the end of the light period and beginning of the dark period), and decreases at night. SD induced significant GHRH depletion, which persisted during recovery. The afternoon rise was delayed, and the nocturnal decline of somatostatin was more rapid than the changes in GHRH. Although the patterns of the diurnal variations in GHRH and somatostatin were similar, there was no significant correlation between them. SD did not alter somatostatin significantly. Comparisons of the present results with previously reported changes in hypothalamic GHRH mRNA suggest that periods of deep nonrapid eye movement sleep (first portion of the light period and recovery sleep after SD) are associated with intense hypothalamic GHRH release.

systemic administration in humans (21, 24, 43) and rats (33). GHRH consistently stimulates NREMS; the promotion of rapid eye movement sleep is variable. GHRH also enhances slow-wave activity in the electroencephalogram (EEG) during NREMS (13, 31, 32). Inhibition of endogenous GHRH by means of a competitive antagonist (34) or immunoneutralization (35) decreases sleep. NREM also decreases in transgenic mice deficient in the somatotropic axis including GHRH (54). NREM is rapidly suppressed in response to somatostatinergic stimulation in the rat (2). Hypophysectomy fails to inhibit the NREM-promoting activity of GHRH (33). It has been suggested that stimulation of GH secretion and NREMS are independent though normally synchronized functions of GHRH. Promotion of NREM is attributed to GHRHergic neurons projecting to the basal forebrain, where GHRH acts as a neurotransmitter-neuromodulator (11, 25, 41).

Long periods of wakefulness elicit sleepiness followed by enhanced sleep. Sleep deprivation (SD) is therefore a widely used tool to stimulate the sleep process. The duration, continuity, and intensity of NREM all increase during recovery (8, 15, 26, 36). Enhancements in NREM precede increases in rapid eye movement sleep after SD (16, 47). The intensity (depth) of NREM is characterized by the slow-wave activity in the EEG (7). In the normal, undisturbed rhythm of sleep-wake activity, high-intensity NREM occurs during the first portion of the sleep period immediately following the circadian active phase (5, 8, 22). Indeed, there is a diurnal rhythm in hypothalamic GHRH mRNA levels (10) and SD-induced changes in hypothalamic GHRH mRNA also occur in the rat (48, 53). However, hypothalamic GHRH and somatostatin contents at various time points of the diurnal cycle and during and after SD have not heretofore been determined. The results indicate that changes in GHRH but not somatostatin correlate with NREM.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (body weights 300–320 g) were acclimated to a 12:12-h light-dark cycle (light on 0800) by housing them in individual cages placed in environmental chambers for at least 2 wk before the experiments. Ambient temperature was regulated at 26 ± 1°C. To study the diurnal rhythm in hypothalamic peptide content the rats were killed at 4-h intervals starting 1 h after light onset. To study the diurnal rhythm in hypothalamic peptide content the rats were killed at 4-h intervals starting 1 h after light onset. In the experiments with SD, the rats were sleep deprived by gentle handling starting at light onset. Rats were killed at the end of 5 (n = 9) and 8 h (n = 9) of SD and after 1 (n = 10) and 2 h (n = 9) of recovery following the termination of SD. The time-matched control rats were undisturbed and killed at hours 4,
7, 9, and 10 of the light period. In addition, one group of rats was killed at light onset (hour 0). To help in the determination of the diurnal rhythms of the peptides, the results obtained from the controls of the SD groups are plotted together with the data collected in the diurnal rhythm experiments so that the peptide contents are depicted for hours 0 (n = 10), 1 (n = 10), 4 (n = 8), 5 (n = 11), 8 (n = 9), 9 (n = 19), and 10 (n = 10) in the light period and hours 13 (n = 12), 17 (n = 11), and 21 (n = 10) in the dark period. In both experiments, only 1 to 3 rats were killed simultaneously at a particular time point in one session, and the sessions were repeated until a sample size consisting of a minimum of eight rats was obtained for each time point. A guillotine was used to kill the rats. The hypothalamus (landmarks: optic chiasma, lateral sulci, mammary bodies, and a depth of 2 mm) was removed within 1 min and stored at −70°C until assayed. The landmarks for the dissection of the hypothalamus corresponded to those used by Katakami et al. (19) and Gil et al. (18) to measure hypothalamic GHRH contents. Calculated with respect to hypotalmic samples, our GHRH values (1,800–2,800 pg/hypothalamus) are in the range with those reported by Gil et al. (18), approximately 1,800–1,900 pg/hypothalamus, but higher than the hypothalamic GHRH content, 900 pg/hypothalamus, found by Katakami et al. (19). The differences can be due to differences in the antisera and the reference peptide.

The frozen samples were weighed and placed in tubes containing 0.5 ml of 2 M acetic acid and then boiled for 5 min (19). The tissues were individually homogenized by means of ultrasound. The homogenates were centrifuged at 15,000 rpm for 20 min at 4°C. Aliquots were taken for protein measurements (9), and the rest of each supernatant was lyophilized. The recoveries of the extraction were 70–75% for GHRH and 90–95% for somatostatin. Radioimmunoassay was used to determine GHRH and somatostatin contents. The kits were purchased from Peninsula Laboratories (standards rat GHRH 90–95% for somatostatin. Radioimmunoassay was used to determine GHRH and somatostatin contents. The kits were purchased from Peninsula Laboratories (standards rat GHRH 43 and somatostatin 1–14). According to the supplier, the antisera to rat GHRH exhibited 100% cross-reactivity with human GHRH, whereas it did not recognize peptides as follows: His3-Nle27-GHRH 1–32, porcine GHRH, human parathyroid hormone 1–34, rat peptide histidine-isoleucine, and human, rat, and porcine vasoactive intestinal peptide. There was a 100% cross-reaction between the antisera to somatostatin and somatostatin 25, somatostatin 28, and [Des-Ala1]-somatostatin, 0.05% cross-reaction with [d-Trp6]-somatostatin, and 0.01% cross-reaction with prosomatostatin 32. The antisera to somatostatin failed to recognize the somatostatin analogs RC-160 and CTP-NH2, human amylin amide, human glucagon, human insulin, porcine neuropeptide Y, substance P, and human, rat, and porcine vasoactive intestinal peptide. The peptides were measured in triplicate with a sensitivity of 8 pg/tube for GHRH and 4 pg/tube for somatostatin. The intra- and interassay coefficients of variation were 4.5 and 5.0 for GHRH and 3.4 and 11.7 for somatostatin, respectively.

The peptide content of the individual samples were averaged for each time point. One-way analysis of variance (ANOVA) was used to detect significant differences among the various time points across the 24-h cycle. The changes in GHRH and somatostatin during and after SD were evaluated with respect to the time-matched control samples by means of two-way ANOVA with the treatment (SD vs. controls) and the time (hours after light onset) as the two factors. Student's t-tests were used to compare mean peptide contents between the light and dark periods, and Pearson Product Moment Correlation was calculated to compare variations in tissue mass and GHRH somatostatin contents. An alpha-level of P < 0.05 was considered to be significant in all tests.

RESULTS

The wet weights of the hypothalamic samples varied between 25 and 30 mg without significant differences among the individual time points. In contrast, significant variations were observed in the protein contents among the samples [F(9,100) = 3.42; P < 0.05]. This finding is consistent with previous reports on diurnal variations in hypothalamic protein contents (1, 28). Although the patterns of variations in GHRH and somatostatin contents expressed as picogram per milligram tissue or nanogram per microgram protein did not differ, only peptide contents expressed as picogram per milligram tissue (weight wet) are presented and will be discussed.

GHRH. Hypothalamic GHRH content exhibited significant diurnal variations [F(9,100) = 3.29; P < 0.05] (Fig. 1). GHRH content was low at light onset. A transient rise in GHRH was observed in hour 1 after light onset, and then GHRH dropped to a minimum occurring by the middle of the day. GHRH content increased gradually during the second portion of the light period. Peak values were observed at the end of the light period and at the beginning of the dark period. GHRH declined steadily during the night. A transient rise in GHRH content was observed in hour 1 after light onset, which was followed by another decline. Minimum GHRH content occurred in the middle of the day. The mean GHRH values during the 12-h light and 12-h dark periods did not differ ([light 74.8 ± 3.19 (SE) pg/mg, dark 81.5 ± 3.2 pg/mg, Student's t-test]. There were no correlations between the wet mass of the
SD-induced changes in hypothalamic GHRH content.

Hypothalamic GHRH contents differed significantly between the control groups and the SD animals \( [F(1,72) = 13.24; P < 0.05] \) (Fig. 1, bars). GHRH content was normal after 4 h of SD and dropped to a low level by the end of hour 8 of SD. GHRH was significantly suppressed after 1 h of recovery. Although GHRH content started to rise by the end of hour 2 of recovery it was still significantly below normal. Significant variations in time and interactions between the treatment and time factors were not obtained.

Somatostatin. Somatostatin content of the hypothalamus also varied significantly across the 24-h day \( [F(9,99) = 4.53; P < 0.05] \) (Fig. 2). Starting from a low value at light onset, a transient rise occurred in somatostatin content 1 h after light onset and then somatostatin declined. These variations were similar to those observed for GHRH. Somatostatin contents stayed low in the afternoon and started to rise only around dark onset. The peak of somatostatin content occurred 1 h after dark onset. Somatostatin declined rapidly at night and dropped to low levels by morning. Mean somatostatin contents during the light period \( [1,576.0 \pm 60.6 \text{ (SE)} \text{ pg/mg}] \) were significantly lower than at night \( (2,031.6 \pm 129.0 \text{ pg/mg}). \) Somatostatin did not correlate with hypothalamic mass. Changes in somatostatin failed to correlate with the variations in hypothalamic GHRH content (Pearson Product Moment Correlation 0.6179; \( P = 0.0569 \)).

Although hypothalamic somatostatin content tended to increase during SD these changes with respect to baseline values did not reach the level of statistical significance (Fig. 2, bars). Also, there were no significant differences in somatostatin among the time points in the SD experiment. Correlations were not found between somatostatin contents and variations in GHRH during and after SD.

**DISCUSSION**

Current results demonstrate diurnal variations and SD-induced changes in hypothalamic GHRH content. In rats the duration and the intensity of NREMS peak at the beginning of the light period. Both of these parameters of NREMS decline toward dark onset. Rats sleep relatively little at night, but the intensity of NREMS increases \( (5, 8, 22) \). The SD protocol used in our experiments induces recovery sleep with intense NREMS for a few hours \((47) \). Therefore, the periods that are likely to be associated with simultaneous increases in GHRH release and synthesis, the first portion of the light period and recovery, correlate with the period of most intense NREMS. It is assumed that the intensity of NREMS is a function of prior wakefulness \((7) \). Enhancements in EEG power densities \( (15) \) and increases in EEG amplitudes \( (16) \) can already be demonstrated in the waking EEG during SD and are regarded as markers of the homeostatic process resulting in the sleep drive. If depletion of hypothalamic GHRH indicates GHRH release at night and during SD then GHRH may be part of the homeostatic process responsible for somnolence. It is noted that the SD-induced variations in GHRH might be due in part to stress associated with the procedure of SD. If the stress component exists, then it is inherently linked to SD and it is also involved in the mechanism of sleep alterations. Irrespective of whether the changes in GHRH release result from the sleep loss per se or from stress, the role of GHRH in the recovery sleep after deprivation is supported by the previous observation that immunoneutralization of GHRH blocks or attenuates the recovery sleep \((35) \).

Promotion of sleep is attributed to GHRHergic neurons residing outside of the arcuate nucleus. These neurons project to various structures in the basal forebrain, including the medial preoptic area \((25, 41) \). Several lines of evidence indicate that this region has a fundamental role in the regulation of NREMS \( (44) \). The basal forebrain is also implicated in the mediation of the effects of GHRH on NREMS, e.g., microinjection of GHRH into the medial preoptic area enhances sleep \((55) \). Studies using in situ hybridization \((48) \) demonstrate that both the diurnal variations and the SD-induced changes in GHRH mRNA occur in extra-arcuate GHRHergic neurons. There is, however, an interesting dissociation between these events: diurnal variations are observed in the periventric-medial GHRHergic neurons, whereas SD increases GHRH mRNA in the paraventricular nucleus. It is possible therefore that different GHRHergic neurons provide circadian and homeostatic inputs for sleep regulation.

Considering the opposite effects of GHRH and somatostatin on both GH secretion and sleep, it was an attractive idea to determine both peptides from the same hypothalamic samples. Unlike GHRH, however, somatostatin is a pleiotropic neurotransmitter in inter-neurons involved in many functions throughout the hypothalamus (reviewed in Ref. 6). Somatostatin content was not significantly altered by SD, although somatostatin displayed diurnal variations. Previously, Nicholson et al. \((30) \) reported that hypothalamic somatostatin content declines during the first portion of the

![Fig. 2. Diurnal (● and lines) and SD-induced (bars) variations in hypothalamic somatostatin contents. See Fig. 1 for additional data.](image-url)
light period. Berelowitz et al. (3) described progressive in vitro somatostatin release during the light period in hypothalamic samples obtained at various time points. Somatostatin concentrations in the cerebrospinal fluid and in hypothalamic portal vessels are higher at night than during the day (4); the large drop in somatostatin content of the hypothalamus might reflect this enhanced release. The diurnal rhythm of somatostatin content in our hypothalamic samples differ from the variations in somatostatin in the suprachiasmatic nucleus (42) or the anterior hypothalamus, which includes the periventricular area, e.g., the major source of the hypophyseotropic somatostatinergic neurons (17). Despite the obvious similarities in their diurnal oscillations, statistically significant correlations could not be demonstrated between the fluctuations of GHRH and somatostatin. Current findings do not exclude the possibility that somatostatin also contributes to sleep regulation. Somatostatinergic stimulation alters sleep (2), and we detected reciprocal changes in the somatostatin and GHRH mRNA levels in the hypothalamus in response to SD (53). The interaction between somatostatin and GHRH is also well documented in the regulation of GH secretion. Our data, however, show that hypothalamic somatostatin contents cannot be simply linked to the activity of the somatotropic axis or sleep without reference to specific nuclei.

Although the kinetics of translation, and those of peptide release, degradation and elimination are not known, comparisons between GHRH mRNA levels and peptide contents may allow some reasonable conclusions. Hypothalamic GHRH mRNA peaks around light onset, declines gradually throughout the light period, and stays at very low levels at night (10). Therefore, the transient rise in GHRH content in the morning may indicate a period of peptide synthesis. In our previous mRNA studies, samples were taken 3 h before light onset and 1 h after light onset. GHRH mRNA peaked 1 h after light onset. An instantaneous translation is unlikely. It is assumed therefore that peak transcription occurs 1 to 2 h earlier than the transient rise in peptide content. The finding that hypothalamic GHRH mRNA is already enhanced at light onset (48) supports this interpretation. A 1- to 2-h interval between the increases in GHRH mRNA and peptide contents corresponds to the kinetics of translation of GHRH in the arcuate nucleus (52). The drop in GHRH content after the morning peak indicates a rapid release exceeding the rate of synthesis until mid-day. The late afternoon rise in GHRH content is attributed to a combination of a continual peptide synthesis and a low rate of release. The decline in GHRH content at night may result from a release or degradation without significant synthesis as indicated by the steady, low level of GHRH mRNA.

Increases in hypothalamic GHRH mRNA are detected after SD starting at light onset and lasting for 6 (48), 8, or 12 h (53). GHRH content decreases significantly by the end of an 8-h SD, and stays low for 1 to 2 h during recovery. Considering that GHRH mRNA is already higher than normal at least 2 h before the termination of the 8-h SD then the low postdeprivation peptide content may occur despite a high rate of synthesis. Unfortunately, it is not known whether translation is altered during SD. Stimulation of transcription during SD may be a regulatory response to the depletion of GHRH sometime between hour 4 when the GHRH content is normal and hour 6 when GHRH mRNA is already enhanced. It is noted that the SD-induced changes in GHRH mRNA may differ between the light and dark periods. Thus Toppila et al. (48) report that a 12-h SD during the dark period fails to stimulate GHRH mRNA. GHRH mRNA, however, increases by light onset due to the diurnal rhythm, and this may mask the effects of SD.

Secretion of GH is pulsatile with surges occurring at 3- to 4-h intervals throughout the day in male rats (46). GH surges are timed to releases of GHRH into the pituitary portal vessels (37). In rats, light has a synchronizing effect on the ultradian rhythm of GH secretion, but there are no diurnal variations in GH release (46, 51). GH secretion tends to be suppressed during SD in humans (40, 45) and rats (20), although there are some inconsistencies among the reports (39), and the deprivation-associated decreases in plasma GH concentrations might depend on age (29). There is therefore no evidence implicating the observed diurnal variations or SD-associated changes in hypothalamic GHRH contents or GHRH mRNA in the regulation of GH secretion.

That the diurnal or SD-induced changes in hypothalamic GHRH contents, and GHRH mRNA are not linked to GH secretion is supported by fundamental observations in an in situ hybridization experiment by Toppila et al. (48). The hypophyseotropic GHRH neurons reside in the arcuate nucleus (11, 41). The arcuate nucleus is also the structure where 3-h oscillations are observed in GHRH mRNA in correlation with GH release (52). Neither the light-dark cycle nor SD alters GHRH mRNA in the arcuate nucleus (48). In contrast, SD results in increases in somatostatin mRNA levels in the arcuate nucleus (48). The somatostatinergic cells in the arcuate nucleus are intranuclear interneurons (6) that inhibit GHRH release (23). It seems therefore that the activity of the hypophyseotropic GHRH neurons is suppressed rather than enhanced during SD.

In addition to the role of hypothalamic GHRH in the regulation of GH secretion and NREMS, GHRH is also implicated in the regulation of food intake (49). Food intake shows diurnal variations in the rat with most feeding occurring at night and a sharp drop in feeding activity around light onset followed by a gradual increase during the rest of the day (38). There are correlations between meal size and intermeal sleep (12). Food intake increases during chronic SD lasting for weeks (14). We are, however, not aware of any reports describing feeding during short-term SD as that used in our experiments, although postdeprivation food intake is not altered by short-term SD (8). Bouts of feeding occurred during SD in our rats, but qualitatively this activity did not seem to be different from feeding occurring periodically during the day. It there-
fore remains to be determined if variations in hypothalamic GHRH contents correlate with feeding activity. In conclusion, the diurnal and SD-induced variations in hypothalamic GHRH content correlate with NREMS. Previous reports on diurnal and SD-associated variations in hypothalamic GHRH mRNA promote understanding of the changes in peptide content in terms of synthesis and release. The findings are consistent with the suggested role of GHRH in the promotion of NREMS.

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