EVIDENCE FOR THE ROLE OF GHRH IN THE REGULATION OF SLEEP

Ph.D. thesis

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PAPERS ATTACHED TO THE THESIS

I. Gardi J, Obál F Jr, Fang J, Zhang J, Krueger JM: Diurnal variations and sleep deprivation-induced changes in rat hypothalamic GHRH and somatostatin contents. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* 277: R1339-R1344, 1999.

II. Gardi J, Szentirmai É, Hajdú I, Obál F Jr, Krueger J M: The somatostatin analog, octreotide, causes accumulation of growth hormone-releasing hormone and depletion of angiotensin in the rat hypothalamus. *Neurosci. Lett.* 315: 37-40, 2001.

III. Obál F Jr, Fang J, Taishi P, Kacsóh B, Gardi J, Krueger JM: Deficiency of growth hormone-releasing hormone signaling is associated with sleep alterations in the dwarf rat. *J. Neurosci.* 21: 2912-2918, 2001.

IV. Gardi J, Krueger JM, Speth RC: Preparation and a simple one-step purification of [His^{1}-mono-^{125}I-Tyr^{10},Nle^{27}]-hGHRH(1-32)-NH_2. *J. Labelled Cpd. Radiopharm.* 45: 13-18, 2002.

V. Gardi J, Speth RC, Taishi P, Kacsóh B, Obál F Jr, Krueger JM: Alterations in GHRH binding and GHRH receptor mRNA in the pituitary of adult *dw/dw* rats. *Peptides* 23: 1497-1502, 2002.

VI. Gardi J, Taishi P, Speth RC, Obál F Jr, Krueger JM: Sleep loss alters hypothalamic growth hormone-releasing hormone receptors in rats. *Neurosci. Lett.* 329: 69-72, 2002.

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of Variance
cAMP	Cyclic Adenosine-Monophosphate
cDNA	Complementary Desoxi-Ribonucleic Acid
EEG	Electroencephalogram
GABA	γ-Amino-Butyric Acid
GH	Growth Hormone
GHRH	Growth Hormone-Releasing Hormone
GPCR	G-Protein Coupled Receptor
HPLC	High Pressure Liquid Chromatography
icv	Intracerebroventricular
IGF-I	Insulin Like Growth Factor-1
mRNA	Messenger Ribonucleic Acid
NREMS	Non-Rapid Eye Movement Sleep
REMS	Rapid Eye Movement Sleep
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sst1-5	Somatostatin Receptor 1-5
TMD	Transmembrane Domain
VIP	Vasoactive Intestinal Peptide

1. INTRODUCTION

GH is synthesized, stored, and secreted by the pituitary somatotrophs. Its secretion is pulsatile in nature. In man, secretory bursts of the hormone occur 4 to 8 times over a 24-h period, with maximal secretion occuring within 1 h after the onset of sleep. Pulsatile secretion of the hormone is believed to be important as regards the physiological action on target tissues. The synthesis and secretion of GH from pituitary somatotroph cells is tightly regulated by several factors, including neurotransmitters such as GABA, neuropeptide Y, peptide hormones such as ghrelin and IGF-I, steroid hormones, and the opioids (1-5). Hypothalamic GHRH and somatostatin are the predominant stimulatory and inhibitory neuroendocrine signals, respectively, for GH secretion by pituitary somatotroph cells (6,7). GHRH stimulates GH secretion and GH synthesis and also promotes somatotroph proliferation (8,9). Somatostatin acts on the pituitary to suppress basal and stimulated GH secretion (10). Each GH secretory episode seems to be initiated by a burst of GHRH release into the hypophyseal portal system, preceded by a reduction of somatostatinergic input both to the pituitary portal circulation and to hypothalamic GHRH neurons (11).

1.1 *GHRH*

GHRH was first isolated from human pancreatic tumors by Guillemin et al. (12) and by Rivier et al. (13) in two forms, as GHRH(1-40)-OH and GHRH(1-44)-NH_2 polypeptides. Both variants are generated from the same precursor (prepro-GHRH), with the full biological activity residing in the first 29 amino acids (14). This 29-residue core has an α-helical conformation (15), and enhancement of the amphipathic α-helical properties of GHRH by alanine replacement results in an increased receptor affinity and potency demonstrated *in vitro* (16). The mature peptide is amidated at the carboxy-terminus in many species, but not in rodents. Hypothalamic and pancreatic GHRH appear to be identical. GHRH belongs to the secretin-glucagon peptide family displaying structural homology with secretin, pituitary adenylate cyclase-activating peptide, VIP, glucagon, glucagonlike peptide-1, gastric inhibitory peptide, and peptide histidine isoleucine/peptide histidine methionine (17). GHRH shares nine residues with VIP, with the greatest similarities occuring in the N-terminal regions of the peptide. The structure of human pancreatic GHRH is

Tyr-Ala-Asp-Ala-Ile-Phe-Tyr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2

In addition to its GH-releasing activity, GHRH is implicated in the control of appetite (18), and sleep (19,20).

Most GHRH containing neurons are found in the arcuate nucleus of the hypothalamus and the pituitary stalk, but neurons of the periventricular area and paraventricular nucleus also contain significant amounts of GHRH. Axons of GHRH neurons of the the arcuate nucleus project to the median eminence and terminate near the capillaries of the primary capillary plexus that in turn is drained by pituitary portal circulation. Release of GHRH into the portal vessels is episodic, contributing to the pulsatile release of GH from the pituitary. GHRH containing perikarya found in the ventromedial nuclei and extrahypothalamic structures do not project to the median eminence but to several other hypothalamic and extrahypothalamic regions, suggesting a possible neuromodulator role of GHRH in the central nervous system. In addition, GHRH has been found in the placenta (21,22), where it might participate in the regulation of fetal GH secretion and is expressed in ovary (23) and testis (24), possibly acting as an intragonadal regulatory factor (25,26). It has been detected in lymphocytes, where it may be involved in immune modulation (27), and in the pancreas and the gastrointestinal tract (28,29), where it possibly regulates the secretion of hormones from both the exocrine and endocrine pancreas (30-32).

1.2 *GHRH receptor*

GHRH receptor expressed predominantly in the anterior pituitary gland, as expected on the basis of its functional role in the regulation of GH secretion. Specific high-affinity binding sites for GHRH have been demonstrated on pituitary membranes using various iodinated GHRH analogs (33-36). This binding was inhibited by guanine nucleotides, suggesting that G-proteins were involved in GHRH action (37). These observations and the finding that cAMP is a key second messenger for GHRH action, suggested that the actions of GHRH were mediated through a GPCR.

GHRH receptor cDNAs from rat (38,39), mouse (38), swine (40), and humans (39,41) have been identified. The amino acid alignment deduced from the human cDNA revealed that the GHRH receptor consists of 423 amino acids containing seven hydrophobic domains with the

potential to serve as membrane-spanning helices. The mouse and rat receptors share 94% identity at the amino acid level and both of these are 82% identical to the human receptor. The protein has a signal sequence and seven TMDs, with a 127-residue-long extension upstream of the first TMD predicted to form an amino-terminal extracellular domain. In addition to the presence of seven TMDs, the predicted GHRH receptor protein has other characteristic features of GPCR, including conserved cysteine residues in the first and second extracellular loops, a cysteine residue in the C-terminal tail that may be palmitoylated, a site for N-linked glycolisation sites in the amino-terminal domain, potential phosphorylation sites in the third intracellular loop, and several residues within the TMDs that are highly conserved among all GPRCs. The GHRH receptor has a high degree of homology to the receptors for GHRH-related peptide hormones including secretin, VIP, glucagon, glucagonlike peptide-1, pituitary adenylate cyclase-activating peptide, and gastric inhibitory peptide receptors, all of which are classified into family B-III of the GPCR superfamily (42). These receptors exhibit a high degree of overall homology, which is highest in the TMDs, and share several distinct structural features, including six cysteines, an aspartate, a trypthophan, and a glycin in the amino-terminal domain that are highly conserved among all the receptors. These receptors are more distantly related to the receptors for parathyroid hormone, calcitonin, and corticotropin-releasing factor, which are also classified into family B of the GPCR superfamily.

In response to GHRH, the GHRH receptor activates adenylate cyclase, resulting in an increase in intracellular cAMP levels (39). It leads to stimulation of the cAMP-dependent protein kinase A, which in turn phosphorylates numerous cytoplasmic and nuclear substrates (43). One of the best characterized protein kinase A nuclear substrates is the transcription factor CREB (cAMP-responsive element binding protein), which stimulates transcription of cAMP-responsive genes after its phosphorylation by protein kinase A (44). GHRH does not stimulate phosphoinositide breakdown in primary pituitary cells (45,46), suggesting that the receptor does not couple to the phospholipase C pathway. In addition to stimulating the adenylate cyclase pathway, GHRH also activates the mitogen-activated protein kinase pathway in both pituitary cells (47,48) as well as in nonpituitary cells expressing the GHRH receptor (49). Activation of the mitogen-activated protein kinase pathway likely mediates the mitogenic activity of GHRH in somatotroph cells (48).

The GHRH receptor mRNA is expressed predominantly in the pituitary gland, can be detected begining with the late embryonic period, and restricted to the anterior lobe of the gland

(38,39). GHRH receptor mRNA has also been found in the placenta (50), the kidney (50,51), and the hypothalamus (52). Using sensitive RT-PCR/Southern blotting assays, the GHRH receptor transcript has been found in an extremely wide range of rat tissues at low levels (51) although the physiological significance of this broad expression remains unclear. GHRH receptor-immunoreactive protein has been demonstrated only in the pituitary and the kidney (53,54).

Several isoforms of the GHRH receptor have been identified in species including the mouse (38,55), rat (39,52,55), pig (40), and human (56-59).

1.3 *Sleep*

Rest-activity rhythms are displayed by each species throughout the animal kingdom. In mammals, two states of sleep are distinguished: REMS and NREMS. The states of sleep are fundamentally different in terms of regulation and EEG activity. The structures involved in the generation of REMS reside in the brainstem. In contrast, forebrain structures (anterior hypothalamus/preoptic region, thalamus) have been implicated in the genesis of NREMS. These areas of the forebrain are the origin of projection pathways that synchronize the activities of distant parts of the brain. Otherwise, the regulation of NREMS might be local (60). Periods of REMS and NREMS alternate during sleep. Besides the duration, NREMS has another measure: intensity (depth of NREMS). The intensity of NREMS is characterized quantitavely by the slow wave activity in the EEG. Deep NREMS with intense slow wave activity is observed during the first sleep cycles of the diurnal rest period. The duration of REMS epochs increases towards the end of sleep resulting in an increased time spent in REMS in the second part of the rest period. These principles of sleep organization are easily recognized in each species with a strong circadian rhythm. Thus, in spite of the phases of the rest period are different in humans and rats (rats are active at night), there is a high degree of similarity in the organization and regulation of sleep between these species (61).

Secretions of many hormones vary in synchrony with sleep-wake activity. These variations are, however, not linked to sleep. Sleep exhibits circadian rhythm and the circadian pacemaker may also modulate hormone production and/or release. That variations in hormone secretions are in fact associated with sleep or they are subject to circadian regulation can be distinguished by means of sleep deprivation. Currently, the biological significance of sleep-associated alterations in hormone secretions is not clear. On the one hand, these changes in hormone release may have importance for endocrinology. On the other hand, hormones may

act as sleep-promoting or arousing substances and might be involved in the regulation of sleep-wake activity.

1.4 *The somatotropic axis in sleep regulation*

Pituitary GH secretion is stimulated by hypothalamic GHRH and is inhibited by somatostatin. GH acts in part directly and in part indirectly via IGF-I in the tissues. IGF-I is both a paracrine/autocrine produced locally and a hormone released from the liver in response to GH. There are also various negative feedback loops in the somatotropic axis. The somatotropic axis is a major humoral mechanism regulating sleep-wake activity. Deep NREMS is associated with GH secretion (62). GHRH promotes sleep, particularly NREMS in both animals (19,20,63) and humans (64-66). GHRH enhances both the duration and the intensity (slow wave activity of the EEG) of NREMS. This is a hypothalamic action of GHRH and independent from the stimulation of GH secretion since hypophysectomy does not block promotion of NREMS by exogenous GHRH (67). Independently from GHRH, GH may increase the intensity of NREMS (68) and the duration of REMS (69-71). NREMS decreases when GHRH is inhibited by means of a competitive receptor antagonist (72) or by the activation of the negative feedback in the somatotropic axis, e.g., after high doses of GH (71), IGF-I (73), or after somatostatinergic stimulation (74,75). Hypothalamic GHRH mRNA content varies with sleep-wake activity (76). Sleep deprivation stimulates GHRH synthesis (77,78), and GHRH is involved in the mediation of the enhancements in sleep following sleep deprivation (79). Microinjection of GHRH into the medial preoptic region is followed by large increases in NREMS whereas GHRH antagonist administered into the same area inhibits NREMS (80). The preoptic region is known to be involved in sleep regulation (81), and it is also receives projections from extraarcuate GHRHergic neurons (82). It is assumed, therefore, that the preoptic region is the NREM-specific somnogenic action site of GHRH. GHRH applied into the preoptic region fails to alter REMS (80). Stimulation of REMS is regarded as an indirect effect of GHRH involving GH (67).

In addition to GHRH, other members of the somatotropic axis may also modulate sleep. Acute administration of GH elicits increases in REMS in humans (71), rats (69), and cats (70). IGF-I may promote NREMS (83), and somatostatin may stimulate REMS (84,85). In addition, acute rises in somatostatin, GH, and IGF-I can suppress sleep via negative feedback inhibition of GHRH (71,73-75). Somatostatin inhibits GHRHergic neurons in the hypothalamus, and thereby it may also decrease NREMS. Somatostatin modulates the activity

of the somatotropic axis in multiple ways. It is delivered to the pituitary gland by the blood where it inhibits GH secretion. Somatostatin also inhibits GHRH-containing neurons in both the arcuate nucleus and the median eminence. Five somatostatin receptors have been determined in the brain (sst1-5). Inhibition of the somatotropic system is attributed to the sst2 receptors (86).

1.5 *Octreotide*

The sequence of octreotide is as follows: DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol) (87). The advantage of using octreotide instead of somatostatin is that octreotide is 20 to 75-fold more potent than somatostatin in inhibiting GH secretion, and it is highly resistant to enzymatic breakdown. Somatostatin is eliminated in a few minutes whereas the half-life of octreotide is 45-110 min in the tissues (88). Octreotide is a strong agonist on the sst2 and sst5 receptors, displays a modest affinity to sst3 receptors, and does not bind to sst1 and sst4 recpetors (89).

Previous experiments in our laboratory indicate that icv administered octreotide induces prompt drinking, vasopressin secretion and rises in blood pressure (75,90). It is well-documented that icv. administration of angiotensin II elicits the same triple response (91). Inhibition of the angiotensin convertase I-enzyme by captropil blocks the octreotide-induced responses (90). It is likely therefore, that octreotide acts via the central angiotensinergic system. Octreotide also alters sleep: octreotide injection elicits an immediate suppression in NREMS followed by enhancements in EEG slow wave activity starting 2 to 3 h post-injection. (75,85). These sleep changes correlate with the effects of octreotide on GH-secretion: octreotide causes prompt inhibition of GH-secretion, and GH-secretion recovers when sleep time is normalized and enhanced slow wave activity is observed in the EEG. It is hypothesized that inhibition of GHRH by octreotide is the cause of the immediate sleep suppression whereas the late enhancements in deep NREMS result from an increased GHRH-activity as the inhibition vanishes.

1.6 *Aims*

The experiments reported herein were carried out in rats with the following specific aims:

1. To study sleep-associated changes in hypothalamic GHRH and somatostatin contents (Paper #1),

2. To analyze changes in hypothalamic GHRH-, angiotensin II-, and vasopressin-content after somatostatinergic stimulation (Paper #2),

3. To characterize GHRH-R mRNA levels and GHRH content of the hypothamus, and its relationship to spontaneous sleep in chronic somatotropic deficiency (Paper #3),

4. To develope a simple, alternative method to label a GHRH analog with ^{125}I, and demonstrating of its utility in a binding assay for GHRH receptors (Paper #4),

5. To characterize GHRH receptor binding and mRNA in the pituitary of dwarf (*dw/dw*) and normal Lewis rats (Paper #5),

6. To study the effects of sleep deprivation on GHRH receptors in the hypothalamus and in the pituitary (Paper #6).

The experiments, including the methods, results and discussion of the data are detailed in the published Papers attached.

2. METHODS

2.1 *Animals*

Adult male outbred Sprague-Dawley rats (body weights 300-320 g), or adult male (approximately 4-month-old) inbred normal Lewis and *dw/dw* Lewis rats were used in the experiments. The rats were housed in individual cages. The cages were placed in recording rooms with a 12:12 h light-dark cycle and with an ambient temperature 26 ± 1 °C. The rooms were sound attenuated. The rats were kept in conditions identical to those in the recording rooms for at least 1-2 weeks before the experiments. Food and water were continously available.

2.2 *Sleep deprivation*

In the experiments with sleep deprivation, the rats were sleep deprived by gentle handling starting at light onset while the rats stayed in their home cage. Whenever behavioral signs of sleep were observed, the rats were aroused by knocking on the cage or touching them.

2.3 *Surgical procedures*

Surgery was carried out under ketamine-xylazine (87 and 13 mg/kg, respectively) anaesthesia. An icv cannula was implanted into the left cerebral ventricle [0.80 mm posterior from bregma, 1.4 mm lateral from midline, and 3.5 mm caudal from the top of the skull according to the rat brain atlas by Paxinos and Watson (92)].

2.4 *Collection of the brain samples*

The rats were sacrificed with a guillotine. The hypothalamus (landmarks: optic chiasma, lateral sulci, mamillary bodies, and depth of 2 mm) and/or the hypophysis was removed within 1 min and stored -70 °C until assayed.

2.5 *Determination of hormones*

The frozen samples were weighed. For extraction, samples were boiled for 5 min in 0.5 ml 2M acetic acid. The tissues were individually homogenized by means of ultrasound. The homogenates were centrifuged at 15000 g for 20 min at 4 °C, aliquots were taken for protein measurement (93), and the rest of each supernatant was lyophilized for the assays.

GHRH, somatostatin, and vasopressin were determined by radioimmunoassay (Bachem/Peninsula Laboratories, San Carlos, CA, USA) whereas angiotensin-II was

measured by enzyme immunometric assay (SPI-BIO, France). The peptides were measured in duplicate or triplicate with a sensitivity of 8 pg/tube for GHRH, 4 pg/tube for somatostatin, 1 pg/tube for vasopressin and 0.2 pg/well for angiotensin-II. The intra- and interassay coefficients of variation were less than 12 % for each assay.

2.6 *Radioiodination of [His^1, Nle^{27}]-hGHRH(1-32)-NH_2*

The chloramine-T method (94) was used for iodination of the GHRH analog. Two-hundred microliters of 500 mM sodium phosphate buffer (pH = 7.4), 25 µl synthetic [His^1, Nle^{27}]-hGHRH(1-32)-NH_2 (1.5 mg/ml), 20 µl $Na^{125}I$ (360 µCi/µl), 20 µl chloramine-T were added into a tube successively. After mixing for 20 s at room temperature the labeling reaction was terminated by addition of 30 µl of $Na_2S_2O_5$ (5 mg/ml), with continued mixing for 20 s. The mixture was loaded onto the chromatographic column.

[His^1, ^{125}I-Tyr^{10}, Nle^{27}]hGHRH(1-32)amide was separated from unreacted ^{125}I and [His^1, Nle^{27}]hGHRH(1-32)amide by reverse phase (C_{18}) HPLC with isocratic elution at a flow rate of 1.6 ml/min. The mobile phase was 30 % acetonitrile and 70 % triethylamine phosphate (166 mM phosphate, pH = 2.35).

2.7 *GHRH receptor binding assay*

For the binding assay, pituitary or hypothalamic samples were immediately harvested and were pooled from 2 to 4 rats to obtain one assay sample.

The GHRH analog [His^1, ^{125}I-Tyr^{10}, Nle^{27}]hGHRH(1-32)amide was used as the radioligand in the binding assay. The samples were weighed and then were homogenized in ice-cold assay buffer (50 mM Tris, 5 mM $MgCl_2$, 0.24 M sucrose, 2 mM EDTA, 2 mg/ml BSA, pH 7.4 for the pituitary or 6.5 for the hypothalamus). 100 µl aliquots were added to tubes containing 50 µl of assay buffer containing the radioligand at different concentrations ranging from 50 to 600 pM. After incubation for 1 h at room temperature, the mixture was transferred to a siliconized microcentrifuge tube and centrifuged for 2 min at approximately 13600 g. The supernatant was aspirated, the bottom of the microcentrifuge tube containing the tissue membrane pellet was chopped off, placed into a fresh tube, and the radioactivity was measured. Nonspecific binding was determined in half of the tubes with the addition 2×10^{-7} M of unlabeled GHRH analog. Nonspecific binding was subtracted from total binding to obtain specific binding.

In competition experiments, membrane homogenates were incubated with approximately 500 pM [His^1, ^{125}I-Tyr^{10}, Nle^{27}]hGHRH(1-32)amide and different concentrations (10^{-10} – 10^{-6} M) of nonradioactive analogs and other peptides.

The binding data were evaluated by computerized nonlinear regression analysis (PRISM, Graphpad Software, San Diego, CA, USA).

2.8 *Quantification of GHRH mRNA and GHRH receptor mRNA*

RT-PCR was used to determine GHRH mRNA and GHRH receptor mRNA levels. Total RNA was extracted from the tissues using RNA STAT-60 or Trizol reagent. Extracted RNA was characterized by formaldehide-containing agarose electrophoresis and by absorbance at 260 nm. First-stranded cDNA was synthesized with oligo-dT priming and Supercript II RNase reverse transcriptase from each sample. The cDNA was amplified by PCR. A detailed description of the RT-PCR procedures can be found in the Papers (#3, #5, #6) attached.

2.9 *Statistics*

The tests used included Student's t-test, one-way and two-way ANOVA, Student-Newman-Keuls test, and Pearson Correleation test. An alpha-level of $P < 0.05$ was considered to be significant in all tests.

Statistics are listed in detail in the Papers attached.

3. RESULTS

3.1 *Diurnal and sleep deprivation-induced variations in GHRH and somatostatin contents of the hypothalamus*

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 experiment with sleep loss, sleep deprivation started at light onset. Hypothalamic samples were obtained after 4 and 8 h of sleep deprivation and after 1 and 2 h of recovery following 8 h of sleep deprivation. The time-matched controls were undisturbed and used for the determination of the diurnal rhythms of the peptides.

GHRH: Hypothalamic GHRH content displayed significant diurnal variations (Fig. 1.) with low levels in the morning followed by a transient rise at 1 h after light onset, and then GHRH dropped to a minimum occuring by the middle of the day. GHRH content increased gradually in the afternoon. Peak values occured at the end of the light period and at the beginning of the dark period. GHRH decreased during the night. The mean GHRH values during the 12-h light and 12-h dark periods did not differ.

Sleep deprivation induced significant depletion of hypothalmic GHRH content (Fig. 1.). GHRH 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. Althought GHRH content started to rise by the end of hour 2 of recovery it was still significantly below normal.

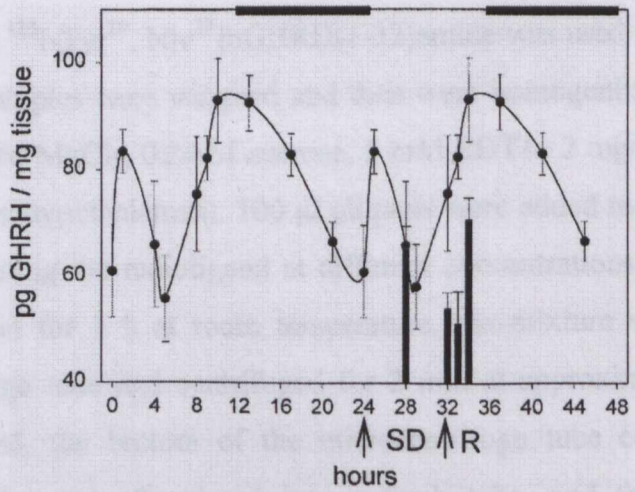

Fig. 1. Diurnal (● and lines) and sleep deprivation (SD)-induced (bars) variations in hypothalamic GHRH content (means ± SE). Diurnal rhythm is double plotted, and effects of SD are shown on *day 2*. Periods between *hour 0* and *hour 12*, as well as *hour 24* to *hour 36* are light periods. Dark periods are marked by horizontal bars. SD started at light onset (*hour 24*) and lasted for 8 h (*hour 32*), as indicated by arrows. GHRH was determined after 4 and 8 h of SD, and after 1 and 2 h of recovery (R) after deprivation.

Somatostatin: Hypothalamic somatostatin content also varied significantly across the 24-h day (Fig. 2.). The patterns of the diurnal variations in GHRH and somatostatin content of the hypothalamus were similar, but there was no significant correlation between them. The afternoon rise was delayed, and the nocturnal decline of somatostatin was more rapid than the changes in GHRH. Mean somatostatin contents during the light period were significantly lower than at night.

SD did not alter somatostatin significantly (Fig. 2.). Correlations were not found between somatostatin contents and variations in GHRH during and after SD.

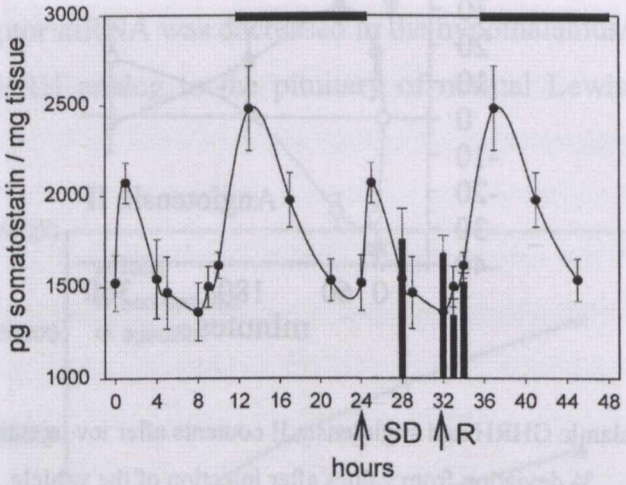

Fig. 2. Diurnal (● and lines) and sleep deprivation (SD)-induced (bars) variations in hypothalamic somatostatin content (means ± SE). See Fig. 1. for additional data.

3.2 *Effects of octreotide on hypothalmic GHRH, angiotensin-II, and vasopressin contents*

One group of rats was injected icv with 0.1 µg octreotide in a volume of 2 µl. The other group received 2 µl vehicle in the same dilution as the octreotide solution. The vehicle containing lactate and mannitol was donated by Novartis. The injections were performed at light onset. Hypothalamic samples were collected 10 min, 1, 3, and 6 h post-injection.

Icv injection of octreotide caused significant changes in GHRH content of the hypothalamus compared to control values obtained in rats injected with the vehicle (Fig. 3.). A tendency to GHRH accumulation was obvious after 10 min but the differences reached statistical significance only at hour 1 post-injection. At that time point, hypothalamic GHRH content was 30% higher in rats injected with octreotide than in the controls. GHRH content started to decline between hours 1 and 3 post-injection and this process continued until hour 6.

Hypothalamic angiotensin II content differed significantly between the rats injected with the vehicle and octreotide, and the difference varied with time (Fig. 3.). Icv octreotide elicited a prompt drop in the angiotensin II content of the hypothalamus. The angiotensin content decreased by about 27% 10 min after injection. It was still below the control values 1 h after injection but these changes were not significant. Thereafter, angiotensin II content returned to normal at hour 3 and increased above baseline at 6 h post-injection. The difference at hour 6 was not significant between the two groups.

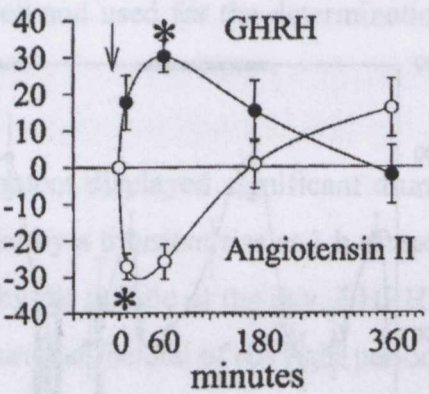

Fig. 3. Changes in hypothalamic GHRH and angiotensin II contents after icv injection of octreotide. Mean ± SE, % deviation from values after injection of the vehicle.
Arrow: injection (time 0); Asterisk: significant difference.

Vasopressin contents of the hypothalamus were not altered siginificantly after octreotide. The values obtained 1 and 3 h after icv octreotide tended to be slightly lower than the vasopressin contents in the controls but these changes did not reach the level of statistical significance. Vasopressin in the pituitary was also measured. Though these sample sizes were too low for statistical analysis, the vasopressin content of the pituitary harvested 10 min after treatment was lower in each of the rats which received octreotide than any of the rats injected with vehicle. There were no differences among the groups in the vasopressin content of the pituitary 1, 3, or 6 h post-injection.

3.3 *GHRH, GHRH mRNA, GHRH receptor mRNA, and GHRH binding in the GHRH receptor-deficient dwarf rat*

The mutant Lewis dwarf (*dw/dw*) rat exhibits GH deficiency and growth retardation which are linked to a malfunction of GHRH signaling. In adult *dw/dw* rats, plasma concentrations of GH, GH content of the pituitary, and GH and cAMP responses to GHRH are greatly

decreased (95,96). GHRH receptor mRNA levels are reduced in the pituitary of *dw/dw* rats (97).

Levels of GHRH mRNA and GHRH receptor mRNA as well as GHRH contents were measured in the hypothalamus/preoptic region, whereas GHRH binding and levels of the short isoform of GHRH receptor mRNA were determined in the pituitary.

Although hypothalamic GHRH mRNA levels were normal, GHRH peptide content in the hypothalamus of the *dw/dw* rats was only one-half of that found in normal Lewis rats. This difference between the two groups was statistically significant. Expression of the short isoform of GHRH receptor mRNA was decreased in the hypothalamus/preoptic region.

The binding of ^{125}I-GHRH analog to the pituitary of normal Lewis rats was specific and saturable (Fig. 4.).

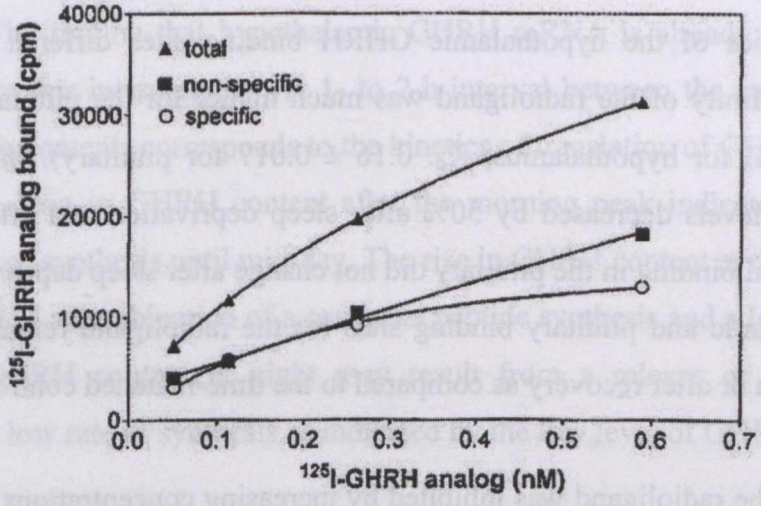

Fig. 4. [His^{1},^{125}I-Tyr^{10},Nle^{27}]hGHRH(1-32) amide binding to pituitary membranes prepeared from normal Lewis rats.

Saturable GHRH binding was undetectable in eight of nine pools of pituitaries harvested from the *dw/dw* rats. Small specific binding was obtained in one of the pools (Table).

Table
GHRH binding parameters (mean ± SE) in pituitary membrane preparations.
K_d: dissociation constant; B_{max}: receptor density
$^{+}$ Specific binding was observed in only one out of nine pools of pituitaries obtained from dw/dw rats, so there is no SE for K_d and B_{max} in this group.

Group	K_d (pM)	B_{max} (fmol/mg protein	B_{max} (fmol/mg tissue)
Lewis (n=10)	241 ± 34.0	22.4 ± 2.60	2.4 ± 0.25
dw/dw (n=1 out of 9)$^{+}$	126	4.3	0.4

The specificity of GHRH binding was demonstrated by diplacement experiments using several peptides. The binding of ^{125}I-GHRH analog was inhibited by increasing concentrations of rat GHRH and human analogs.
GHRH receptor mRNA was detectable in all *dw/dw* pituitaries examined, averaging 21% that of normal Lewis rats.

3.4 *Effects of sleep deprivation on GHRH receptors in the hypothalamus and in the pituitary*
Hypothalamic and pituitary GHRH binding and GHRH receptor mRNA were determined in Sprague-Dawley rats at the termination of an 8-h sleep deprivation and after 2 h of recovery following sleep deprivation. Control samples were collected from undisturbed rats at a time point corresponding to the termination of sleep deprivation, and at the time point corresponding to sleep deprivation + 2 h recovery.

The characteristics of the hypothalamic GHRH binding sites differed from those of the pituitary. The affinity of the radioligand was much higher for the pituitary membranes (K_d: 3.21 ± 0.641 nM for hypothalamus; K_d: 0.16 ± 0.017 for pituitary). In the hypothalamus, GHRH binding levels decreased by 50% after sleep deprivation and after 2 h recovery. In contrast, maximal binding in the pituitary did not change after sleep deprivation. The affinities of the hypothalamic and pituitary binding sites for the radioligand remained unaltered after sleep deprivation or after recovery as compared to the time-matched controls.

The binding of the radioligand was inhibited by increasing concentrations of rat GHRH(1-43) and by human GHRH analogs in both the pituitary and the hypothalamic samples. Vasoactive intestinal peptide did not compete with the ^{125}I-GHRH analog in the pituitary membrane preparations at a concentration as high as 1 μM. In contrast, the IC_{50} of VIP (24.69 nM) to hypothalamic GHRH binding sites was in the same range as that of the rat GHRH (14.15 nM). Hypothalamic GHRH receptor mRNA levels significantly decreased in the sleep-deprived rats compared to controls. In contrast, pituitary GHRH receptor mRNA levels did not differ between the two groups.

4. DISCUSSION

4.1 *Sleep-associated variations in hypothalamic GHRH and somatostatin contents*

Our results demonstrate diurnal variations and sleep deprivation-induced changes in hypothalamic GHRH content.

Although the kinetics of translation, and those of peptide release, degradation and elimination are not known, comparison 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 (76). Therefore, the transient rise in GHRH content in the morning may indicate synthesis of the peptide. In our previous mRNA studies, samples were taken 3 h before light onset and 1 h after light onset. Peak of GHRH mRNA occured 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 (77) 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 (98). The drop in GHRH content after the morning peak indicates a rapid release exceeding the rate of synthesis until mid-day. The rise in GHRH content at the end of the light period 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 with disproportionately low rate of synthesis as indicated by the low level of GHRH mRNA.

Hypothalamic GHRH mRNA increased after sleep deprivation starting at light onset and lasting for 6 (77), 8, or 12 h (78). GHRH content decreases significantly by the end of an 8-h sleep deprivation, and stays low for 1 to 2 h of recovery. Considering that GHRH mRNA is already higher than normal at least 2 h before the termination of the 8-h sleep deprivation then the low postdeprivation peptide content may occur in the face of a high rate of synthesis. Taken together, it suggest an intense hypothalamic GHRH release. Unfortunately, it is not known whether translation is altered during sleep deprivation. Stimulation of transcription during sleep deprivation 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.

In rats, the duration and the intensity of NREMS peak at the beginning of the light period. Both of these parameters of NREMS decline towards dark onset (99-101). The sleep deprivation protocol used in our experiments induces recovery sleep with intense NREMS for a few hours (102). Therefore, the periods of increased in GHRH release and synthesis, the first portion of the light period and recovery after sleep deprivation, correlate with the period of most intense NREMS. If depletion of hypothalamic GHRH indicates GHRH release at night and during sleep deprivation then GHRH may be part of the homeostatic process responsible for somnolence.

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 interneurons involved in many functions throughout the hypothalamus (103). Sleep deprivation did not alter somatostatin significantly, although somatostatin displayed diurnal variations. Previously, Nicholson *et al.* (104) reported that hypothalamic somatostatin content declines during the first portion of the light period. Berelowitz *et al.* (105) 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 (106). The large drop in somatostatin content of the hypothalamus might reflect this enhanced release. The diurnal rhythm of somatostatin content in our hypothalamic samples differed from the variations in somatostatin in the suprachiasmatic nucleus (107) or the anterior hypothalamus, which includes the periventricular area (108). Our results do not exclude the possibility that somatostatin also contributes to sleep regulation. Somatostatinergic stimulation alters sleep (85), and there are reciprocal changes in the somatostatin and GHRH mRNA levels in the hypothalamus in response to sleep deprivation (78). 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.

4.2 *Effects of icv octreotide on GHRH, angiotensin II, and vasopressin contents of the hypothalamus*

The GHRH content of the hypothalamus increased significantly after icv administered octreotide. This immediate accumulation of GHRH in the hypothalamus may indicate either an increase in synthesis or an inhibition of release. Interpretation of GHRH accumulation *in*

vivo as a sign of release inhibition by octreotide is in agreement with previous *in vitro* data demonstrating inhibition of GHRH release by somatostatin (109). Histological observations indicate that a subgroup of GHRH-containing neurons expresses somatostatin receptors and is innervated by somatostatinergic fibers (110,111). Some of these somatostatinergic neurons project to and inhibit GHRH-containing neurons via sst2 type somatostatin receptors (112). Octreotide is a potent agonist on sst2 and sst5 receptors (86). Our results suggest that stimulation of sst2 receptors inhibits GHRH release and icv-administered octreotide reaches the sst2 receptors mediating this action. GHRH promotes the duration and the intensity of NREMS, and suppression of endogenous GHRH actions by means of immunoneutralization of GHRH (79) or by means of a competitive antagonist (72) is followed by decreases in duration and intensity of NREMS. Inhibition of GHRH release may explain the octreotide-induced suppression of sleep reported previously. Our data also show that the GHRH accumulated upon exposure to octreotide was gradually released 1 h post-injection. This excess GHRH may mediate the enhancements in the intensity of NREMS observed during this period (75).

Icv somatostatinergic stimulation by octreotide elicits prompt and excessive decreases in hypothalamic angiotensin II content. The depletion of angiotensin II suggests a release of the peptide from the the hypothalamus. Somatostatin-induced vasopressin secretion and rises in blood pressure were previously reported in the literature. By using octreotide, experiments in our laboratory supported these findings and revealed that icv administration of octreotide also induces characteristic behavioural responses (75). 1-2 min post-injection the rats start drinking, and water consumption lasts 6-10 min. Drinking is followed by scratching, grooming and eating (113). Drinking, vasopressin release and increase in blood pressure are well-documented responses to icv administration of angiotensin II. Hence, octreotide elicits the same triple response as angiotensin II does. Icv injection of the angiotensin I-converting enzyme inhibitor, captopril, and the angiotensin receptor antagonists, saralasin and losartan, inhibit octreotide-induced drinking, vasopressin secretion and rises in blood pressure (75). These observations suggest that intracerebral angiotensin II either has a permessive role, or mediates, octreotide-induced drinking and vasopressin secretion. Somatostatin is an inhibitory neurotransmitter and, therefore, it cannot directly stimulate angiotensinergic neurons. In agreement with the notion that somatostatin may inhibit an inhibitory system involved in thirst (114), we suggested that somatostatin may suppress a tonic, GABAergic inhibition of angiotensinergic neurons (90). GABA inhibits the dipsogenic, pressor and vasopressin

responses of angiotensin II (115) whereas somatostatin releases these activities by inhibiting GABAergic transmission. Our findings do not exclude the possibility that hypothalamus angiotensin may also stimulate a particular subset of somatostatinergic neurons which modulate sodium appetite (114).

Vasopressin content of the hypothalamus was not altered significantly by icv injection of octreotide while octreotide elicited immediate decrease in vasopressin content of the pituitary. This decrease is in agreement with previous experiments reporting significant rises in the plasma level of vasopressin as soon as 5 min after icv somatostatinergic stimulation (90,116). Hypothalamic magnocellular neurons are the source of pituitary vasopressin (117). It was anticipated, therefore, that vasopressin secretion can also be detected as a drop in hypothalamic vasopressin contents. One and 3 h after octreotide injection, mean hypothalamic vasopressin content were in fact lower than in the control rats but these changes were not significant. On the one hand, vasopressin is stored in the nerve terminals of posterior pituitary, and thus vasopressin release may occur without significant alterations in hypothalamic vasopressin. On the other hand, vasopressin is also found in hypothalamic neurons (mainly in the suprachiasmatic nucleus), which do not project to the pituitary. Therefore, determination of total vasopressin contents of the hypothalamus may not accurately reflect the activity of the magnocellular neurons.

4.3 *GHRH signaling in dw/dw rats*

Hypothalamic GHRH mRNA levels were normal but GHRH content was significantly less in the *dw/dw* rats than in intact Lewis rats. The decreased hypothalamic GHRH content is interpreted as a sign of enhanced GHRH release due to a failure of the GH-mediated negative feedback. This interpretation is supported by earlier data. *In vitro* observations suggest that the release of GHRH is enhanced in the *dw/dw* rats (118). Depletion of GHRH is observed in rats and mice with various GH deficiencies (119,120) and in rats after hypophysectomy (121,122).

GHRH receptor mRNA significantly decreased in the pituitary of *dw/dw* rats (55,123). A short and a long GHRH receptor mRNA species described in the rat pituitary (38,39). The short isoform is the predominant transcript and a shorter (α) and a longer (β) subtype of this isoform have been distinguished (55).

GHRH receptor mRNA is expressed in the rat hypothalamus (52). Our results support this finding and it seems that the short GHRH receptor mRNA isoform is also the dominant in the hypothalamus/preoptic region. The significantly decreased GHRH receptor mRNA levels in the *dw/dw* rats indicate that the GHRH signaling mechanism has the same defect in the hypothalamus/preoptic region as the previously described deficit in the pituitary of this strain of rats.

The *dw/dw* rats spend less time in NREMS (light and dark period) and REMS (light period) than do the control Lewis rats. GHRH promotes NREMS by targeting neurons in the preoptic region (67,80). Decreases in central GHRHergic transmission may explain the permanent NREMS loss in the *dw/dw* rat secondary to GHRH receptor deficiency.

We could not detect GHRH binding in eight pools of pituitaries of dwarf rats, and it was weak in one pool. The extent of this reduction in binding was at least moderately greater than the decrease in the levels of GHRH receptor mRNA determined by us (Paper #3), and in other studies (55,123). Disperate changes in GHRH binding and GHRH mRNA, have been observed previously also by others (124). The reductions in GHRH receptor binding and mRNA may reflect somatotroph hypoplasia in the *dw/dw* rat pituitary (97). Decrease in GHRH receptor mRNA may also be a consequence of a defect in GHRH signaling. For example, decreased binding of GHRH to GHRH receptors may decrease GHRH-induced promotion of further GHRH receptor synthesis. Alterations in translation of GHRH receptor mRNA can also decrease receptor protein synthesis. Mutations of proteins, which mediate the function of a particular receptor, may also alter binding (39). In conclusion, in addition to the possible defects in GHRH receptor transcription or a failure of the signal transduction mechanism, translational and/or posttranslational modifications of the GHRH receptor should also be considered as potential causes of the GH deficiency in the *dw/dw* rat.

4.4 *Effects of sleep deprivation on hypothalamic and pituitary GHRH receptors*

Both GHRH binding and GHRH receptor mRNA levels decreased in the hypothalamus after sleep deprivation, whereas there were no alterations in the pituitary. We detected a single class of GHRH binding sites in the hypothalamus with a much lower affinity than GHRH binding sites of the pituitary. Specific high affinity binding sites for GHRH have been reported on pituitary membranes (33,125,126). GHRH binding sites are also found in human cancer cells (59,127), in the rat renal medulla (125), in the granulosa cells of the ovary (25),

but they possess different characteristics from binding sites in the pituitary. Intrahypothalamic GHRH binding also clearly differed from GHRH binding in the rat pituitary. The GHRH binding sites in the hypothalamus displayed a similar affinity for VIP than for GHRH. GHRH is a member of the secretin-glucagon peptide family, and cross-reactivity is often observed between receptors for individual peptides in the family. VIP receptors display siginificant GHRH binding (128). However, it is unlikely that the GHRH binding sites in our hypothalamic samples are VIP receptors, because the affinity of the VIP receptors is much higher for VIP than for GHRH in the brain (129) and other tissues (130,131). A special binding site, coined the VIP/GHRH receptor, has been described in granulosa cells of the ovary, on which both VIP and GHRH act as physiological ligands (25). The hypothalamic binding sites found in our experiments might be similar to those receptors.

Hypothalamic GHRH receptor mRNA decreased to the same extent as GHRH binding after sleep deprivation. It is an important observation suggesting that the hypothalamic binding sites function as GHRH receptors. The primers used for determining GHRH receptor mRNA in the hypothalamus detect the major functional GHRH receptor mRNA in the pituitary (126). Tumor cells (59,127) and also pituitary (126) express splice variants of the GHRH receptors. It is possible, therefore, that the different binding characteristics of pituitary and hypothalamic GHRH receptors may result from alternative splicing of the mRNA.

Hypothalamic GHRH is implicated in physiological sleep regulation and in sleep responses to sleep deprivation (132). Our data suggest an approximately 50% down-regulation of hypothalamic GHRH receptors after 8 h of sleep deprivation and the number of receptors does not seem to recover within the first 2 h upon completion of sleep deprivation. Prolonged GHRH exposure down-regulate GHRH receptors in the pituitary (133). Sleep deprivation causes significant GHRH release resulting in a robust GHRH depletion by the end of 8 h of sleep deprivation, and the GHRH release is likely continue during recovery sleep (Paper #1). The changes in GHRH binding and GHRH receptor mRNA levels of the hypothalamus, therefore, are consistent with a sleep deprivation-associated GHRH release.

CONCLUSIONS

Comparisons of the variations in hypothalamic GHRH contents with previously reported changes in GHRH mRNA levels of the hypothalamus suggest that the time of the day where maximum GHRH synthesis and release occur corresponds to the period of deepest NREMS. Experiments with sleep deprivation support this conclusion showing that sleep deprivation for 8 h results in excessive depletion of hypothalamic GHRH with very low GHRH contents at the termination of the deprivation. Another finding that indirectly supports an excessive GHRH release induced by sleep deprivation, is a 50% reduction of GHRH binding and GHRH mRNA in the hypothalamus after 8 h of sleep deprivation. It is characteristic of GHRH receptors that massive exposure to GHRH elicits down-regulation, therefore, a robust intrahypothalamic GHRH release during and after sleep deprivation is a likely explanation for the down-regulation of hypothalamic GHRH receptors.

The somatostatin analog, octreotide, promptly inhibits hypothalamic GHRH release coinciding with sleep suppression. After 1-3 h postinjection, when intensity of NREMS increases, the accumulated GHRH is gradually released from the hypothalamus. Therefore, a possible explanation for the biphasic sleep response to octreotide is that octreotide suppresses sleep via inhibiting GHRH release, and this period is followed by supranormal GHRH release and resulting in deep NREMS. In addition, our experiments revealed, that somatostatin may also be involved in the control of intracerebral angiotensinergic system regulating water balance.

The dwarf (*dw/dw*) rats exhibit both intracerebral and pituitary deficits in GHRH receptors and they sleep less than the normal Lewis rats. These results suggest that somatotropic functions and sleep regulation are intimately related.

Our results demonstrate that GHRH receptors exist in the hypothalamus and they respond differently to sleep deprivation than the GHRH receptors in the pituitary.

These observations support the hypothesis that hypothalamic GHRH has a major role in the physiological regulation of sleep.

SUMMARY

Sleep is regulated by interdependent humoral and neuronal mechanisms. The somatotropic axis is a major hormone system that influences sleep-wake activity. Hypothalamic GHRH is one of the best documented sleep-promoting substance. Somatostatin mediates negative feedback in the somatotropic axis by inhibiting both GHRH and GH. The *aim* of our work was to extend our knowledge on the interaction between GHRH and sleep including experiments to **a;** determine of hypothalamic GHRH content in correlation with sleep, after somatostatinergic stimulation, and in chronic somatotropic deficiency. **b;** characterize hypothalamic and pituitary GHRH receptors in the dwarf rats, and after sleep deprivation. *Results:* **a;** GHRH peptide contents in the hypothalamus display diurnal variations that correlate with sleep-wake activity, and respond to sleep deprivation. These changes suggest enhanced GHRH release during the period of deep NREMS, i.e., during the first portion of the diurnal rest period and during recovery sleep after sleep deprivation. Intense GHRH release, however, starts already during sleep deprivation. Hypothalamic somatostatin contents also vary across the 24-h day, but sleep deprivation does not appreciably alter the total somatostatin content of the hypothalamus. The somatostatin analog, octreotide, causes a rise in GHRH content of the hypothalamus 1 h post-injection followed by a (1-6 h) gradual depletion of accumulated GHRH. During our experiments with icv-administered octreotide, we observed prompt drinking behavior. The serendipitous observation prompted additional studies regarding its underlying mechanism. Hypothalamic angiotensin II decreases promptly after octreotide treatment, while vasopressin content of the hypothalamus is not altered. Hypothalamic GHRH content is less in the *dw/dw* rats than in normal Lewis rats. **b;** Pituitary GHRH binding is almost abolished and both pituitary and hypothalamic GHRH receptor mRNA are decreased in the *dw/dw* rats. GHRH binding and GHRH receptor mRNA levels are reduced in the hypothalamus of sleep-deprived rats, whereas there are no alterations in the pituitary. *Conclusions:* Sleep-related changes in hypothalamic GHRH contents and GHRH receptors suggest enhanced GHRH release during the period of deep NREMS, i.e., during the first portion of the diurnal rest period and during recovery sleep after sleep deprivation. The octreotide-induced alterations in hypothalamic GHRH may explain previously reported changes in sleep. Our experiments suggest that angiotensin may mediate octreotide-induced drinking, vasopressin secretion and rises in blood pressure via sst2 receptors. The present findings provide evidence for a deficit in GHRH receptors in both hypothalamus and pituitary

of the *dw/dw* rats. These mutant rats sleep less, therefore, our results support that GHRH involved in sleep regulation.

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