SOMATOSTATIN, GROWTH HORMONE-RELEASING HORMONE, GALANIN AND THEIR HYPOTHALAMIC MESSENGER RIBONUCLEIC ACIDS IN THE REGULATION OF SLEEP IN RATS

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Academic Dissertation

To be publicly discussed, by the permission of the Medical Faculty of the University of Helsinki, in the auditorium of the Institute of Biomedicine, Department of Physiology (Siltavuorenpenger 20 J) on November 5th 1999, at 12 noon.

HELSINKI 1999

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ISBN 951-45-8724-3 (PDF version)
Helsingin yliopiston verkkojulkaisut, Helsinki 1999

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ABBREVIATIONS

AMP	adenosine monophosphate
ANOVA	analysis of variance
AUC	area under curve
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
dATP	deoxyadenosine triphosphate
EDTA	ethylenediaminetetra-acetic acid
EEG	electroencephalography
EMG	electromyography
EOG	electro-oculography
GABA	gamma-aminobutyric acid
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRP	growth hormone-releasing peptide
GHS	growth hormone secretagogue
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IGF	insulin-like growth factor
mRNA	messenger ribonucleic acid
NPY	neuropeptide Y
p.o.	peroral
PBS	phosphate-buffered saline
PRL	prolactin
REM	rapid eye movement
s.c.	subcutaneous
SSC	saline-sodium citrate
SSR	somatostatin receptor
SWS	slow wave sleep
TED	tris-EDTA-dithiothreitol
tRNA	transfer ribonucleic acid
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide

LIST OF ORIGINAL PUBLICATIONS

I Toppila, J., Stenberg, D., Alanko, L., Asikainen, M., Urban, J. H., Turek, F. W. and Porkka-Heiskanen, T. REM sleep deprivation induces galanin gene expression in the rat brain. *Neurosci. Lett.* 1995, 183: 171-174.

II Toppila, J., Asikainen, M., Alanko, L., Turek, F. W., Stenberg, D. and Porkka-Heiskanen, T. The effect of REM sleep deprivation on somatostatin and growth hormone-releasing hormone gene expression in the rat hypothalamus. *J. Sleep Res.* 1996, 5: 115-122.

III Toppila, J., Alanko, L., Asikainen, M., Tobler, I., Stenberg, D. and Porkka-Heiskanen, T. Sleep deprivation increases somatostatin and growth hormone-releasing hormone messenger RNA in the rat hypothalamus. *J. Sleep Res.* 1997, 6: 171-178.

IV Toppila, J., Niittymäki, P., Porkka-Heiskanen, T. and Stenberg, D. Intracerebroventricular and locus coeruleus microinjections of somatostatin antagonist decrease REM sleep in rats., 1999, (Submitted)

INTRODUCTION

The regulatory role of endogenous peptides in the control of sleep and wakefulness was first indicated by the classical studies in Japan and France at the beginning of this century. It was observed that cerebro-spinal fluid (CSF) of a sleep deprived dog promoted sleep when injected intracerebroventricularly (i.c.v.) to a recipient dog (Ishimori 1909, Legendre and Piéron 1910). A hypothesis was put forward that sleep promoting substances accumulated in CSF during wakefulness. In further studies it was found that heating or ultrafiltration abolished the observed sleep promoting effect (Legendre and Piéron 1912), suggesting that the substance was structurally a thermolabile macromolecule, possibly a peptide.

To date several peptides have been found which affect sleep when injected systemically or directly into the brain (Borbély and Tobler 1989). However, administration of pharmacological doses of a peptide does not necessarily indicate that the peptide has an endogenous function in the regulation of sleep. In addition it is required that the secretion rhythm of the sleep promoting substance is associated with the sleep-wake rhythm. Blockade of the action of the endogenous peptide should also have an opposite effect on sleep, and manipulation of the spontaneous sleep-wake rhythm e.g. by sleep deprivation should affect the activity of this peptide system. According to current knowledge there are three putative neuroendocrinological systems which may be associated with specific vigilance states: association of growth hormone secretion with slow wave sleep (SWS), the hypothalamo-pituitary-adrenal axis with wakefulness, and the VIP/prolactin system with REM sleep (Steiger and Holsboer 1997). In addition, there are several other neuropeptides and macromolecules which affect the structure of sleep, but their endogenous function in sleep regulation is not completely clear (Inoué and Krueger 1990).

Secretion of GH and the amount of deep SWS decrease similarly during normal aging (Van Cauter *et al.* 1998). Decreased SWS may be one contributing factor to poor sleep quality and increased prevalence of sleep disorders in the elderly. Also in some pathological conditions, such as fatal familiar insomnia, the deterioration of sleep is coupled with a simultaneous decrease in GH-secretion (Portaluppi *et al.* 1995). It is possible that this simultaneous decrease in both GH secretion and SWS is generated by a common neural mechanism, which may be related to the GH regulatory neuropeptide system. Better knowledge about this mechanism could create new strategies for the development of more physiological methods for the management of poor sleep quality associated with normal aging and pathological conditions.

REVIEW OF THE LITERATURE

Structure and regulation of sleep

Sleep of terrestrial mammals consists of two main sleep stages: rapid eye movement (REM) sleep and non-REM sleep which can be further divided into light and deep non-REM sleep. These stages can be recognized by their typical characters in sleep polygraphy, which includes electrophysiological recording of brain cortex activity by electroencephalography (EEG), eye movements by electro-oculography (EOG) and skeletal muscle tonus by electromyography (EMG). During light non-REM sleep there are typical activity bursts called sleep spindles in EEG and deep non-REM sleep can be recognized by synchronized EEG activity with high amplitude and low frequency delta waves, and for this reason deep non-REM sleep is also called slow wave sleep (SWS). Widely used human sleep stage classification criteria categorizes non-REM sleep into four stages (S1-4) where S2 is light and S4 deep non-REM sleep. S1 represents transition between wake and light sleep and S3 is an intermediate stage between light and deep non-REM sleep (Rechtschaffen and Kales 1968). Electrophysiologically REM sleep differs from non-REM sleep in several ways. During REM sleep, EEG is highly desynchronized corresponding to the EEG recorded during wakefulness. There is also almost a total loss of tonus in the skeletal musculature interrupted by occasional twitches especially in the facial muscles which can be recognized in EMG. In EOG there are bursts of rapid eye movements which has given the name for this vigilance stage. Because non-REM and REM sleep differ in several characteristics, these sleep stages can be regarded as two independent vigilance states together with wakefulness.

According to a current and widely accepted model of sleep regulation, sleeping is controlled by two separate components: circadian process C which affects the appropriate timing of sleep and homeostatic process S which accounts for a sufficient amount of sleep (Borbély 1982). This is a two process model. The circadian process C is mainly controlled by the rhythmic activity of the suprachiasmatic nucleus in the hypothalamus. For the homeostatic process S, no single locus has been found in the CNS. It seems rather to be controlled by several neural systems including monoamines, neuropeptides, and cytokine transmitters which are localized in the hypothalamus, basal forebrain and brain stem nuclei (Borbély and Tobler 1989). There is another theory, which models the rhythm between REM sleep and the other vigilance states. According to this reciprocal interaction theory, REM sleep is controlled by feedback regulatory connections between monoaminergic and cholinergic nuclei in the pontine brainstem (Hobson *et al.* 1975). Monoaminergic nuclei of this regulatory system consist of the noradrenergic locus coeruleus and serotonergic raphe nuclei. These nuclei are most active during wakefulness, decrease their activity during non-REM sleep and are almost totally inhibited during REM

sleep. Cholinergic laterodorsal and pedunculo-pontine tegmental nuclei (LDT, PPT) are most active during REM sleep. Several factors such as neuropeptides may also have modulatory effects on this REM sleep regulatory system.

Association of GH secretion with sleep

Growth hormone (GH, somatotropin) is a polypeptide hormone consisting of 191 amino acids in humans and is secreted by somatotropic cells of the anterior pituitary. Unlike the other pituitary hormones, which affect a specific target organ, GH affects several tissues throughout the body. The main effects of GH are stimulation of growth in bone metaphyses during growing, anabolic effect in the muscles, conservation of proteins and carbohydrates, and mobilization of fat for energy sources (lipolysis). The effects of GH are partly mediated by somatomedins of which the most important are insulin-like growth factors (IGF) 1 and 2. Although somatomedins are secreted locally by several tissues, the plasma IGF-1 content is mostly originated from the liver and kidneys.

Secretion of GH from the anterior pituitary is pulsatile. The secretion bursts are flanked by almost undetectable levels of plasma GH. GH pulses occur more frequently and the basal level of plasma GH is higher in females than males who have fewer GH pulses but which are of a higher amplitude (Van Cauter *et al.* 1998). In humans there is typically one high secretion pulse and a few lower ones during the 24-h day-night span (Van Coevorden *et al.* 1991, Van Cauter *et al.* 1998). Soon after the development of radioimmunological hormone assay methods in the 1960's, it was found that the main secretion pulse was closely associated with the beginning of the sleep phase when the amount of SWS is highest (Takahashi *et al.* 1968, Honda *et al.* 1969, Sassin *et al.* 1969, Van Cauter *et al.* 1992). Sampling and measurements of plasma GH at frequent (up to 30 s) intervals combined with the deconvolution procedure, which mathematically estimates the rate of secretion of a hormone from the plasma concentrations, have revealed a close association of GH secretion with SWS phases (Van Cauter *et al.* 1992, Holl *et al.* 1991). Delay, advance or interruption of a sleep phase will shift the main GH secretion pulse correspondingly (Golstein *et al.* 1983, Van Cauter *et al.* 1992). At least in humans GH secretion is also controlled by an endogenous circadian rhythm. When the sleep period is shifted from its normal time, some GH is still secreted during the early night according to the endogenous clock (Van Cauter *et al.* 1992). GH secretion is highest during growing and early adulthood. In humans the secretion rate starts to decrease during the fourth decade of life. During aging the daytime secretion pulses diminish first, while the sleep associated GH pulse persists longer (Van Cauter *et al.* 1998).

In animals it is more difficult to find a correlation between GH secretion and sleep because many animal species have typically several sleep phases of variable lengths during the 24-h day-night span. However, elevated plasma GH levels during sleep have been demonstrated in several mammals (reviewed by Van Cauter *et al.* 1998). In the rat, which is a widely used animal model in neuroscience, the GH secretion is pulsatile with an approximately 3-h cycle. This rhythm is associated with an ultradian sleep-wake rhythm with the same cycle length, so that the GH pulses precede the sleep maxima by about 24 min (Mitsugi and Kimura 1985). Short term (3 h) total sleep deprivation during the light phase resulted in a decrease of GH secretion during the deprivation in the rat (Kimura and Tsai 1984).

The effect of injected GH on sleep

Systemic administration of GH by an intraperitoneal injection has been found to increase especially REM sleep in cats and rats (Stern *et al.* 1975, Drucker-Colín *et al.* 1975). When endogenous GH was immunoneutralized by an injection of GH antiserum, both SWS and REM sleep decreased in the rat (Obál *et al.* 1997). In humans, intramuscular bolus injection of GH before sleep onset was found to increase REM sleep and to decrease SWS (Mendelson *et al.* 1980). However, a more recent study failed to repeat this finding (Kern *et al.* 1993).

GH regulatory hypothalamic peptides

Pituitary secretion of GH is controlled by two hypothalamic peptides: growth hormone-releasing hormone (GHRH) which stimulates GH secretion, and somatostatin (somatotropin-release inhibiting factor, SRIF) which inhibits it. Somatostatin was isolated and chemically characterized from the ovine hypothalamus in the early 1970's (Brazeau *et al.* 1973). Somatostatin is a 14 amino acid cyclic peptide linked by an internal disulfide bridge (Fig. 5). Somatostatin is widely distributed in the body, e.g. in the brain, intestine and pancreas. In addition to GH secretion, it inhibits the secretion of several other hormones such as TSH, insulin, glucagon, and also pancreatic secretion. The somatostatin gene is 1.2 kilobases in length and have one 630 base intron within the coding sequence. Somatostatin mRNA is about 600 base pairs long and codes a 116 amino acid precursor peptide preprosomatostatin (Goodman *et al.* 1985). GHRH was isolated and chemically characterized from a pancreatic tumor in a patient with high GH levels in 1982 (Guillemin *et al.* 1982). It is a linear peptide containing 43 amino acids in rats and 44 in humans, but in the blood circulation also shorter forms exist. The rat GHRH gene spans nearly 10 kilobases in the genome and has five exons which encode a mRNA

of approximately 700 nucleotides. GHRH mRNA is translated into 104 amino acid precursor peptide of the rat GHRH (Mayo *et al.* 1985, Gonzales-Cresbo and Boronat 1991).

Peptide transmitter is synthesized by translation from mRNA in ribosomes and modified from precursor peptides in the Golgi apparatus. Transmitter peptide is then transported to axon terminals by axonal transport mechanisms. Except in the case of small molecule transmitters, there are no re-uptake mechanisms for peptide transmitters in presynaptic terminals and new transmitter is generated by translation from mRNA (Brownstein 1994). Although translation and modification of active peptide transmitter from its precursors are regulated by many cellular mechanisms, the amount of specific transmitter mRNA in a cell can be regarded as a rough estimate of the synthesis rate of the peptide transmitter. The rate of synthesis is in turn normally coupled to the release rate of a transmitter and to the activity of a peptidergic neuron.

Somatostatin cells in the CNS

Somatostatin containing neurons are present widely in the CNS, e.g. in many hypothalamic nuclei, preoptic area, hippocampus and cortex. Somatostatin secreting cells are also found in the wall of the gastrointestinal tract. The widespread distribution of somatostatin cells in the CNS strongly suggests that somatostatin may also have functions other than the inhibition of GH secretion. The somatostatin cells that control GH release are located in the anterior part of the periventricular nucleus of the hypothalamus (Ishikawa *et al.* 1987, Merchenthaler *et al.* 1989), from where they project to the median eminence. There somatostatin is released to the pituitary portal vein system from neurosecretory axon terminals and finally to its receptors in the somatotrophic cells of the pituitary gland (Fig. 1). To date five main types of somatostatin receptors have been cloned (SSR1-5). Due to alternative mRNA splicing, there are two variants of SSR2, named SSR2A and B (Florio *et al.* 1996). All SSRs reduce the production of cyclic AMP. There are also several other intracellular transduction mechanisms which may couple to SSRs (Florio *et al.* 1996). Although all types of SSRs are present in the pituitary, inhibition of GH secretion is mediated mainly by SSR2 in humans and rats. This action involves inhibition of cytoplasmic Ca^{2+} influx. There is a strong expression of SSR1 in the periventricular area and the median eminence. This receptor type is possibly an auto-receptor in hypophysiotropic somatostatin cells, where somatostatin reduces its own release by an ultra short-loop feedback mechanism (Helboe *et al.* 1998). Somatostatin cells located in the dorsomedial part of the arcuate nucleus probably inhibit GHRH cells in the same nucleus but do not project into the median eminence (Willoughby *et al.* 1989, Merchenthaler *et al.* 1989) (Fig.1). SSRs are found in the noradrenergic cells of the locus coeruleus where they inhibit the activity of these neurons

(Inoue *et al.* 1988, Pérez *et al.* 1994, Mounier *et al.* 1996). The locus coeruleus has projections to the hypothalamus where noradrenaline stimulates the release of somatostatin in the periventricular nucleus (Liposits 1993, Mounier *et al.* 1996). This mechanism may partly mediate the attenuated GH release during wakefulness when the neurons in the locus coeruleus are active (Fig. 15).

GHRH cells in the CNS

Unlike somatostatin, the distribution of GHRH neurons in the CNS is restricted to the hypothalamus (Sawchenko *et al.* 1985). The most important GHRH cell group controlling the GH secretion is in the arcuate nucleus (Daikoku *et al.* 1986) (Fig.1). GHRH containing cells have also been found in the paraventricular nucleus, dorsomedial nucleus, and in the periventromedial hypothalamic area (Sawchenko *et al.* 1985). Hypophysiotropic GHRH cells in the arcuate nucleus also express tyrosine hydroxylase (TH) and have dopamine as a co-transmitter. Dopamine is released into the portal circulation of the pituitary, where it regulates at least the secretion of prolactin and TSH (Niimi *et al.* 1992). There are possibly autoregulatory connections within the arcuate GHRH cells (Horváth and Palkovits 1988) (Fig. 1). Two types of GHRH cells exist which have partly different morphology and possibly also different projections (Daikoku *et al.* 1986). Most of arcuate GHRH cells project to the median eminence and are hypophysiotropic, while the GHRH cells outside of the arcuate nucleus, especially in the periventromedial hypothalamic area project to other hypothalamic nuclei and neighboring brain areas such as the preoptic hypothalamic area and the basal forebrain (Sawchenko *et al.* 1985). These cells may account for the possible neuroregulatory effects of GHRH. A G-protein mediated receptor for GHRH has been isolated and cloned (Mayo 1992). In addition to the pituitary gland, the GHRH receptor is expressed in several hypothalamic nuclei and in the preoptic area (Takahashi *et al.* 1995).

Autoregulation of GH-axis in the brain

Plasma GH content regulates the activity of hypophysiotropic somatostatin and GHRH cells. An elevated GH level in plasma increases the synthesis and release of somatostatin and decreases the synthesis and release of GHRH (Zeitler *et al.* 1990). Hypophysectomy decreases the activity of somatostatin cells and increases GHRH cell activity (Minami *et al.* 1993). GH receptors are present in periventricular somatostatin cells (Burton *et al.* 1992). Only a minority (<10%) of GHRH cells in the arcuate nucleus expresses the GH receptor, which may indicate

that the effect of GH on GHRH is mediated indirectly by somatostatin, IGFs, or neuropeptide-Y (NPY) (Burton *et al.* 1995, Korbonits *et al.* 1996, Chan *et al.* 1996). (Fig. 1)

Somatostatin and GHRH cells also affect each other via intrahypothalamic connections without the involvement of GH (Horváth *et al.* 1989, Zeitler *et al.* 1991) (Fig.1). Somatostatin cells in the periventricular nucleus inhibit the activity of GHRH cells in the arcuate nucleus, and also inhibit the release of GHRH from the axon terminals in the median eminence (Dickson *et al.* 1994). Arcuate somatostatin and GHRH cells possibly have a reciprocal regulatory connection within the nucleus, where somatostatin cells inhibit GHRH cells and GHRH stimulates somatostatin (Horváth *et al.* 1989). GHRH cells in the arcuate are possibly connected to the periventricular nucleus where they stimulate cells, but this regulation pathway may be mediated indirectly by NPY or galanin (Chan *et al.* 1996) because very few arcuate GHRH cells have been found to project directly to the periventricular nucleus (Willoughby *et al.* 1989). Nevertheless, GHRH fibers and GHRH receptors are present in the periventricular nucleus (Horváth *et al.* 1989, Takahashi *et al.* 1995), which suggests that also other than arcuate GHRH cells may regulate the somatostatin cells in periventricular nucleus.

Feedback regulation by GH and intrahypothalamic connections generate rhythmic alteration in the expression of mRNA in hypophysiotropic somatostatin and GHRH cells, so that the maximal mRNA expression in somatostatin cells is 180° out of phase with the maximal expression of GHRH mRNA (Zeitler *et al.* 1991). This oscillation may explain the GH secretion rhythm in rats and GH pulses in humans.

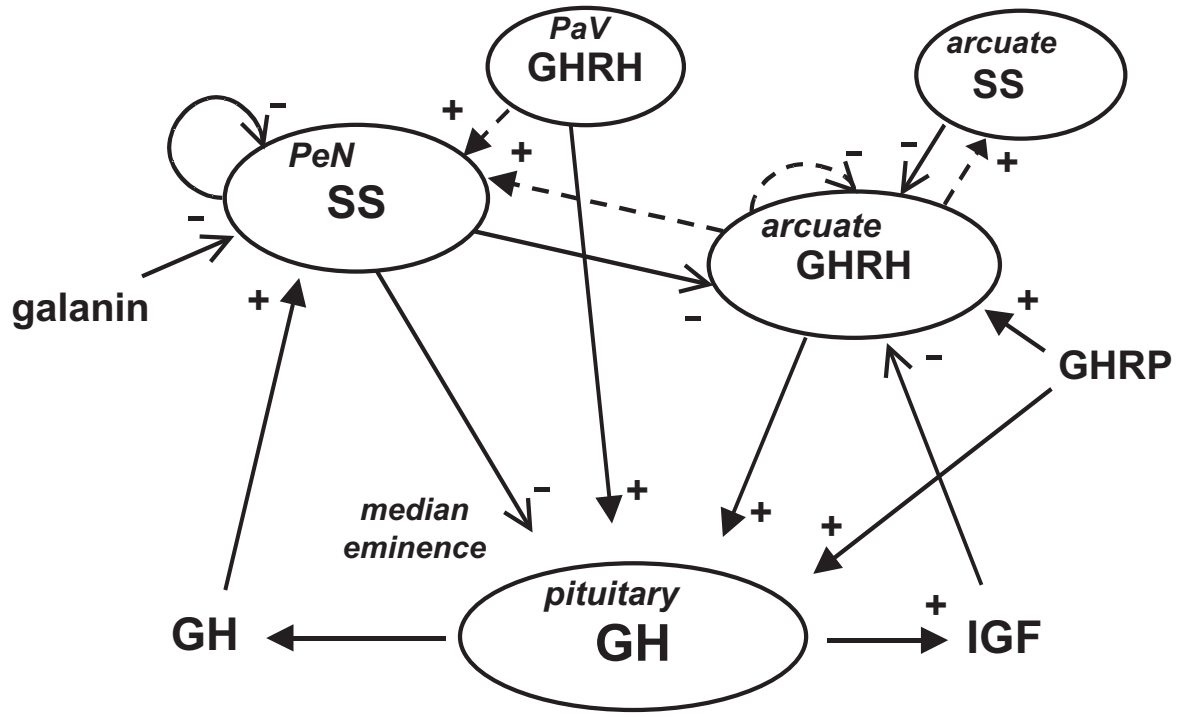

Fig. 1 Schematic illustration of the hypothalamic regulatory system of the pituitary GH release. SS: somatostatin, PeV: periventricular nucleus, PaV: paraventricular nucleus.

The effect of injected GHRH on sleep

I.c.v. injection of GHRH increases the amount of SWS, slow wave activity and REM sleep in rats and rabbits (Ehlers *et al.* 1986, Nisticò *et al.* 1987, Obál *et al.* 1988). A similar effect has also been found after systemic injection of GHRH in rats (Obál *et al.* 1996). The observed stimulatory effect of systemic GHRH on REM sleep is abolished by hypophysectomy. This does not affect the SWS promoting effect, which suggests that the effect of GHRH on SWS is primary and not mediated by GH (Obál *et al.* 1996).

In humans repeated i.v. injections of GHRH have been found to increase the amount of SWS during the early sleep phase (Steiger *et al.* 1992, Marshall *et al.* 1996) or after a single injection in the middle of the sleep phase after the third REM sleep episode (Kerkhofs *et al.* 1993). A single i.v. injection in the early sleep phase increases the amount of REM sleep but has no effect on SWS (Kerkhofs *et al.* 1993). Continuous infusion (Sassolas *et al.* 1986, Kern *et al.* 1993, Marshall *et al.* 1996), bolus injection before or at the sleep onset (Garry *et al.* 1985, Kupfer *et al.* 1991) and repeated i.v. boluses during the late sleep phase (Schier *et al.* 1997) failed to affect the sleep structure although these treatments increased plasma GH.

Blocking the action of GHRH by i.c.v. injection of a GHRH antagonist (Obál *et al*. 1991) or antibody (Obál *et al.* 1992) decreased the amount of spontaneous sleep in rats, and administration of a GHRH antibody also decreased sleep rebound after sleep deprivation. The amount of GHRH mRNA is highest in the morning when the amount of non-REM sleep is usually at its highest in rats (Bredow *et al.* 1996). A GHRH-GH-IGF deficient dwarf mouse strain has a reduced amount of non-REM sleep (Zhang *et al.* 1996), which also suggests that GHRH facilitates non-REM sleep.

The effect of injected somatostatin on sleep

A long term intracerebroventricular (i.c.v.) infusion of somatostatin was found to increase and depletion of somatostatin stores by cysteamine to decrease REM sleep in rats (Danguir 1986). However, i.c.v. or epicortical administration of a high dose (10μg) of somatostatin in rats increased wakefulness during the first two hours after the injection (Havlicek *et al.* 1976, Rezek *et al.* 1976). Intraperitoneal (i.p.) or subcutaneous (s.c.) injection of the long-acting somatostatin analog octreotide (SMS 201-995, OCT) causes a delayed facilitation of REM sleep in rats (Danguir and De Saint-Hilaire-Kafi 1988b, Beranek *et al.* 1997). A selective REM sleep facilitatory effect has also been found in aged rats after oral or i.p. administration of octreotide (Danguir 1989). I.p. injection of octreotide counteracts the suppression of REM sleep

caused pharmacologically by scopolamine (Danguir and De Saint-Hilaire-Kafi 1988b) or by desipramine (Danguir and De Saint-Hilaire-Kafi 1989). Immunoneutralization of endogenous somatostatin by i.c.v. infusion of somatostatin antibody decreases REM sleep (Danguir 1988) and microinjection of somatostatin antibody into the nucleus of the solitary tract antagonizes carbachol-induced REM sleep in rats (Danguir and De Saint-Hilaire-Kafi 1988a).

Somatostatin appears to stimulate REM sleep in laboratory animals, whereas human studies have yielded inconsistent results of the effects of somatostatin on sleep. Intravenously (i.v.) infused somatostatin at the beginning of sleep did not affect the structure of human sleep, although it suppressed the GH secretion peak (Kupfer *et al.* 1992). Repeated i.v. boluses of somatostatin caused only a non-significant tendency of enhanced REM sleep in young males (Steiger *et al.* 1992). When somatostatin was injected into elderly subjects according to the same protocol, REM sleep decreased and the sleep structure deteriorated (Frieboes *et al.* 1997).

GH-secretagogues

GH-secretagogues (GHS) are a class of synthetic oligopeptides, such as growth hormone-releasing peptide 6 (GHRP-6), GHRP-2 and non-peptide compounds (MK-677, L-692,429, L-629,585), which are potent stimulating factors of GH release (Bowers *et al.* 1984, Bowers 1994). The GHS receptor, which differs from the GHRH receptor, has been cloned (Howard *et al.* 1996) and it is found to be expressed in the pituitary, arcuate nucleus and ventromedial hypothalamus (Guan *et al.* 1997). Approximately 25% of the GHRH cells in the arcuate nucleus and periventromedial hypothalamus express GHS receptor mRNA (Tannenbaum *et al.* 1998). GHSs stimulate GH secretion both directly from the pituitary (Cheng *et al.* 1993) and indirectly by affecting the activity of GHRH cells in the arcuate nucleus (Dickson *et al.* 1995, Dickson and Luckman 1997). The present hypothesis is that the GHS receptor is a receptor for an unknown endogenous substance controlling GH secretion.

Pharmacological studies of the effects of GHSs on human sleep suggest that these compounds could facilitate sleep. Repeated i.v. boluses of GHRP-6 during early night increased the amount of light S2 sleep, although the amount of wakefulness, SWS or REM sleep was not affected (Frieboes *et al.* 1995). An i.v. bolus of GHRP-2 after the third REM sleep episode did not affect sleep parameters significantly while causing a marked GH secretion pulse (Moreno-Reyes *et al.* 1998). Oral administration of MK-677 at bedtime for 7-14 days was found to increase the amount of deep SWS (S4) and REM sleep in young (18-30 years) and REM sleep in elderly (65-71 years) subjects (Copinschi *et al.* 1997). GH secretion was not affected while IGF-1 levels increased as a consequence of GHS treatment in the young subjects. In the old

subjects administration of MK-766 increased both GH secretion and IGF-1 levels (Copinschi *et al.* 1997). The effects of systemic or local injections of GHS on sleep have not been studied in experimental animals according to the current literature. I.c.v. injection of GHRP-6 paradoxically decreased GH secretion in rats (Yagi *et al.* 1996). This may be due to stimulation of hypothalamic somatostatin cells because pretreatment with somatostatin antibody blunted this effect. I.c.v. injection of GHSs stimulated electrical activity and Fos protein production in the arcuate GHRH cells in rats (Dickson *et al.* 1995). Immunoneutralization of endogenous GHRH strongly decreased the effect of injected GHRP on GH secretion (Yagi *et al.* 1996), which suggests that the effect of GHS is mostly mediated by GHRH and not by its direct effect on somatotrophic cells in the pituitary.

There is a special interest in GHS as a possible remedy against age related attenuation of GH secretion (Merriam *et al.* 1997). Spontaneous secretion of GH decreases strongly during the fourth decade of life and this could partly explain the simultaneous reduction of SWS (Van Cauter *et al.* 1998). As presented earlier, many of the known GHSs are effective when administered orally. Since they stimulate both GHRH and GH secretion they could improve SWS and REM sleep and also have such beneficial effects as conservation of muscle mass and decreased accumulation of fat.

The role of galanin as a possible sleep regulating neuropeptide

Galanin is a 29 amino acid peptide isolated first from the porcine intestine (Tatemoto *et al.* 1983). The human galanin has 30 amino acids. The rat galanin mRNA is about 900 base pairs and it encodes a 124 amino acid precursor peptide, from which the 29 amino acid galanin is finally cleaved (Vrontakis *et al.* 1987). Galanin is present in the gastrointestinal tract and widely in the CNS where it has been found to participate in many brain functions such as feeding, memory consolidation, nociception and hormone release (Bartfai *et al.* 1993). Galanin is usually co-localized with a non-peptide or with another peptide transmitter: with acetylcholine in the preoptic area, with GHRH and dopamine in the hypophysiotropic cells of the arcuate nucleus, with CRH in the paraventricular nucleus, with histamine in the posterior hypothalamus, and with noradrenaline in the locus coeruleus (Melander *et al.* 1986, Skofitsch and Jacobowitz 1985). The post-synaptic effects of galanin are usually inhibitory and mediated by three types of galanin receptors (Smith *et al.* 1998). Systemic or intracerebroventricular injection of galanin increased GH secretion while immunoneutralization or i.c.v. injection of galanin antagonist decreased it (Bauer *et al.* 1986, Ottlecz *et al.* 1988, Gabriel *et al.* 1993). Since galanin does not affect GH release from pituitary cells in vitro, it is probable that galanin affects

the GH regulatory system in the hypothalamus (Ottlecz *et al.* 1988). Galanin immunoreactive fibers are found in the periventricular nucleus (Liposits *et al.* 1993) and periventricular somatostatin cells express galanin receptors (Chan *et al.* 1996). This suggests that galanin increases GH secretion by inhibiting somatostatin release (Tanoh *et al.* 1993) (Fig.1). Since very few GHRH cells in the arcuate nucleus express galanin receptors (subtype 1), it is possible that GHRH is indirectly involved in this mechanism (Chan *et al.* 1996).

Since galanin is expressed in brain areas known to regulate vigilance states, e.g. in the preoptic area, posterior hypothalamus, and locus coeruleus, galanin might directly affect sleep as a sleep modulatory neuropeptide or by the hypothalamic GH regulatory system. The noradrenergic locus coeruleus is one possible site for a direct effect of galanin on sleep. Galanin immunoreactive fibers are found in the locus coeruleus (Skofitsch and Jacobowitz 1985) and locus coeruleus cells express galanin receptors which inhibit noradrenergic cells like somatostatin in the same nucleus (Sevcik *et al.* 1993). Recent findings in human subjects indicate that i.v. administration of galanin increases sleep (Murck *et al.* 1999).

AIMS OF THE STUDY

According to the literature GHRH may act as a slow wave sleep facilitating factor when injected systemically or i.c.v. Corresponding studies with somatostatin have demonstrated that somatostatin may facilitate REM sleep, but the results of the studies are divergent showing some discrepancy between the findings in experimental animals and humans. It should be noted that the effects of somatostatin receptor antagonist on sleep structure have not been studied. Further, there is no earlier knowledge about the effects of total sleep deprivation or selective REM sleep deprivation on the gene expression of GHRH and somatostatin in the hypothalamus. Furthermore the proposed sleep regulatory role of galanin, which is known to affect the hypothalamic GH-regulatory system, is almost completely unstudied. For these reasons this work concentrated on the present series of experiments:

I To measure the effects of selective REM sleep deprivation on somatostatin, GHRH and galanin mRNA expression in hypothalamic cells, and to measure the effect of recovery sleep on these neuropeptide mRNAs after REM sleep deprivation.

II To measure the effect of short term total sleep deprivation on somatostatin, GHRH and galanin mRNA expression in hypothalamic cells, and to compare the differences in the effects of REM sleep deprivation and total sleep deprivation on the mRNA expression of these neuropeptides.

III To study the effects of blocking endogenous somatostatin by administering a somatostatin antagonist on spontaneous sleep and rebound sleep after REM sleep deprivation, and to compare these effects with those observed after administrations of somatostatin and galanin.

IV To study the role of the locus coeruleus as one of the possible sites of sleep modulation by administering somatostatin, somatostatin antagonist and galanin locally into the cells of locus coeruleus.

MATERIALS AND METHODS

Experimental animals

Adult male Hannover Wistar (I, II, IV) and Sprague Dawley (III) rats, weight 270-390g, total number 158 were used. The rats were bred in the colony of the Department of Physiology of the University of Helsinki (I,II), at the University of Zürich (III) and at the Experimental Animal Center of the University of Helsinki (IV). The animals were kept in 12/12h light/dark rhythm with free access to standard rat chow and tap water. The animal experiment protocols and animal housing conditions were approved by the provincial administrative board in accordance with the laws of Finland and the European convention for the protection of experimental animals (N:o 1360/1990).

REM sleep deprivation (I, II, IV)

REM sleep deprivation was achieved by the platform method first described by Jouvet for cats (Vimont-Vicary *et al.* 1966) and Morden for rats (Morden *et al.* 1967). The rats were kept on small platforms (diameter 6.5- 7.5 cm) surrounded by water of approximately 5 cm depth on the cage floor. Food and fresh drinking water were freely available during the REM sleep deprivation. Under these conditions REM sleep is suppressed almost totally (Porkka-Heiskanen *et al.* 1995) (IV) because the animal is not able to maintain its balance on the platform during REM sleep-associated muscle hypotonia. In the in situ histochemistry experiments (I, II) rats (n=6/group) were deprived in the same waterbath-cage system for 24 h (I, II) or 72 h (II) with 7-9 platforms i.e. the rats were able to move from platform to platform but were unable to sleep on any of them. The rats in the REM sleep rebound group (n=6) were first deprived for 72 h, and then they were moved to their usual cage for 24 h. Groups of control animals were kept for corresponding times on large platforms (diameter 11 cm) which allow the same conditions as the small platforms otherwise, except that almost the same amount of REM sleep is produced as in the baseline conditions (Porkka-Heiskanen *et al.* 1995). An additional control group consisted of animals housed together and taken directly from their regular cage (“home controls”). In plasma GH measurement (II) and i.c.v. injection (IV) experiments deprivation was produced in single rat cages containing two platforms per rat.

Total sleep deprivation (III)

Total sleep deprivation was achieved using the gentle handling method (Franken *et al.* 1993). Rats were kept awake by introducing and removing different objects e.g. pieces of wood and cardboard boxes from the cage. The aim of this method is to achieve sleep deprivation with minimal stress by using the natural curiosity of rats i.e. their ability to forget sleeping when they examine new objects in the cage. The rats were observed continuously, and were woken up by tapping the cage or by touching them lightly if they were falling asleep regardless of the objects. Dim red illumination allowed observation during the dark phase. During total sleep deprivation, the vigilance state was not monitored by on-line EEG, but the criteria for sleep behavior were identical with those used previously (Tobler and Borbély 1990, Franken *et al.* 1993) and sleep deprivation was produced in the same laboratory (Institute of Pharmacology, University of Zürich).

In situ hybridization protocol (I-III) (Fig. 2)

At the end of REM (I, II) or total sleep deprivation (III) the rats were decapitated with a guillotine, their brains immediately removed and frozen on a piece of solid carbon-dioxide. 20 µm coronal sections were cut through the hypothalamus with a cryotome. Sections were thaw-mounted on gelatin coated slides and stored at -70°C. Before the hybridization the sections were post-fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 5 min at +4°C, washed in cold PBS (0.1 M Na_2HPO_4, 0.15 M NaCl, pH 7.4) for 2 min, acetylated 10 min in 0.15% triethanolamine containing 2% acetic anhydride, dehydrated with graded ethanols and delipided for 5 min in chloroform. 48 base oligonucleotide probes were made by oligonucleotide synthesizer (Northwestern University, Evanston, IL, U.S.A.). The galanin probe consisted of bases 221-268 of galanin mRNA (Vrontakis *et al.* 1987), the somatostatin probe consisted of bases 361-408 of the rat somatostatin mRNA (Goodman *et al.* 1985), and the GHRH probe contained the bases 323-370 of the rat GHRH mRNA (Gonzales-Cresbo and Boronat 1991). The probes were labeled at the 3' end with ^{35}S-dATP (Amersham, Buckinghamshire, UK) using terminal transferase (TdT, Pharmacia Biotech Europe GmbH). The purified probe was mixed with 1% yeast tRNA (Boehringer Mannheim GmbH, Germany) solution and TED (10 mM Tris, 1 mM EDTA, 10 mM dithiotreitol, pH 8.0) buffer, heated to 70°C for 3 min and cooled on ice. The final concentration of the probe in the hybridization buffer (50% formamide, 10% dextran, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 1x Denhardts and 10 mM dithiotreitol) was 3 pmol/ml, and the specific activity was 3500 Ci/mmol for galanin (I), 1220-1610 Ci/mmol for somatostatin (II, III) and 1250-1750 Ci/mmol for GHRH (II, III).

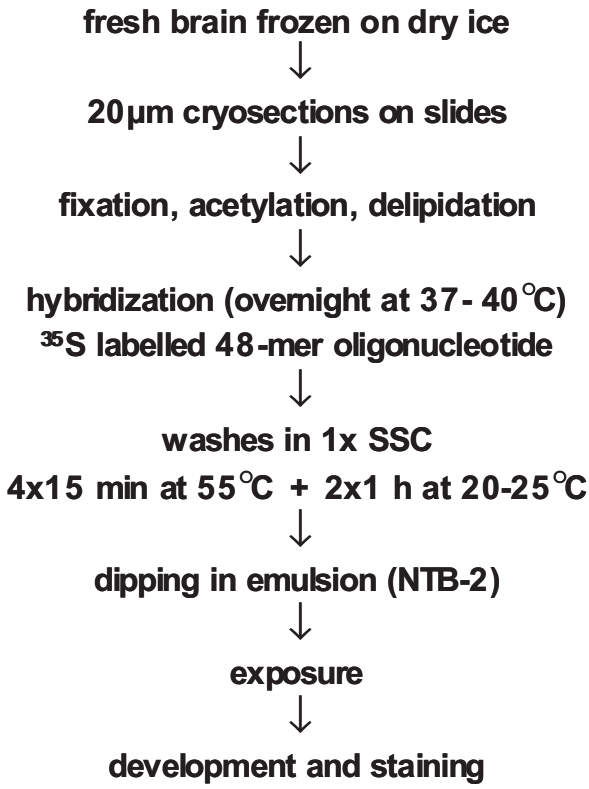

Fig. 2 Schematic presentation of the sequence of the consecutive steps in the in situ hybridization.

Sections were covered with 45 µl of hybridization mixture, covered with silanized cover slips (50x24 mm), and incubated overnight at 37 - 40°C. The slides were washed in 1xSSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 4 x 15 min at 55 °C, followed by 2 x 1 h washes at room temperature. After dehydration with graded ethanols containing 6% ammonium acetate, slides were dipped in Kodak NTB-2 emulsion diluted 1:1 with 0.6 M ammonium acetate. The slides were exposed in light-proof boxes at +4°C for 21 days (galanin), 15-18 days (somatostatin) and 29-30 days (GHRH). After exposure the emulsion was developed with D-16 developer (+16°C diluted 1:1) and Kodak T-max fixer, and the sections were lightly stained with cresyl violet acetate for visualization in bright and dark field photo microscopy (Nikon Labophot, magnification 40 - 400 x). The sections were matched according to the rat brain atlas of Paxinos and Watson (1986) for analysis. The cells containing mRNA of somatostatin, GHRH or galanin were identified in the dark field as clusters of bright silver granules around stained blue nuclei on dark red background. If necessary, the cluster was checked in the bright field as dark granules around a stained violet nucleus. The specifity of the hybridization was tested by treatment of the sections with ribonuclease (10 µg/ml, Pharmacia Biotech) and the addition of 100 x the amount of unlabeled probe into the hybridization mixture. Both of these treatments extinguished the signal in the sections.

Cell count and computer densitometry

The amount of mRNA was measured by counting the number of mRNA expressing cells in specific nuclei (I-III) or by computer image-analysis densitometry (III). For the cell count, all cells in the area of the specific nucleus which expressed more than five times the background granule density were included and counted visually by the microscoper. Both measurements (cell count and densitometry) were made in 4-6 sections at regular intervals along the rostrocaudal projection of the studied nucleus and the mean of the measured sections was calculated.

For the computer densitometry, a Sony CCD video camera mounted in a Nikon Labophot microscope with a combined dark/light field condenser was used. The video signal was A/D converted by a LG-3 frame grabber card (Scion Corporation, Frederic, MD, U.S.A.) and image analysis was done by a NIH Image program running in a Macintosh Centris 650. During the measurements the analyzer was unaware of the group status of an individual rat. The sections were analyzed in dark field illumination. The nucleus was encircled by free-hand drawing of a border around the outermost mRNA-expressing cells in the nucleus. The mean density and the area of the selection were measured. The mean density of the background was measured from a rectangular area of corresponding size in the immediate vicinity of the nucleus. The mean density of the background was subtracted from the mean density of the nucleus and the resulting difference was multiplied by the area of the nucleus. Multiplication of the signal density with the area compensates for the influence of the background between the cells to the signal value.

Linearity of the densitometry was tested by regression analysis of measured density values (n=10) compared with the number of silver granules counted in the same areas. For comparing the slope of regression values between different sections these measurements were done in four sections and the slope values were tested by analysis of variance (ANOVA). In the regression analysis the r^2 values were between 0.94 and 0.77 and no significant difference was detected between the slope values of the different sections ($F_{(3,36)}$=1.27, $P > 0.1$).

Analysis of somatostatin cells (II, III)

Expression of somatostatin mRNA was measured in the arcuate and periventricular nuclei of the hypothalamus. In the arcuate nucleus the somatostatin cells were identified as a densely packed cell group situated just laterally of the ventral part of the third ventricle (Fig. 3b). In the REM sleep deprivation study (II) somatostatin mRNA was measured bilaterally from 6 sections between stereotactical levels -2.1 and -3.6 mm from the *bregma.* In the total sleep deprivation

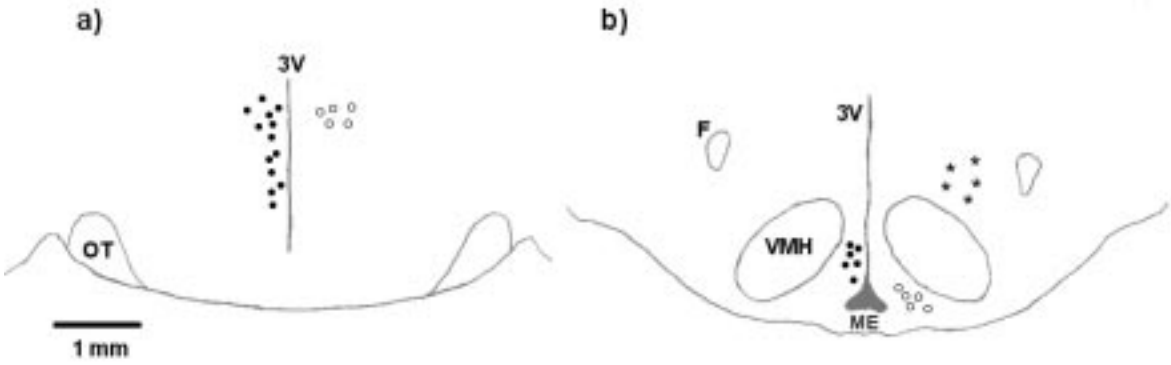

Fig. 3 Schematic drawing of the rat hypothalamus approximately from the coronal levels -1.8 (a) and -3.3 (b) from the *bregma*. **a)** location of somatostatin cells in the periventricular nucleus (●) and GHRH cells in the paraventricular nucleus (o). **b)** location of the somatostatin cells (●) and the GHRH cells (o) in the arcuate nucleus and the GHRH in the periventromedial hypothalamic area (*). 3V: third ventricle, F: fornix, ME: median eminence, OT: optic tract, VMH: ventromedial nucleus.

study (III) 4 sections were analyzed between -3.1 and -3.5. In the periventricular nucleus the somatostatin-expressing cells were grouped bilaterally beside the third ventricle (Fig. 3a). Expression of somatostatin mRNA was analyzed in 4 sections between -1.4 and -1.8.

Analysis of GHRH cells (II, III)

Expression of GHRH mRNA was measured in the paraventricular and the arcuate nuclei. In the total sleep deprivation study (III) the GHRH cells of the periventromedial hypothalamic area were also analyzed. The paraventricular nucleus was identified as the area where the GHRH mRNA- expressing cells were clustered lateral of the third ventricle (Fig. 3a). GHRH cells were analyzed bilaterally in 4 sections between the levels -1.8 and -2.2 from the *bregma*. GHRH cells in the arcuate nucleus were identified as a GHRH mRNA-expressing group of cells situated ventrolaterally of the third ventricle (Fig. 3b). Analysis was made bilaterally of 6 sections between the levels -1.9 and -3.4 (REM sleep deprivation) (II) and 4 sections between -3.05 and -3.45 (total sleep deprivation) (III). GHRH cells in the periventromedial hypothalamic area were a sparsely distributed group of cells situated dorsolaterally from the arcuate GHRH cells near the border of the ventromedial hypothalamic nucleus (Fig. 3b). Analysis was made bilaterally in 4 sections between levels -3.2 and -3.6.

Analysis of galanin cells (I)

Galanin mRNA expressing cells were counted in the medial preoptic area and the periventricular nucleus in the anterior hypothalamus. In the medial preoptic area galanin

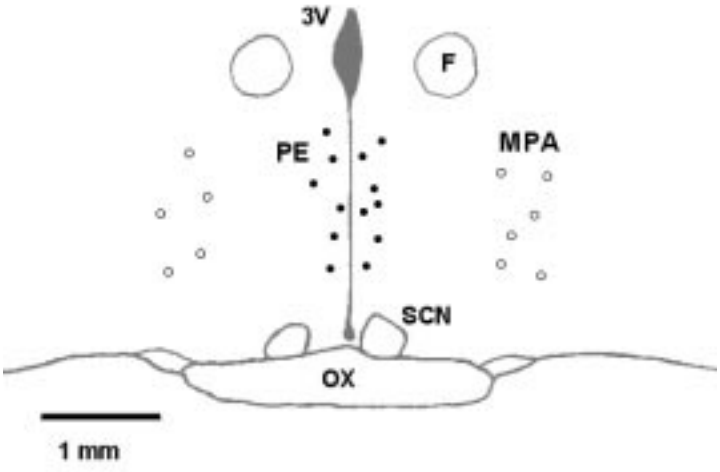

Fig. 4 Schematic drawing of the rat anterior hypothalamus approximately from the coronal level -0.92 from the *bregma*. Location of galanin cells in the periventricular nucleus (PE) (●) and in the medial preoptic area (MPA) (o). F: fornix, OX: optic chiasm, SCN: suprachiasmatic nucleus, 3V: third ventricle.

expressing cells were scattered in the anterior hypothalamus between anterior commissure and optic chiasm (Fig. 4). Cell counts were made between the stereotactical coronal levels -0.04 and -1.04 mm from the *bregma.* Galanin cells in the periventricular nucleus were clustered bilaterally beside the third ventricle (Fig. 4). Counts were performed in 5 sections between -0.56 and -1.04 mm from the *bregma*. Cells within a distance of 1 mm lateral from the third ventricle were included.

Plasma GH measurement during REM sleep deprivation (II)

Rats were provided with a silastic jugular cannula under combined medetomidine (Domitor^{R}, Orion-yhtymä, Espoo, Finland, 75 µg/kg s.c.) and pentobarbital (Mebunat^{R}, Orion, 45mg/kg i.p.) anesthesia. After the operation the cannula was heparinized, and the rats were allowed 48 h of recovery before the experiment. Five rats were deprived of REM sleep for 24 h by the platform method. A control group consisted of six rats kept on the large platforms. During deprivation hours 25-31 samples (200 µl)were collected from the cannula at 20 minute intervals. Blood was similarly collected from rats (n=4+4) that stayed in their home cages. REM sleep deprivation and large platform groups were compared to respective home control groups in two separate radioimmunoassays for GH. In the analysis laboratory the detection limit for GH-assay was 2.5 ng/ml and the intra- and inter assay coefficient of variations were 4.5% (intra) and 15.2% (inter) (Laartz *et al.* 1994). Reagents for the GH assay were provided by the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK, Bethesda, Maryland, U.S.A.) and RP-2 was used as a GH reference.

Animal surgery for i.c.v. and local microinjections (I, IV)

Two dental screw electrodes for electroencephalography (EEG) were implanted into the scull ipsilaterally above the frontoparietal cortex and two steel wire electrodes for electromyography (EMG) were inserted into neck muscles under combined medetomidine (Domitor^{R}, Orion, 0.1 mg/kg s.c.) and pentobarbital (Mebunat^{R}, Orion, 30 mg/kg i.p.) anesthesia. The leads of the electrodes were soldered into a connector, which was secured in place with dental acrylate. For intracerebroventricular injections (I, IV) a guide cannula (outer diameter 0.7 mm) was implanted into the lateral ventricle according to stereotactical coordinates: 0.8 mm posterior from the *bregma*, 1.4-1.5 mm lateral from the midline, 6.0 mm (I) or 3.8 mm (IV) ventral from the level of the *bregma* (Paxinos and Watson 1986). After the surgery the rats were allowed to recover for 5-7 days before the recordings. After the operation and during the first post-operative day rats received 20 µg/kg buprenorphine s.c. (Temgesic^{R}, Reckitt & Colman, Hull, England) for analgesia (IV). During the recovery and experiments the animals were kept in single animal boxes. Before the experiments the placement of the cannula was checked by injecting 0.2 µg of angiotensin II (Sigma Chemical Co., St. Louis, MO, U.S.A. product: A9525) into the cannula. Animals that did not show a clear drinking response were not used in the experiments. After the experiments the cannula placement was also verified by microscopy of 20 µm histological cryosections made of the dissected and frozen brains.

Injection procedure (I, IV)

In the i.c.v. experiments a steel injection cannula (outer diameter 0.4 mm) extending 0.5 mm beyond the guide was used. The cannula was connected by polyethylene tubing to a Hamilton syringe which was fitted into a CMA/100 microinjection pump (Carnegie Medicin, Stockholm, Sweden). In the microinjection experiments a cannula made of fused silica capillary (Composite Metal Services Ltd., The Chase, Hallow, Worcestershire, England.) (outer diameter 0.12 mm, extending 2 mm beyond the guide) was used. The injection system was first filled with distilled water and then a sufficient amount of injection solution was driven into the tubing separated from the water by an air bubble. In the i.c.v. injections a 2 µl volume was injected in 4 min (0.5 µl/min) and in the locus coeruleus microinjections 0.25 µl in 5 min (0.05 µl/min).

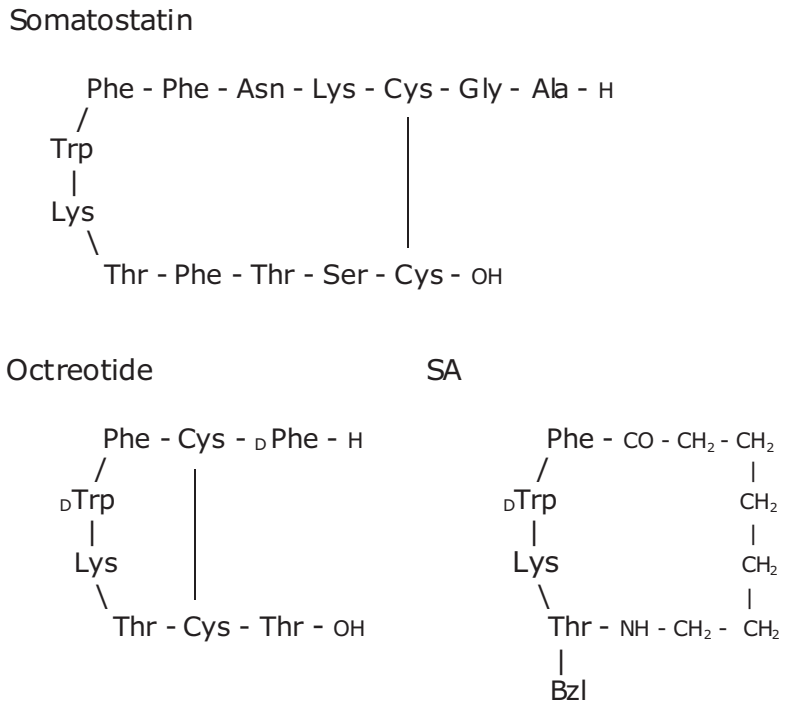

Fig. 5 Structure of somatostatin, octreotide (synthetic somatostatin analog) and somatostatin antagonist (SA) used in this study. Bzl: benzoate.

I.c.v. injections of somatostatin and somatostatin antagonist (IV)

In the i.c.v. somatostatin antagonist experiment, rats (n=5) received in a random order either 2 µl of artificial cerebrospinal fluid (aCSF) or 0.5 nmol of somatostatin antagonist, cyclo-(7-aminoheptanoyl-phe-d-trp-lys-thr(bzl)) (Sigma C4801, Fig. 5) dissolved in 2 µl of artificial CSF or 2 nmol of somatostatin antagonist in the same volume. In the somatostatin experiment 5 rats received artificial CSF, 0.5 nmol or 2 nmol of somatostatin (Sigma S9129) according to the same protocol. Injections were given between 9:30 and 10:30 after sleep onset.

I.c.v. somatostatin antagonist after REM sleep deprivation (IV)

24 h REM sleep deprivation was performed by the platform method starting and ending at 9:00 am. After the deprivation, rats were put into their home cages and an injection of artificial CSF or 0.5 nmol of somatostatin antagonist was given to 4 rats after the onset of sleep. A 2 nmol dose of somatostatin antagonist was studied using different rats (n=5) with the same protocol. Every rat received both treatments (artificial CSF and somatostatin antagonist) and there were 3-6 non-experiment days before the same rats were deprived and injected again.

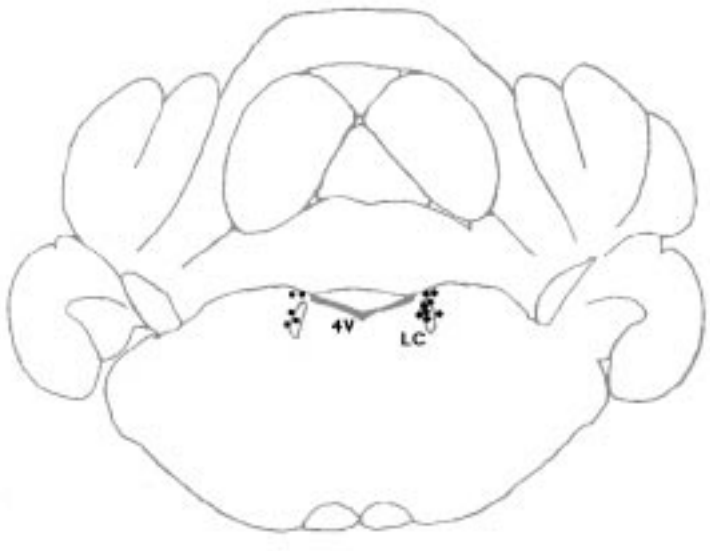

Fig. 6 Schematic illustration of the microinjection sites in the brainstem at the coronal level -9.3 - 9.8 from the *Bregma* according to photomicroscopic verification. ◆ (right): somatostatin / somatostatin antagonist injections (n=8), ● (left): galanin / somatostatin (n=5) (for clarity, plotted on the left side, actual injections were performed to the right). 4V: fourth ventricle, LC: locus coeruleus.

I.c.v. injections of galanin (I)

In the i.c.v. galanin experiments (I) rats received 0.06, 0.6 or 6 nmol of porcine galanin (Sigma G5773) at 9:00 am or 0.6 nmol at 8:00 pm (5-6 rats / treatment). Control injections consisted of saline vehicle. Every rat received altogether 2 treatments (1 dose of galanin and 1 saline control).

Microinjections into locus coeruleus (IV)

In the locus coeruleus microinjection experiments 8 rats received unilaterally 0.25 nmol of somatostatin, somatostatin antagonist or 0.25 µl of artificial CSF in a random order (Fig. 6). Injections were given in the morning between 9:30 and 10:00am after sleep onset. Another group of rats (n=5) received 0.25 nmol of rat galanin (Bachem AG, Bubendorf, Switzerland, product: H7450) or combined injections of 0.25 nmol of galanin + 0.25 nmol of somatostatin or 0.25 µl of artificial CSF according to the same protocol.

Sleep polygraphy (I, IV)

The EEG and EMG signals were amplified with an 8 channel Beckman type T electroencephalograph (Offner division, Chicago, U.S.A.). The amplified signal was plotted on paper (I) or collected and digitized by a CED 1401 plus interface (Cambridge Electronic Design

Ltd, Cambridge, England) with a sampling rate of 62 Hz. Data were displayed and stored by a Spike2 program (Cambridge Electronic Design Ltd) running in a desktop PC. During the recordings rats were remote-observed by means of a video camera and monitor. Collected data were categorized visually into wakefulness, non-REM sleep and REM sleep in 30-s epochs according to the standard criteria (Timo-Iaria *et al.* 1970). Briefly, wakefulness was defined as low amplitude and mixed intermediate/high frequencies in EEG and high amplitude EMG with artifacts caused by body movements. Non-REM sleep was defined as high amplitude slow waves in the EEG and intermediate EMG amplitude, and REM sleep was defined as low/intermediate amplitude of predominant theta frequency in EEG combined with low amplitude EMG with occasional twitches. Transition from non-REM to REM sleep ("pre-REM sleep", high amplitude theta bursts mixed into slow waves in EEG) was allocated equally between non-REM and REM sleep.

Proportions of vigilance states per 2 h time intervals were calculated from the 6 h (IV) 8 h (I) recording. In experiment IV the first half hour after the injection was excluded because of the arousal caused by the injection procedure.

Statistics

The effect of REM sleep deprivation on mRNA expression (I, II) was evaluated with one-way analysis of variance (ANOVA) between the home cage condition and 24 h or 72 h on small or large platforms. Significant results were further analyzed by pairwise comparisons between the treatment groups with Student-Newman-Keuls method (I) or Duncan's test (II). The effects of rebound sleep after 72 h REM sleep deprivation (II) were calculated separately by two-way ANOVA for the 72 h deprivation and 72 h deprivation + 24 h rebound groups. The factors were treatment (small or large platform) and length of rebound (0 or 24 h).

The effects of total sleep deprivation on mRNA expression (III) were tested by two-way ANOVA. The factors were treatment (deprivation or control) and time of decapitation (9:00 after 12 h treatment or 15:00 after 6 h treatment). If interaction between the factors was detected ($P > 0.1$ for interaction) all four groups were compared by one-way ANOVA followed with paired comparisons by Newman-Keuls method.

The plasma GH measurements (II) were evaluated by integrating the area under the curve (AUC), and the difference between platform and corresponding home cage control groups was compared using the *t*-test for independent measurements.

In the i.c.v. somatostatin, somatostatin antagonist and in the microinjection experiments (IV) differences in the proportions of the vigilance states between the treatments were compared by one-way ANOVA for repeated measurements. Significant results were compared pairwise between the treatments by Newman-Keuls test or by Bonferroni *t*-test (somatostatin and somatostatin antagonist microinjections). In the i.c.v. galanin injection (I) and i.c.v. somatostatin antagonist after REM sleep deprivation (IV) experiments the treatments were compared by paired *t*-test. In all statistical tests $P < 0.05$ was set as a limit of statistical significance.

RESULTS

The effect of REM and total sleep deprivation on somatostatin mRNA

REM sleep deprivation for 24 h increased the number of somatostatin mRNA expressing cells significantly in the arcuate nucleus when compared to 72 h REM sleep deprivation and control groups ($F_{(4, 22)}$=6.72, P = 0.001, Duncan P < 0.05) (II) (Fig. 7a). After 72 h of REM sleep deprivation there was no difference in the number of somatostatin mRNA expressing cells in the arcuate nucleus when compared to the corresponding large platform control or home group. In the periventricular nucleus the number of somatostatin cells was significantly higher after 72 h of REM sleep deprivation compared to large platform controls ($F_{(4, 18)}$=3.42, P = 0.03, Duncan P < 0.05) but neither treatment group differed from the home control group (II) (Fig. 7b). 24 h REM sleep deprivation did not affect the number of somatostatin mRNA expressing cells in the periventricular nucleus. After a 24 h recovery after 72 h REM sleep deprivation the number of somatostatin mRNA expressing cells in the arcuate and periventricular nuclei did not differ from the control and 72 h deprivation groups (II) (Fig. 7a-b).

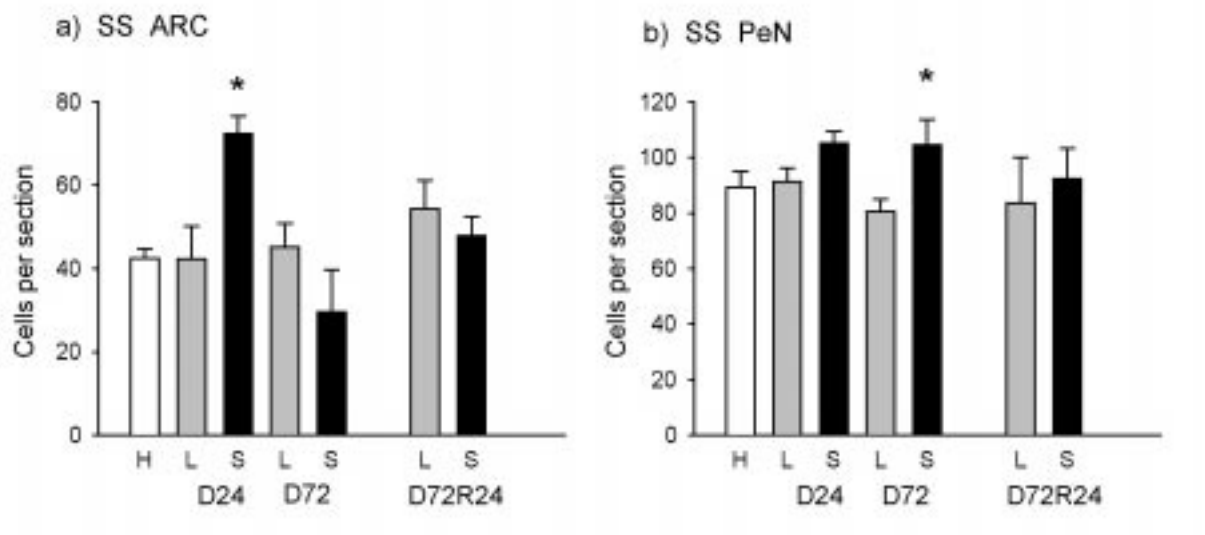

Fig. 7 The average number of somatostatin (SS) mRNA-expressing cells in the rat arcuate (ARC) (a) and periventricular (PeN) (b) nuclei. H: home control, S: REM sleep deprivation with the platform method (small platforms), L: large platform controls. D24 and D72: deprivation for 24 and 72 hours, D72R24: deprivation for 72 h followed by 24 h of rebound sleep in normal conditions. Vertical bars indicate the standard error of means (SE). * Duncan's test P < 0.05 after one-way ANOVA at significance level P < 0.05.

Both 6 and 12 h total sleep deprivation increased the amount of somatostatin mRNA in the arcuate nucleus measured by densitometric analysis when compared to the control groups ($F_{(1,20)}$= 5.84, P = 0.03) (III) (Fig. 8a). Cell count showed a similar but non-significant trend between the deprivation and control groups ($F_{(1,20)}$= 3.04, P = 0.10). In the periventricular nucleus, total sleep deprivation did not affect the amount of somatostatin mRNA when compared to controls ($F_{(1, 20)}$= 0.01, P = 0.92)(III) (Fig. 8b).

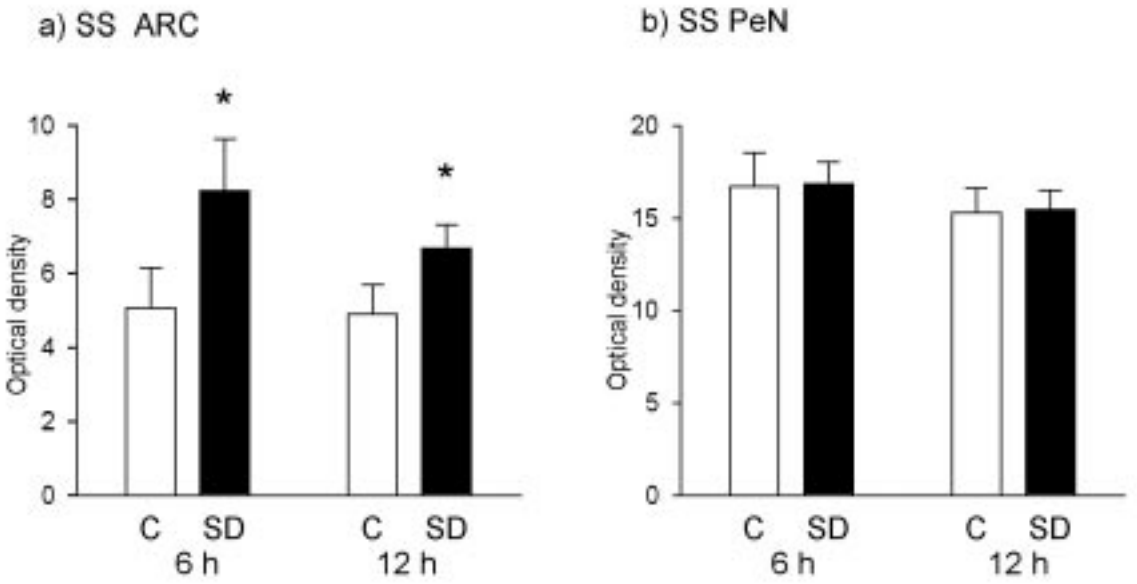

Fig. 8 Mean optical density of somatostatin (SS) mRNA visualized by in situ hybridization in the arcuate (ARC) (a) and periventricular (PeN) (b) nuclei. Groups: control (C), sleep deprivation by gentle handling (SD) either for 6 h during the light phase (decapitated at 15:00) (6 h) or 12 h during the dark phase (decapitated at 9:00) (12 h). * The amount of somatostatin mRNA increased as a consequence of total sleep deprivation in the arcuate nucleus (two-way ANOVA: $P = 0.03$ for SD vs. control).

The effect of REM and total sleep deprivation on GHRH mRNA

REM sleep deprivation for 24 and 72 h on the small platforms decreased the number of GHRH mRNA expressing cells in the paraventricular nucleus (II) (Fig. 9b). 72 h on the large platforms also decreased the number of GHRH mRNA cells when compared to home and 24 h large platform groups ($F_{(4, 19)} = 9.01$, $P = 0.0003$, Duncan $P < 0.05$). In the arcuate nucleus the number of GHRH mRNA cells tended to be lower in all platform treatment groups when compared to the home controls. ($F_{(4, 21)} = 2.57$, $P = 0.07$) (II) (Fig. 9a). 24h rebound sleep after 72 h deprivation had no effect on the number of GHRH mRNA expressing cells in the

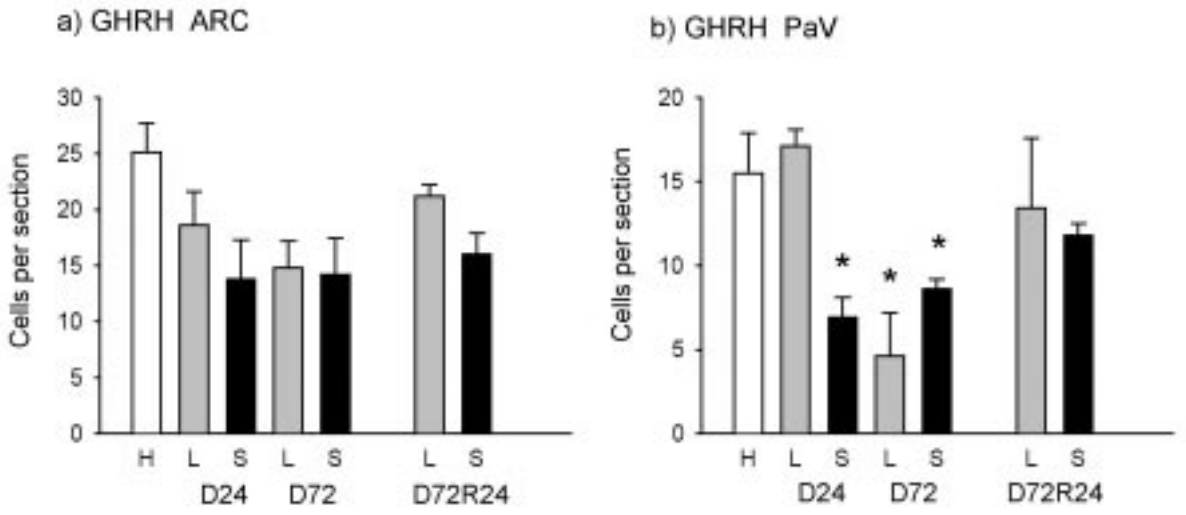

Fig. 9 The average number of GHRH mRNA-expressing cells in the rat arcuate (ARC) (a) and paraventricular (PaV) (b) nuclei. H: home control, S: REM sleep deprivation with the platform method (small platforms), L: large platform controls. D24 and D72: deprivation for 24 and 72 hours, D72R24: deprivation for 72 h followed by 24 h of rebound sleep in normal conditions. Vertical bars indicate the standard error of means (SE). * Duncan's test $P < 0.05$ after one-way ANOVA at significance level $P < 0.05$.

paraventricular or arcuate nuclei when compared to 72 h deprivation or large platform controls (II) (Fig. 9).

Total sleep deprivation for 6 h during the light phase increased the amount of GHRH mRNA in the paraventricular nucleus measured by densitometric analysis when compared to controls and 12 h deprivation during the dark phase ($F_{(3, 20)}$= 3.57, P = 0.03, Newman-Keuls: $P < 0.05$) (III) (Fig. 10a). 12 h total sleep deprivation did not affect the amount of GHRH mRNA when compared to controls. Cell count from the same areas showed a similar but non-significant result ($F_{(3, 20)}$= 1.71, P = 0.20). In the arcuate nucleus 6 or 12 h sleep deprivation did not affect the amount of GHRH mRNA ($F_{(1,20)}$=0.43, P=0.52) (III) (Fig.10b). In the periventromedial area the amount of GHRH mRNA was significantly higher in the morning (9:00am) than in the afternoon (3:00pm) ($F_{(1,20)}$= 5.17, P = 0.03) (III) (Fig.10c). 6 or 12 h sleep deprivation did not have any effect when compared to the control treatments ($F_{(1, 20)}$= 0.15, P = 0.71). Cell count showed again a similar but non-significant trend in the difference between 9:00am and 3:00pm groups in the periventromedial area ($F_{(1, 20)}$= 3.55, P = 0.07).

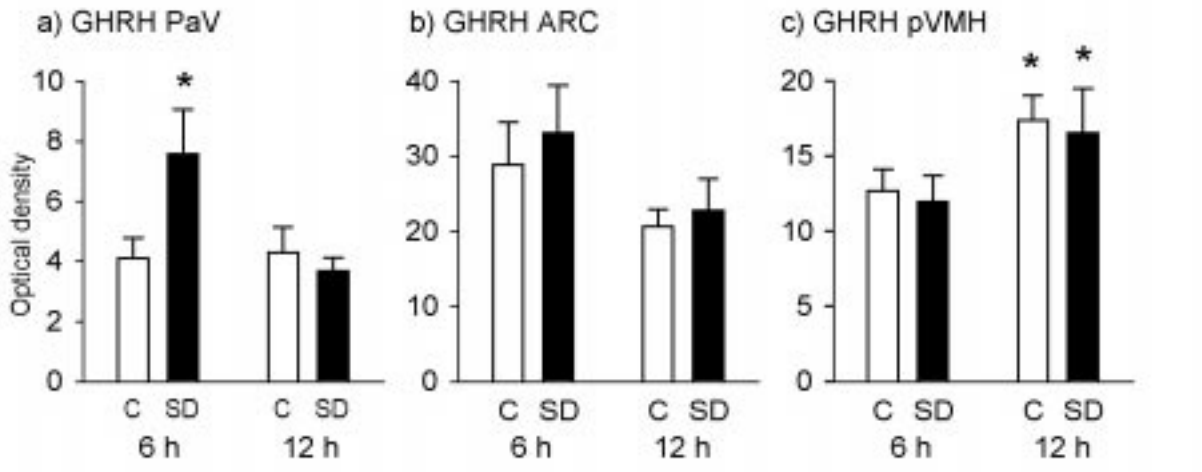

Fig. 10 Mean optical density of GHRH mRNA visualized by in situ hybridization in the paraventricular (PaV) (a) and arcuate (ARC) nuclei (b) and in the periventromedial (pVMH) area (c). Groups: control (C), sleep deprivation by gentle handling (SD) either for 6 h during the light phase (decapitated at 15:00) (6 h) or 12 h during the dark phase (decapitated at 9:00) (12 h). * The amount of GHRH mRNA increased after 6 h total sleep deprivation in the arcuate nucleus when compared to all other groups (one-way ANOVA P = 0.03, Newman-Keuls $P < 0.05$) (a). The amount of GHRH mRNA was higher in the rats decapitated in the morning (9:00) than in the afternoon (15:00) in the periventromedial area (two-way ANOVA: P = 0.03 for 9:00 (12 h) vs. 15:00 (6 h)) (c).

The effect of REM and total sleep deprivation on galanin mRNA

After 24 h REM sleep deprivation on the small platforms the number of galanin mRNA expressing cells increased in the medial preoptic area and the periventricular nucleus when compared to home and large platform controls (I) (Fig. 11). There was no significant difference in the number of galanin mRNA expressing cells between the two control groups (home and large platforms). 6 hour total sleep deprivation during the first half of the light phase and 12 hour

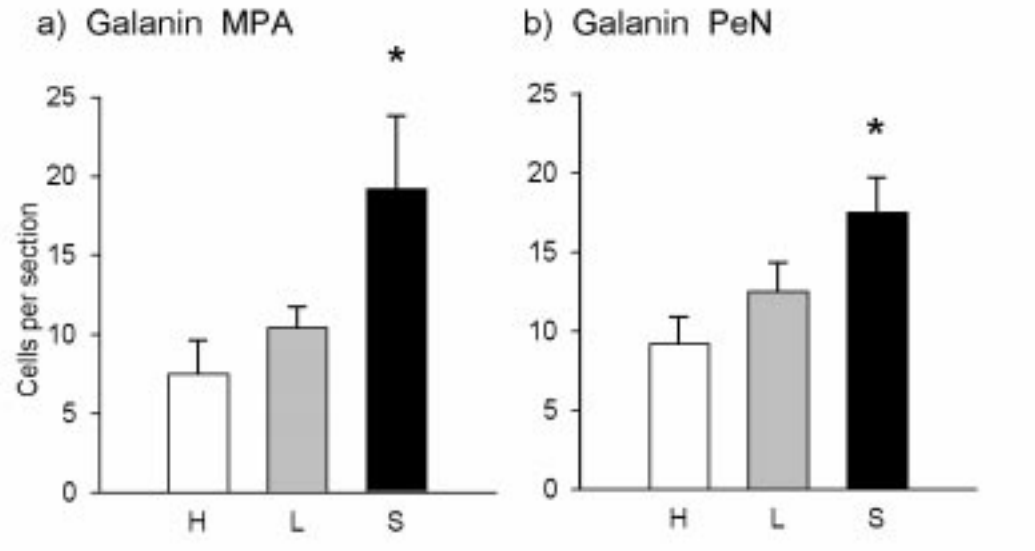

Fig. 11 Number of galanin mRNA-expressing cells in the medial preoptic area (MPA) (a) and in the periventricular nucleus (PeN) (b) after REM sleep deprivation or control conditions. S: 24 h REM sleep deprivation on small platforms, L: large platform controls, H: home controls. * one-way ANOVA $P < 0.05$, Newman-Keuls: S vs. L and H $P < 0.05$.

deprivation during the dark phase did not affect the number of galanin mRNA expressing cells in the medial preoptic area and the periventricular nucleus (Toppila *et al.* 1996).

The effect of REM sleep deprivation on plasma GH

Secretion of GH occurred in typical secretion pulses at approximately 3 h intervals. These pulses were generally much lower in the REM sleep deprived (Fig. 12b) and large platform rats (data not shown) than in the home controls (Fig. 12a). The plasma GH content approximated by the integrated area under the curve (AUC) was significantly lower during REM sleep deprivation (small platforms) and also during large platform treatment when compared to the

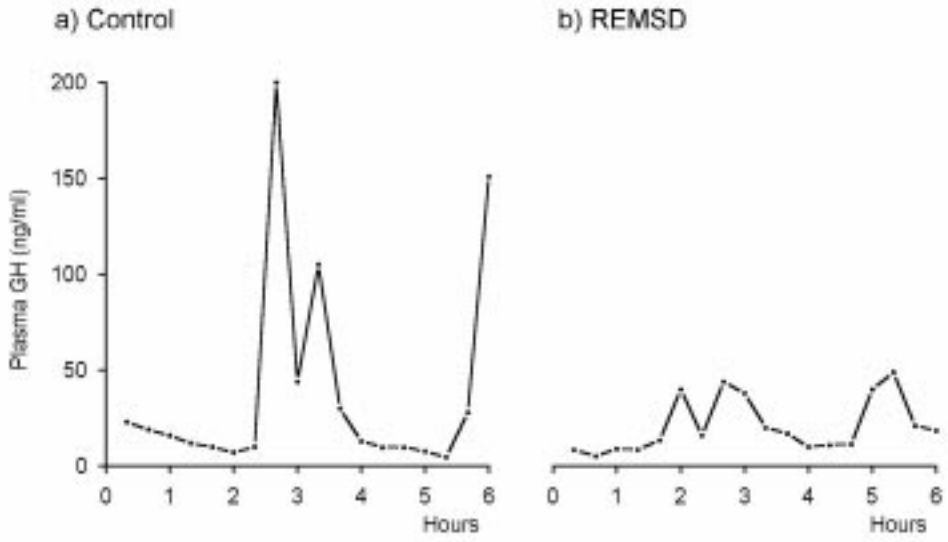

Fig. 12 The effect of REM sleep deprivation on the rat plasma GH profile. (a) untreated animal, (b): REM sleep deprivation with the platform method. The samples were collected between 24-30 h of REM sleep deprivation. The amplitude of GH pulses is lower in the REM sleep deprivation animal.

corresponding home control group (small platforms: $t_{(7)} = 2.49$, $P = 0.04$, large platforms: $t_{(8)} = 2.89$, $P = 0.02$) (II).

I.C.V. INJECTIONS OF SOMATOSTATIN, SOMATOSTATIN ANTAGONIST, AND GALANIN

Somatostatin and somatostatin antagonist

I.c.v. injection of 0.5 and 2 nmol of somatostatin antagonist reduced the amount of REM sleep during the post injection period from 0.5-2 hours when compared to artificial CSF controls (ANOVA: $F_{(2, 8)} = 4.66$ $P < 0.05$, Newman-Keuls: $P < 0.05$) (IV). (Fig. 13a) There was no difference between the two doses of somatostatin antagonist. Later, during the periods 2-4 and 4-6 hours, there was no significant difference in the amount of REM sleep between the treatments. Somatostatin antagonist did not affect the amount of non-REM sleep during the 6 h

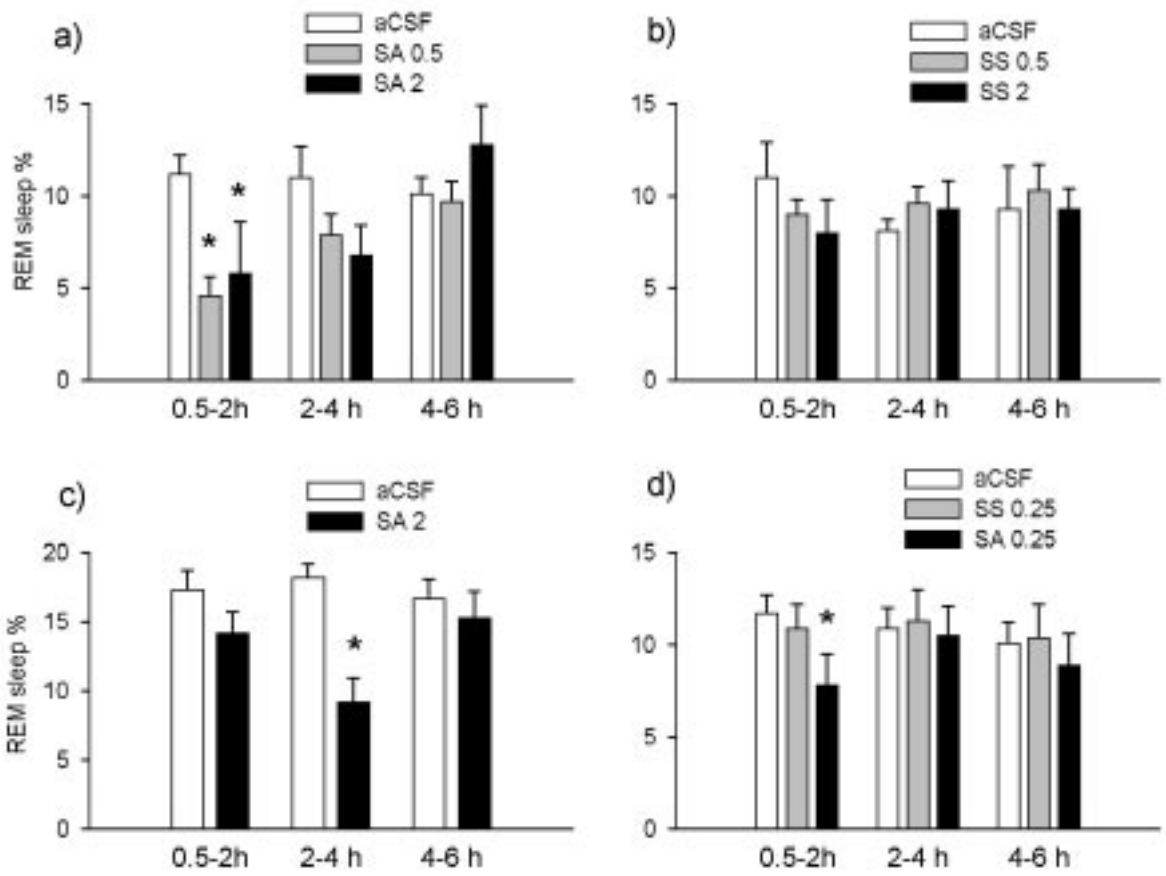

Fig. 13 The effect of injected somatostatin (SS) and somatostatin antagonist (SA) on mean proportions of REM sleep during three post-injection time periods. **a:** i.c.v. injection of artificial CSF (aCSF) (vehicle control), SA 0.5 or 2 nmol (n=5), * $F_{(2, 8)} = 4.66$, $P < 0.05$, Newman-Keuls: SA 0.5 and SA 2 vs. aCSF $P < 0.05$. **b:** i.c.v. injection of aCSF, somatostatin 0.5 or 2 nmol (n=5). **c:** i.c.v. injection of aCSF or 2 nmol of SA after 24h REM sleep deprivation (n=5). * Paired *t*-test: $t_{(4)} = 4.36$, $P < 0.05$. **d:** microinjection of aCSF, somatostatin 0.25nmol or SA 0.25nmol (n=8), * ANOVA $F_{(2, 10)} = 8.09$, $P < 0.01$, Bonferroni's t-test: SA vs.aCSF $t_{(6)} = 5.10$, $P < 0.01$. Vertical bars indicate the standard error of means (SE).

post-injection recording period. I.c.v. injected boluses of corresponding doses of somatostatin did not affect the amount of REM (Fig. 13b) or non-REM sleep during any post-injection time period when compared to artificial CSF controls (IV).

Somatostatin antagonist after REM sleep deprivation

After 24 h REM sleep deprivation an i.c.v. injection of 2 nmol of somatostatin antagonist reduced REM sleep during the time period 2-4 h post injection when compared to artificial CSF ($t_{(4)} = 4.36$, $P < 0.05$) (IV) (Fig. 13c). During the periods 0.5-2 and 4-6 h there was no significant difference in the amount of REM sleep. Non-REM sleep was not significantly affected. 0.5 nmol of somatostatin antagonist after REM sleep deprivation did not significantly affect the amount of REM or non-REM sleep during the 6 h recording of recovery sleep when compared to artificial CSF injection (IV).

Galanin

I.c.v. injection of 0.06, 0.6 or 6 nmol of galanin did not affect the daytime proportions of REM or non-REM sleep when compared to controls during the 8 h post-injection recording (I) (Tab. 1). During the first 8 h of the dark phase, when the amount of natural sleep is low in the rat, 0.6 nmol of galanin i.c.v. did neither affect the amount of REM or non-REM sleep when compared to control injection (I) (Tab. 1).

	REM sleep		non-REM sleep	
	%	SD	%	SD
Light				
Saline	10.0	1.8	51.9	3.7
Galanin 0.06	9.6	2.0	52.9	7.2
Galanin 0.6	12.8	2.6	48.7	5.0
Galanin 6	12.8	2.4	49.7	7.8
Dark				
Saline	5.0	0.5	27.7	2.0
Galanin 0.6	5.0	0.9	21.7	1.9

Tab. 1 The effects of i.c.v. injections of 0.06-6 nmol of galanin on sleep compared to saline controls. Sleep polygraphy was recorded 8 h during light (started ar 9:00 am) or dark (started at 8:00 pm) phase (lights 8:00 am - 8:00 pm). SD = standard deviation. n = 5-6 per group.

Microinjections of somatostatin, somatostatin antagonist and galanin into the locus coeruleus

The accepted sites of the microinjection were in the locus coeruleus or in the immediate vicinity of the nucleus not penetrating the fourth ventricle (Fig. 6). Microinjection of 0.25 nmol of somatostatin antagonist into the locus coeruleus reduced REM sleep during the post injection period 0.5-2 hours (ANOVA: $F_{(2, 10)}$= 8.09, $P < 0.01$, Bonferroni $t_{(6)}$= 5.10, $P < 0.01$) (IV) (Fig. 13d). Non-REM sleep was not significantly affected. A corresponding dose of somatostatin did not affect post-injection proportions of sleep phases when compared to artificial CSF (IV). Microinjection of 0.25 nmol of galanin or a combined injection of 0.25 nmol of galanin and 0.25 nmol of somatostatin did not affect REM or non-REM sleep during the 6 post-injection hours when compared to artificial CSF injection (IV).

DISCUSSION

GENERAL DISCUSSION

According to earlier studies there are several endogenous and synthetic compounds that affect sleep when injected systemically or directly into the CNS. The sleep modulatory mechanism of these substances can be either the direct effect on sleep regulatory brain mechanisms or an indirect effect mediated by e.g. metabolism, thermoregulation or the immune system. When studying brain mechanisms that regulate sleep, it is important to get knowledge about not only the sleep modulatory effects of pharmacological manipulation of a neuronal system, but also about the reciprocal effects of the sleep-wake rhythm and sleep deprivation on the activity of the studied neuronal system. In the case of the GH-axis and its effects on sleep there was already established knowledge about the synchronization of GH-secretion and sleep-wake rhythm, and about the sleep modulatory effects of pharmacological manipulation of the GH-axis, while the effects of sleep deprivation on the activity on the GH-regulating neuropeptides in the brain were mostly unknown.

Our results showed that REM sleep deprivation increased somatostatin and decreased GHRH mRNA in the hypothalamus, while total sleep deprivation (combined lack of slow wave and REM sleep) increased both GHRH and somatostatin mRNA in the hypothalamus. Earlier injection studies have revealed that injection of GHRH was capable to induce SWS, suggesting that GHRH is a possible SWS facilitatory neuropeptide (Obál *et al.* 1996). Our finding that GHRH mRNA increases during SWS loss but not during REM sleep deprivation when SWS is possible, is generally in accordance with the findings of the injection studies. According to the injection studies, somatostatin could facilitate REM sleep but these results obtained from animal and human studies are not as consistent as the results of the effect of GHRH on SWS. Our results that the lack of REM sleep achieved either by REM or total sleep deprivation will lead to increases in somatostatin mRNA in the hypothalamus, and the finding that i.c.v. or local injection of somatostatin antagonist will reduce REM sleep give further support to the hypothesis that somatostatin facilitates REM sleep in the brain. We also found that REM sleep deprivation increases the expression of galanin mRNA in the anterior hypothalamus although total sleep deprivation did not affect galanin mRNA. I.c.v. or local microinjection of galanin into the locus coeruleus did not affect the amount of sleep. This may indicate that galanin could mediate some of the effects of REM sleep deprivation in the brain, but does not itself affect sleep.

THE POSSIBLE ROLE OF GHRH IN THE REGULATION OF SWS

The GHRH mRNA expression varied essentially between the hypothalamic GHRH mRNA expressing nuclei after total or REM sleep deprivation. In the paraventricular nucleus total sleep deprivation increased while REM sleep deprivation decreased GHRH mRNA. On the other hand in the arcuate nucleus there were no significant changes after either total or REM sleep deprivation. In the periventromedial hypothalamic area there was a difference in the amount of GHRH mRNAs between morning and afternoon samples. It is of interest that sleep deprivation was ineffective in this respect. Thus it is possible that the GHRH nuclei in the hypothalamus have partly different functions (Fig. 14). In the following chapters the effects of sleep deprivation on different GHRH nuclei in the hypothalamus are discussed separately.

Paraventricular nucleus

After 24 h REM sleep deprivation the amount of GHRH mRNA was reduced in the paraventricular nucleus and this effect was also found after 72 h deprivation. However, at the 72 h time point the amount of GHRH mRNA was similarly decreased in the large platform controls. This may be due to the possibility that rats staying on the large platforms also experienced partial reduction of sleep although this was not not found in an earlier study from our laboratory (Porkka-Heiskanen *et al.* 1995). The effects of sleep deprivation may occur more slowly during staying on the large platforms than during staying on the small platforms. In the latter case 24 h REM sleep deprivation was already enough to reduce the amount of GHRH mRNA.

Total sleep deprivation for 6 h during the light phase increased the amount of GHRH mRNA in the paraventricular nucleus, which was an opposite effect to that seen in the REM sleep deprivation experiment. Total sleep deprivation for 12 hours during the dark phase did not affect GHRH mRNA in the paraventricular nucleus when compared to controls. The higher effect of the shorter deprivation may be due to the fact that rats will mostly sleep during the first half of the light phase, when the 6 h deprivation was performed, and thus more sleep was lost when compared to situation in the 12 h deprivation during the dark phase, when rats normally are mostly awake (Franken *et al.* 1991, Tobler and Borbély 1990). In a recent study the effects of total sleep deprivation on GHRH mRNA in the hypothalamus were measured by a reverse transcription polyclonal chain reaction (RT-PCR) method, and an increase in the GHRH mRNA

in the whole hypothalamus was observed after 8-12 h total sleep deprivation (Zhang *et al.* 1998). This finding agrees with our present findings in the specific hypothalamic nucleus.

We found that total sleep deprivation increases, while REM sleep deprivation decreases, the amount of GHRH mRNA in the paraventricular nucleus. This may be due to the different degree of sleep deprivation obtained by the total and the REM sleep deprivation methods. During the total sleep deprivation both non-REM and REM sleep are inhibited, but during REM sleep deprivation non-REM sleep was reduced only partly. A decrease of 38% in non-REM sleep during the first 24 h was observed, while REM sleep was inhibited almost totally (Porkka-Heiskanen *et al.* 1995). Our finding suggests that total inhibition of non-REM sleep activates the GHRH cells in the paraventricular nucleus. Reduction of GHRH mRNA in the paraventricular nucleus during REM sleep deprivation may be due to the inhibition which is mediated by somatostatin or by other transmitters which mediate the effects of REM sleep deprivation or stress. Unfortunately the afferent connections of the paraventricular GHRH cells are poorly known. Nevertheless, it is possible that this subgroup of GHRH cells mediates the SWS facilitatory effect of GHRH as suggested by the pharmacological studies (Fig. 14). The paraventricular GHRH cells are hypophysiotropic (Liposits 1993), but the nucleus has also projections to other brain areas such as preoptic area and basal forebrain (Sawchenko *et al.* 1985), where cholinergic and GABAergic cells regulate wakefulness and sleep (Szymusiak 1995) (Fig.14). Some cells in the preoptic area express GHRH receptors (Takahashi *et al.* 1995), but the transmitter identities of these cells are unknown. In a recent study it was found that local microinjections of GHRH into the preoptic area increased sleep and that microinjections of GHRH antagonist decreased spontaneous sleep and rebound sleep after short term sleep deprivation (Zhang *et al.* 1999). This supports the hypothesis that the preoptic area may be the target area for the SWS facilitatory effect of the endogenous GHRH.

Arcuate nucleus

In our study neither REM nor total sleep deprivation affected the amount of GHRH mRNA in the arcuate nucleus. It is known that the arcuate GHRH cells are mostly hypophysiotropic and that there are very few projections to other areas than the median eminence among these cells (Merchenthaler *et al.* 1985). Thus, it is possible that regulation of GH secretion is the main function of the arcuate GHRH cells and that the mRNA expression of these cells is thus not particularly sensitive to sleep loss (Fig. 14). Because secretion of GH is reduced during both REM (II) and total sleep deprivation (Kimura and Tsai 1984), it is probable that the arcuate GHRH cells are somehow inhibited by hypothalamic somatostatin cells, which increase mRNA

expression during sleep deprivation. Increased somatostatin activity may reduce the release of GHRH into the pituitary portal circulation, while the synthesis of GHRH remains unaltered, which is indicated by the unchanged amount of the arcuate GHRH mRNA after both total and REM sleep deprivation.

Periventromedial hypothalamic area

In the periventromedial hypothalamic area the amount of GHRH mRNA was higher in the rats decapitated in the morning (9:00 am) than in the afternoon (3:00 pm), while there was no difference between the sleep deprivation and control groups. The amount of GHRH mRNA in the whole of the hypothalamus samples has in another study using Northern blot analysis also been found to be higher in the morning (Bredow *et al.* 1996). One possibility is that this variation of GHRH mRNA may be associated with the daily sleep-wake distribution of the rat. Rats typically have the highest amount of non-REM sleep during the first half of the light phase (Borbély and Neuhaus 1978), at the same time when the amount of the periventromedial GHRH mRNA was found to be high. The periventromedial GHRH cells are not hypophysiotropic, but have projections to the preoptic area (Sawchenko *et al.* 1985), which could mediate the sleep association of these cells similarly as in the case of the paraventricular GHRH cells (Fig.14). The afferent connections of the periventromedial GHRH cells are unclear. It is possible, though at this point only hypothetical, that the activity of these cells is controlled by the suprachiasmatic or ventromedial nuclei, which activity oscillates in an endogenous circadian

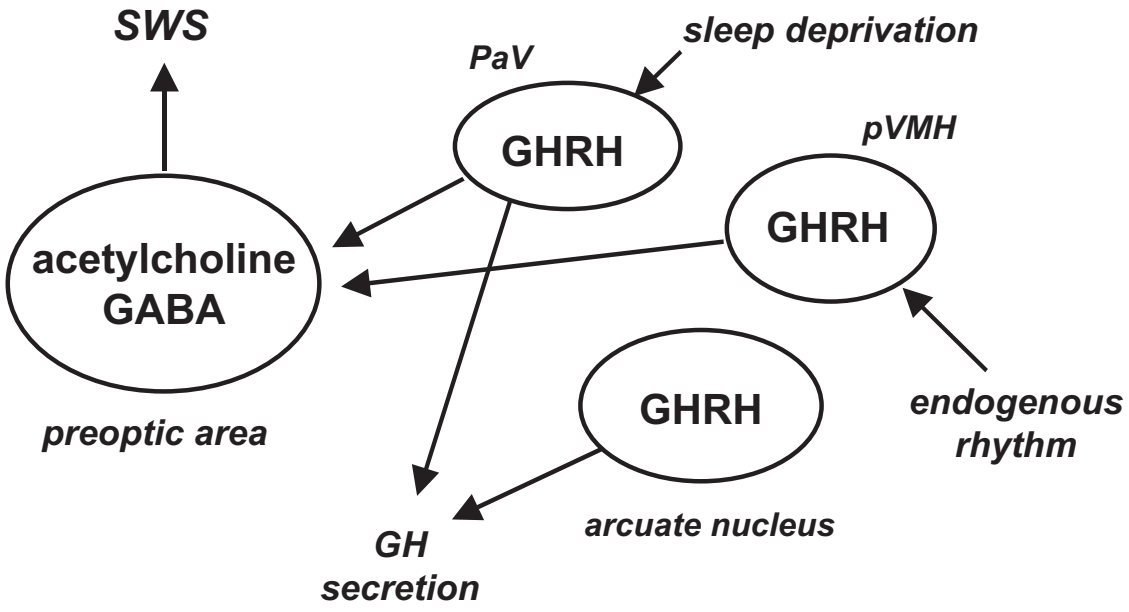

Fig. 14 Schematic illustration of the possible roles of the hypothalamic GHRH cell populations. PaV: paraventricular nucleus, pVMH: periventromedial hypothalamic area.

rhythm. The circadian association of sleep distribution may be partly mediated by this neuronal pathway.

THE POSSIBLE ROLE OF SOMATOSTATIN IN THE REGULATION OF REM SLEEP

Arcuate nucleus

According to our findings, both 24 h REM sleep deprivation and 6-12 h total sleep deprivation increased the amount of somatostatin mRNA in the arcuate nucleus. After the longer 72 h REM sleep deprivation there was no difference in the arcuate somatostatin mRNA between the REM sleep deprivation and control groups. The observed activation of the arcuate somatostatin cells as a consequence of REM sleep loss caused by REM or total sleep deprivation is in accordance with the injection studies suggesting that somatostatin facilitates REM sleep. It is known that the arcuate somatostatin cells are not hypophysiotropic, but project locally in the arcuate nucleus where they inhibit the hypophysiotropic GHRH cells (Merchenthaler *et al.* 1989, Willoughby *et al.* 1989). The amount of somatostatin mRNA oscillates in the arcuate nucleus in association with the GH secretion rhythm (Zeitler *et al.* 1991). By their effect on the hypophysiotropic GHRH cells in the same nucleus, these somatostatin cells may mediate the inhibition of GH secretion which is found during REM (II) and total sleep deprivation (Kimura and Tsai 1984). During the longer REM sleep deprivation the amount of somatostatin mRNA in the arcuate nucleus returned to the level of the control group. This may be a consequence of the feedback regulation caused by the decreased plasma GH, which may inhibit somatostatin cells in the arcuate nucleus.

Periventricular nucleus

In the periventricular nucleus the amount of somatostatin mRNA increased after 72 h REM sleep deprivation when compared to large platform control treatment but not when compared to unhandled animals allowed normal sleep. The durations of total sleep deprivation of 6 and 12 h did not affect the amount of the periventricular somatostatin mRNA. The periventricular nucleus is the most important source of hypophysiotropic somatostatin by its projection to the median eminence (Merchenthaler *et al.* 1989). There are findings that this somatostatin cell group projects also to other brain areas such as arcuate nucleus and locus coeruleus (Dickson *et al.* 1994, Liposits 1993). Because sleep deprivation decreases GH secretion, it would be logical that the activity of the periventricular somatostatin cells increases during the deprivation. It is

possible that the transmitter synthesis rate and the stores are so high in this massive somatostatin cell population that an induction of mRNA expression is not needed during short sleep deprivation. Another possibility is that these cells regulate primarly GH secretion as do the GHRH cells in the arcuate nucleus, but that the cells are not sensitive to short sleep deprivation.

Connections to locus coeruleus

Some somatostatin cells in the hypothalamus are connected to cells in the locus coeruleus (Palkovits *et al.* 1982, Liposits 1993, Mounier *et al.* 1996). Somatostatin immunoreactive fibers have been found in the locus coeruleus (Sutin and Jacobowitz 1988). Expression of somatostatin receptors SSR1-3 has been observed in this nucleus in various studies (Señarís *et al.* 1994, Pérez *et al.* 1994, Schindler *et al.* 1997). Somatostatin affects noradrenergic cells in the locus coeruleus by decreasing their electrical activity (Inoue *et al.* 1988). Noradrenergic locus coeruleus cells have a reciprocal efferent connection to hypothalamic somatostatin cells (Liposits 1993, Mounier *et al.* 1996), by which the locus coeruleus stimulates somatostatin release. Because somatostatin inhibits the locus coeruleus, and inhibition of the locus coeruleus is needed during REM sleep episodes, it is possible that the locus coeruleus mediates at least partly the effects of somatostatin on REM sleep (Fig. 15).

Injections of somatostatin and somatostatin antagonist

I.c.v. injection of somatostatin antagonist decreased the amount of spontaneous daytime REM sleep in rats and also of rebound REM sleep after 24 h REM sleep deprivation. Local microinjection of somatostatin antagonist into the locus coeruleus decreased the amount of spontaneous daytime REM sleep. The cells in the locus coeruleus are also capable of mediating the actions of i.c.v. somatostatin antagonist, because the nucleus is situated in the vicinity of the fourth ventricle. While somatostatin antagonist was effective, an i.c.v. or locus coeruleus microinjection of somatostatin did not affect the amount of daytime REM sleep. There are at least three possible causes which may explain this discrepancy between somatostatin and somatostatin antagonist. First, the half-life of endogenous somatostatin may be much shorter than the half-life of synthetic somatostatin antagonist. The fact that in an earlier study (Danguir 1986) an increased amount of REM sleep was found during continuous long-term infusion of somatostatin supports this hypothesis. However, in another study (Lin *et al.* 1991) it was found that the duration of the effect of somatostatin and somatostatin antagonist on the baroreceptor reflex was almost equal. The second possibility is the ceiling effect. During the first half of the

light phase, when the amount of sleep is highest in the rats, inhibition of the locus coeruleus caused by endogenous somatostatin may be strong enough, so that somatostatin injection is ineffective to cause any further inhibition to increase the amount of REM sleep. In the study of Danguir (1986), the proportions of sleep phases were calculated in 24 h time periods, which does not reveal, whether the enhancement of REM sleep was concentrated in the light or dark phase. The third possibility would be different receptor affinity profiles of somatostatin and somatostatin antagonist. Periventricular somatostatin cells are autoinhibited through SSR1 receptors, which are located in the pericarya and axon terminals of these cells (Helboe *et al.* 1998). Due to this autoinhibiton, i.c.v. injection of somatostatin paradoxically increases GH secretion (Mounier *et al.* 1996). I.c.v. injected somatostatin inhibits hypophysiotropic somatostatin release, while the i.c.v. somatostatin is unable to affect somatotropes in the pituitary directly. Somatostatin terminals in the locus coeruleus may also be autoinhibited by SSR1 and thus either i.c.v. or local locus coeruleus microinjection of somatostatin may inhibit the release of endogenous somatostatin, which may blunt the postsynaptic effect of somatostatin mediated by SSR2 or 3. Somatostatin antagonist may have higher affinity on postsynaptic somatostatin receptors in the locus coeruleus. It is as well possible that the somatostatin antagonist which we used has also some antagonist effect on VIP receptors (Chen *et al.* 1993). Microinjection of VIP into the oral pontine tegmentum, close to the locus coeruleus, has been found to increase REM sleep (Bourgin *et al.* 1999). As mentioned earlier, i.c.v. injection of somatostatin paradoxically increases GH secretion (Mounier *et al.* 1996). According to this, one could expect that the effect of i.c.v. injected somatostatin on REM sleep is possibly explained by indirect enhancement of GH secretion. However, this hypothesis is not supported by the finding that i.p. injection of the somatostatin analog octreotide increases REM

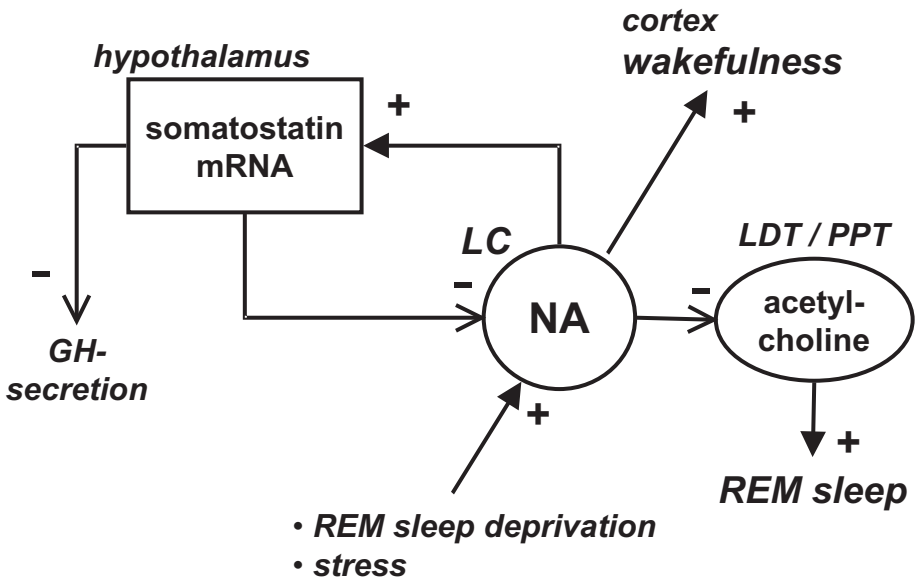

Fig. 15 Schematic illustration of the reciprocal connection between hypothalamic somatostatin cells and noradrenergic (NA) cells in the locus coeruleus (LC) and the possible connection of somatostatin to the REM sleep regulatory system in the brainstem (LDT: laterodorsal tegmental nucleus, PPT: pedunculopontine tegmental nucleus, ACh: acetylcholine).

sleep, while it decreases GH secretion by direct inhibition of pituitary somatotropes (Beranek *et al.* 1997). Novel, more potent and specific somatostatin antagonists have been synthesized (Bass *et al.* 1996, Hocart *et al.* 1998), but they are not yet commercially available. These compounds may reveal the identity of the receptor responsible for the effect somatostatin on REM sleep.

CONTRIBUTION OF GALANIN IN SLEEP REGULATION

REM sleep deprivation for 24 h increased the amount of galanin mRNA in the periventricular nucleus and the median preoptic area, while 6 or 12 h total sleep deprivation did not affect the amount of galanin mRNA in the same regions (Toppila *et al.* 1996). The galanin cells in the medial preoptic area, which are sensitive to REM sleep deprivation are intermingled with or in the immediate vicinity of the GABA and cholinergic cells which regulate cortical and thalamic activity and the state of vigilance (Szymusiak 1995). Galanin is usually co-expressed in the cells with another peptide or a non-peptide transmitter (Bartfai *et al.* 1993). The co-transmitter identity of the REM sleep deprivation sensitive cells is not clear. I.c.v. injection of galanin increases GH secretion (Melander *et al.* 1987), which is possibly mediated by inhibition of somatostatin cells in the periventricular nucleus, which in turn reverses the inhibition of GHRH and GH release. This may be one mechanism which could mediate the hypothetical effects of galanin on sleep. However, we found that i.c.v. injection of galanin at doses which should affect GH release did not affect day or night-time sleep in the rats. The periventricular nucleus is connected reciprocally with the locus coeruleus (Liposits 1993), where galanin receptors in noradrenergic cells decrease the activity of these cells (Seutin *et al.* 1989). During REM sleep deprivation continuous and/or increased activity of the locus coeruleus may activate galanin cells in the periventricular nucleus. However, galanin microinjections into the locus coeruleus did not affect the amount of daytime REM sleep in rats either alone or when combined with somatostatin. It is possible that these negative findings are caused by the short half-life of these peptides, or by the ceiling effect during daytime, when the amount of sleep is high in the rats anyway. However, these findings together with the data of i.c.v. injections suggests that galanin does not affect sleep when injected i.c.v. or locally into the locus coeruleus. In a quite recent study a high dose (10 nmol) of galanin i.c.v. decreased locomotor activity in rats, but the same effect was also obtained by an injection of galanin antagonist M-35 (Ericsson and Ahlenius 1999). The increased galanin mRNA in the medial preoptic area and the periventricular nucleus after REM sleep deprivation may mediate the effects of REM sleep deprivation to other neuronal systems, while it possibly has no direct feedback connections to sleep regulating systems. Galanin mRNA has been found to increase in

response to various neuronal stimuli such as lesions and blockade of axonal transport by colchicine (Cortés *et al.* 1990a, Cortés *et al.* 1990b). REM sleep deprivation could be a stimulus which belongs to this category.

CONTRIBUTION OF STRESS IN SLEEP DEPRIVATION EXPERIMENTS

A certain amount of stress is inevitably associated with any kind of sleep deprivation procedure. In order to be able to correct for the stress caused during REM sleep deprivation by the platform method, control groups of rats were kept for the same time on large platforms. It is suggested that this treatment causes approximately the same amount of immobilization than the REM sleep deprivation treatment on the small platforms, while REM sleep is not reduced. The effect of REM sleep deprivation by the platform method on plasma corticosterone has been measured in our laboratory and there was no significant difference between the REM sleep deprivation and the large platform control groups during 8-72 h deprivation (Porkka-Heiskanen *et al.* 1995). Sleep deprivation by gentle handling has been regarded less stressful than the platform method and it does not affect blood corticosterone levels significantly (Murison *et al.* 1982)

Stress activates waking mechanisms in the brain, the same ones which can be assumed to be activated during sleep deprivation. The noradrenergic locus coeruleus is one important neuronal system supporting wakefulness and alertness in the brain. Exposure to REM sleep deprivation but also stress increases the amount of tyrosine hydroxylase mRNA in the locus coeruleus (Angulo *et al.* 1991, Porkka-Heiskanen *et al.* 1995, Basheer *et al.* 1998). On the contrary, total sleep deprivation produced by the same protocol as in the somatostatin, GHRH, and galanin mRNA studies did not affect tyrosine hydroxylase mRNA in the locus coeruleus (Alanko *et al.* 1996). After stress exposure similar REM sleep rebound in the REM sleep amounts has been found as after REM sleep deprivation (Rampin *et al.* 1991). It is well known that all kinds of stress exposure activate the hypothalamo-pituitary-adrenal axis which leads to increased levels of glucocorticoids in the plasma by increased release of CRH and ACTH. There is possibly a similar association between wakefulness and hypothalamic CRH release as between SWS and GHRH (Steiger and Holsboer 1997, Chang and Opp 1998). I.c.v. injection of CRH increases wakefulness and decreases SWS, while i.c.v CRH antagonist decreases wakefulness, increases SWS and has no effect on REM sleep (Ehlers *et al.* 1986, Chang and Opp 1998).

Stress affects also the GH axis. Acute and repeated stress caused by immobilization increases somatostatin release in the median eminence (Benyassi *et al.* 1992, Benyassi *et al.* 1993) and decreases plasma GH (Marti *et al.* 1996, Marti *et al.* 1993). However, in humans several kinds

of stressful conditions have been found to increase plasma GH (reviewed by Reichlin 1987). During acute stress the GHRH release in the median eminence is decreased with a slight delay, which possibly indicates that this effect is indirect and mediated by somatostatin (Aguila *et al.* 1991). Sleep deprivation and stressful situations are usually related to increased wakefulness, when the noradrenergic system in the locus coeruleus is activated (Fig. 15). This may stimulate hypothalamic somatostatin cells which in turn inhibit GHRH cells in the hypothalamus and GH release from the pituitary. This hypothesis is supported by the finding that the β-adrenergic antagonist propranolol reverses the blunted GH response to GHRH caused by glucocorticoid injections (Devesa *et al.* 1995). Reciprocally, hypothalamic somatostatin may inhibit noradrenergic cells in the locus coeruleus by a feedback mechanism, but if the stimulus supporting wakefulness is strong enough, it will overcome the inhibitory effect of somatostatin, while the inhibition may make the maintaining of prolonged wakefulness more difficult. When the stimulus maintaining wakefulness is over, an activated somatostatin system may promote sleep rebound.

CONCLUSIONS

I Lack of REM sleep increases somatostatin mRNA and lack of SWS increases GHRH mRNA in the rat hypothalamus. This together with the earlier pharmacological studies supports the hypothesis that endogenous somatostatin facilitates REM sleep and GHRH facilitates SWS.

II Sleep deprivation affects mostly the somatostatin cells in the arcuate nucleus and GHRH cells in the paraventricular nucleus, which are not the main hypophysiotropic nuclei of these neuropeptides. Thus, it is possible that sleep regulatory and GH-controlling functions in the hypothalamus are partly mediated by different somatostatin and GHRH cell populations.

III REM sleep deprivation increases galanin mRNA in the anterior hypothalamus but injected galanin does not affect the sleep structure. It is possible that galanin mediates the effects of REM sleep deprivation in the CNS but does not itself affect sleep via the areas examined in the present work.

IV Intracerebroventricular and local microinjection of somatostatin antagonist into the locus coeruleus decreases the amount of REM sleep, which supports the hypothesis that the effect of somatostatin on REM sleep is at least partly mediated by the locus coeruleus.

SUMMARY

The large night-time growth hormone (GH) pulses are associated with slow wave sleep (SWS) phases in humans and a similar association has been found also in some other mammalian species. According to previous pharmacological studies an administration of growth hormone-releasing hormone (GHRH) promotes sleep, especially SWS, in humans and laboratory animals. Similar studies in rats have shown that somatostatin promotes REM sleep. The neuropeptide galanin increases GH-secretion and induces sleep in humans. The cells synthetising GHRH, somatostatin and galanin are mainly located in the hypothalamic areas. In order to study the possible endogenous role of GHRH, somatostatin and galanin in sleep regulation we measured the effect of REM and total sleep deprivation on the expression of mRNA of these neuropeptides in the rat hypothalamus by in situ hybridization. We also measured the effect of galanin, somatostatin and somatostatin antagonist on sleep by a polygraphic method in the course of which these peptides were injected intracerebroventricularly (i.c.v.) or locally into the locus coeruleus of rats. We observed that selective REM sleep deprivation increased the amount of somatostatin mRNA in the arcuate nucleus and decreased GHRH mRNA in the paraventricular nucleus. Combined loss of REM sleep and SWS achieved by total sleep deprivation increased both somatostatin and GHRH mRNA in these hypothalamic nuclei. Because the paraventricular (GHRH) and arcuate (somatostatin) nuclei are not the main hypophysiotropic nuclei of GHRH and somatostatin, control of sleep and wakefulness and GH regulatory functions of these neuropeptides are presumably performed by different cell populations in the hypothalamus. REM sleep deprivation increased the amount of galanin mRNA in the hypothalamus, while total sleep deprivation had no effects. I.c.v. or locus coeruleus microinjections of galanin did not have effects on sleep. These findings suggest that galanin may mediate some effects of REM sleep deprivation in the brain, but does not regulate REM sleep by itself. I.c.v. or local injection of somatostatin did not affect daytime sleep in rats, but a somatostatin antagonist reduced REM sleep when injected i.c.v. or locally into the locus coeruleus. I.c.v. injection of somatostatin antagonist also reduced REM sleep when REM sleep propensity was elevated after REM sleep deprivation. The present findings support the hypothesis that endogenous GHRH promotes SWS while somatostatin promotes REM sleep. We hypothesize that the locus coeruleus may partly mediate the effects of somatostatin on REM sleep.

ACKNOWLEDGMENTS

This work was carried out at the Institute of Biomedicine, Department of Physiology, University of Helsinki. I wish to express my gratitude to the Chairmen of the Institute: Professor Olli Jänne and Professor Ismo Virtanen, and the Department Head, Professor Dag Stenberg for placing the facilities of the Department at my disposal.

I am deeply grateful to the supervisors of this work: Professor Dag Stenberg and Docent Tarja Porkka-Heiskanen for their unfailing interest, guidance and support during the progress of this work and the facilities to carry it out. I wish to thank my colleagues in the research group: Lauri Alanko M.D., Marjut Asikainen M.D. and Pia Niittymäki M.B. for their collaboration and support. I wish also thank Ms. Sari Levo-Siitari for her invaluable help in experimental animal and in situ hybridization work.

I am grateful to the reviewers of this manuscript: Professor Juhani Leppäluoto and Professor Leena Rechardt for their valuable comments and constructive criticism.

I wish to express my deepest thanks to all the personnel of the Department of Physiology, especially to Ms. Anne Nenonen for secretarial assistance, Ms. Seija Turunen and Ms. Nina Katajamäki for their assistance in collecting the literature, Ms. Kaija Hakkarainen for the experimental animal care and Docent Ilkka Linnankoski and Dr. Ernst Mecke for the revision of the English language of the manuscript.

I wish to express my gratitude to the collaboration laboratories: Professor Fred W. Turek and his group, especially Ms. Yi-Rong Ge in the Department of Neurobiology and Physiology at Northwestern University, Evanston, IL, U.S.A., and Professor Irene Tobler and her group at the Institute of Pharmacology, University of Zürich, Switzerland.

I owe my deepest thanks to my parents Maija-Liisa and Antti, sister Sanna and brother Ville, and all of my relatives and friends, especially the crews of s/y *Brainstorm*, s/y *Marleena* and s/y *Jasmiina*, for their continuous encouragement, interest and support during the progress of this work.

For the financial support I wish express my thanks to the Finska Läkaresällskapet, the Paulo Foundation, and the Finnish Sleep Research Society.

Helsinki, September 1999

Jussi Toppila

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