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THE MECHANISMS OF SLEEP SUPPRESSION AND DRINKING ELICITED BY INTRACEREBRAL SOMATOSTATINERGIC STIMULATION

PHD THESIS

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PUBLICATIONS INVOLVED IN THE THESIS

I. Hajdu I, Obál F Jr, Gardi J, Laczi F, Krueger JM. **Octreotide-induced drinking, vasopressin and pressure responses: role of central angiotensin and acetylcholine.** *Am J Physiol Regul Integr Comp Physiol* 279: R271-R277, 2000

II. Hajdu I, Obál F Jr, Fang J, Krueger JM, Rollo CD. **Sleep of transgenic mice producing excess rat growth hormone.** *Am J Physiol Regul Integr Comp Physiol* 282: R70-R76, 2002

III. Hajdu I, Szentirmai E, Obál F Jr, Krueger JM. **Different brain structures mediate drinking and sleep suppression elicited by the somatostatin analog, octreotide, in rats.** *Brain Res* 994, 115-123, 2003

(see appendix)

SUMMARY

We made experiments with a somatostatin agonist, octreotide (OCT) to study its effects on sleep and water consumption.

1. Intracerebroventricularly (icv) administered OCT elicited dose-dependent drinking in rats.
2. Pretreatments with inhibitors of angiotensin (captopril, saralasin, loasartan) and acethylcholine (atropine) blocked the drinking response.
3. Icv OCT stimulated vasopressin secretion, which response was prevented by pretreatments with captopril and atropine in rats.
4. Icv OCT raised blood pressure, which action was also prevented by captopril and atropine.

Previous experiments showed that OCT causes sleep suppression with consecutive sleep enhancement in rats. In present experiments we used transgenic MT-rGH mice, which produce excess growth hormone with low GHRH levels.

5. These mice display robust increases in rapid eye movement (REM) sleep, slight enhancements in non-REM sleep, and normal sleep response to sleep deprivation.
6. Sleep response to OCT is attenuated in MT-rGH mice.

Finally we localized intracerebral structures eliciting drinking or sleep suppression in rats by microinjection technique.

7. Drinking was elicited by OCT injected into the subfornical organ and periventricular nucleus. Injecting OCT into the anterior hypothalamic/ medial preoptic region and arcuate nucleus caused sleep suppression. In the far lateral points of the hypothalamus OCT stimulated non-REM sleep.

The results are consistent with hypothesis that sleeping and drinking responses are mediated by independent mechanisms and brain structures. Drinking – with vasopressin secretion and rise in blood pressure – is mediated by angiotensin and acetylcholine linked to the angiotensinergic dypsogenic circuit in the brain. Sleep suppression by OCT is mediated by inhibition of GHRHergic brain structures. Sleep promoting effect of OCT in the lateral hypothalamus may be associated with inhibition of cholinergic basal forebrain projections. Depressed sleep response to OCT in the MT-rGH mice supports the importance of GHRH in the effects of OCT on sleep.

INTRODUCTION

1. Background

In homoiothermic species, like humans, three states of vigilance are distinguished: wakefulness, rapid eye movement or paradoxical sleep (REMS) and non-REMS (NREMS). The sequence of the states of vigilance is called the hypnogram. The information generally used for the determination of the state vigilance is as follows 1. Qualitative description of the electroencephalogram (EEG); 2. Quantitative description of the EEG by means of spectral analysis or other mathematical tools which provide a measure for the depth or intensity of NREMS (e.g. the intensity of NREMS correlates with delta power); 3. Records of motor activity or the electromyogram (EMG); 4. Records of eye movements. There are a number of other physiological parameters, which help distinguishing among the states of vigilance. Our laboratory routinely records cortical brain temperature, which displays characteristic changes in each state (37).

In the rat or the mouse, the EEG during wakefulness consists of irregular theta waves (4-8 Hz) of low amplitude, and the animal displays motor activity. Cortical brain temperature is slowly increasing during wakefulness. NREMS is characterized by high amplitude, slow delta waves. The amplitude increases, the frequency decreases as the depth of sleep is increasing. The animal is immobile, the skeletal muscle tone is decreased, and the brain temperature slowly declines. In REMS, theta waves dominate the EEG; these are highly regular waves with higher amplitudes than the theta waves in wakefulness. Skeletal muscle tone is ceased except the activity in the respiratory and external eye muscles. The rapid eye movements occur in bursts together with twitches in skeletal muscles.

The three states alternate periodically during the day. In humans, the long period of wakefulness during daylight is followed by rest at nighttime. During sleep, NREMS is interrupted by REMS episodes in every 60-90 minute. The period including one NREMS and one REMS episode is called the sleep cycle. The rodents used in our experiments are nocturnal animals: they are active at nighttime and sleep mostly during the day. Their sleep

cycle is much shorter than the cycle in humans, it is between 10-12 min. In humans, wakefulness and sleep generally appear in longer solid blocks; in rodents these states are more fragmented. Nevertheless, most aspects of human and rodent sleep are very similar. The common features include that the deepest NREMS occurs in first sleep cycle after sleep onset, and sleep becomes progressively shallower towards the end of the rest period. This change in NREMS intensity is due to a dissipation of the need for sleep during sleep. The time spent in REMS increases in the second portion of the rest period because the duration of REMS periods increases as NREMS propensity decreases. Finally, fundamentals of sleep regulation discovered so far apply to both human and rodent sleep.

The principles of sleep regulation have been described in a quantitative model by Alexander Borbély (8). Two major processes are implicated in sleep regulation: a circadian (Process C) and a homeostatic process (Process S). Briefly, Process C provides an oscillating threshold, which determines how easy is to fall asleep. The suprachiasmatic nucleus is the center of the circadian regulation. Rats maintained in an environment without time cues display regular rest (sleep) – activity periods close to 24-h periodicity. The circadian rhythm disappears after destruction of the suprachiasmatic nucleus (20; 54). Neurons in this nucleus show spontaneous, rhythmic firing pattern (27). This nucleus is also responsible for harmonizing circadian rhythms with longer acting influences like seasonal variations in daily illumination, and hormonal factors like melatonin. The homeostatic process determines sleep need as the function of previous wakefulness. After long periods of wakefulness the intensity (EEG slow wave activity) and duration of NREMS are enhanced (1). The homeostatic process explains why the deepest NREMS occurs in the first sleep cycle. The periods of wakefulness can be extended by means of sleep deprivation (SD), and thus SD is capable of enhancing sleep intensity until saturation. SD is the major experimental tool when studying the function of homeostatic sleep regulation. After shorter SD (hours to several days) the body primarily endeavors to compensate NREMS, and when the NREMS rebound declines, the REMS time begins to rise (8). It is unclear whether REMS has an intensity dimension.

Neural and humoral mechanisms are involved in the regulation of sleep-wake activity. The basal forebrain and thalamus play essential roles in the regulation of NREMS whereas the structures generating REMS reside in the brainstem. The major humoral mechanisms implicated in the regulation of NREMS are as follows: adenosine, prostaglandin D2 (PGD2), cytokines [interleukin-1, tumor necrosis factor and growth factors (e.g. NGF)], and growth hormone (GH)-releasing hormone (GHRH). It is noted, however, that only PGD2 is believed to act as a classical humoral factor, adenosine and cytokines/growth factors function as paracrine/autocrine substances whereas GHRH is a neurotransmitter in the basal forebrain sleep-promoting circuit (reviewed in (33)).

GHRH is also part of the somatotropic axis [GHRH-GH-insulin-like growth factor-I (IGF I) + somatostatin + ghrelin]. In addition to the somatotropic axis a number of hormones are capable of modulating sleep-wake activity. Thus, the hypothalamo-ptuitary-adrenal axis suppresses NREMS whereas vasoactive intestinal peptide (VIP) and prolactin enhance REMS. The role of the somatotropic system in sleep regulation is the major research area of our laboratory.

2. *Somatotropic system and its role in sleep regulation*

GH derives from the anterior lobe of pituitary. Secretion of GH is pulsatile. The production and secretion of GH are stimulated by the hypothalamic GHRH. The actions of the GH are in part direct (lipolysis, stimulation of protein synthesis), and are in part mediated by IGF-I (protein synthesis, cell division). IGF-I is an autocrine/paracrine substance produced locally in the tissues, and it is also a hormone released from the liver in response to GH. Like GHRH, somatostatin is a hypothalamic neurohormone and a neurotransmitter. Somatostatin inhibits GHRH neurons in the hypothalamus, and GH secretion in the pituitary (6).

That there are links between sleep and the somatotropic system has been known for decades. It was discovered at the end of the 60's, that a robust GH release – which amounts to 2/3 of the daily GH output in young male human subjects – occurs during the first NREMS episode

immediately after falling asleep (48; 52). It was also noticed that neither GH was the cause of NREMS nor NREMS elicited GH secretion (41). An unknown mechanism was hypothesized which synchronizes these events. It seems that this mechanism corresponds to GHRH: GHRH is capable of stimulating both GH secretion and NREMS. GHRH promotes NREMS in each species tested, in rats, mice, rabbits, and humans (reviewed in (34). NREMS increases after systemic administration of GHRH (intravenous, intraperitoneal (ip), intranasal), after injection into the brain intracerebroventricularly (icv) or into the anterior hypothalamus / medial preoptic region (AH/MPO). Blocking GHRH by competitive antagonist or antibodies suppresses NREMS (35; 36). Mutant mice and rats with a deficiency of GHRH receptors spend less time in NREMS (30; 31), and transgenic mice with decreased GHRH production also display less spontaneous NREMS (60). GHRH also may play role in the rebound-like NREMS rise following SD; the sleep response to short-term sleep loss can be prevented by anti-GHRH antibodies (36). Some observations suggest that GH has its own NREMS-promoting activity (2; 24), but this has not been not confirmed by other laboratories (23; 28; 51). IGF-I may also enhance NREMS (32).

Somatostatinergic stimulation decreases the duration of NREMS (4; 5). Somatostatin may promote REMS (13). During SD, hypothalamic GHRH mRNA expression increases while somatostatin mRNA level decreases (59) suggesting that somatostatin has reciprocal relationship with GHRH.

3. Role of transgenic and mutant animals in the research of the somatotropic system

Transgenic and mutant animal models with hormone deficiencies or excess production of hormones have an important contribution to an understanding of endocrine functions and diseases. There are several strains of rats and mice with alterations in the somatotropic axis (16). Our team uses these models to study sleep in conditions with chronic alterations of the somatotropic system. I had the occasion to work with the MT-rGH transgenic mice as a result of collaboration with a Canadian scientist, Dr. David Rollo, who provided the mice. The transgenic MT-rGH mice are named “Supermice” because they secrete rat GH (rGH) in enormous quantity and therefore, they are giant mice (39; 40). The transgene is a fusion gene

composed of the coding region of the rGH gene and the promoter region of the mouse metallothionein (MT) gene. There are several copies of the transgene in a single chromosome. The transgene is expressed practically in each tissue, e.g. the gastrointestinal tract, kidneys, skin, gonads, but particularly in the liver. Circulating GH concentration is 100-800-fold higher than normal. Secretion of GH from the pituitary is blocked, the acidophil cells degenerate. Through negative feedback, high circulating GH levels suppress GHRH, and increase somatostatin secretion. Production of IGF-I is increased by 2-3 fold resulting in gigantism: the Supermice's body weight is double of normal littermates. We used these mice as a model of GH excess and GHRH deficiency to study spontaneous sleep and the sleep response to somatostatinergic stimulation.

4. Previous observations with somatostatinergic stimulation in our laboratory

For injections, instead of somatostatin, we use the somatostatin analog, octreotide (OCT). OCT has a half-life of 45-120 minutes while somatostatin is cleaved in minutes. In addition OCT is 20-75-fold more potent in blocking GH secretion (3; 42). Because of these attributes and its antiproliferative action, OCT is used as medicine in the treatment of pituitary adenomas (acromegaly) and hormone secreting gastrointestinal tumors. Of the five somatostatin receptor subtype, OCT has the highest affinity to the sst2 and sst5 subtypes though it also binds weakly to the sst3 receptor. Sst2 receptors are responsible for inhibiting somatotropic activity; this subtype can be found in the entire brain (43).

We recorded two fundamentally different classes of responses after administration of OCT in rats (4; 5):

A) During the sleep experiments, we noticed that the rats drank water regularly after systemic injection of OCT. The drinking response was particularly consistent when OCT was administered icv. Analysis of the rat's behavior after OCT injection revealed a characteristic sequence of events: OCT first elicited drinking which started in 1-2 min and lasted for approximately 10 min. Drinking was followed by periods of scratching and finally by eating. Icv somatostatin was previously reported to elicit vasopressin secretion and to rise blood pressure (9; 44). The triple response, consisting of drinking, vasopressin secretion, and increases in blood pressure, is characteristic of angiotensinergic stimulation (11). Therefore, the effects of angiotensinergic blockade were tested on the OCT-induced responses. Pretreatment with the angiotensin convertase enzyme inhibitor, captopril verified the involvement of central angiotensin in the OCT-elicited drinking *(fig. 1.).*

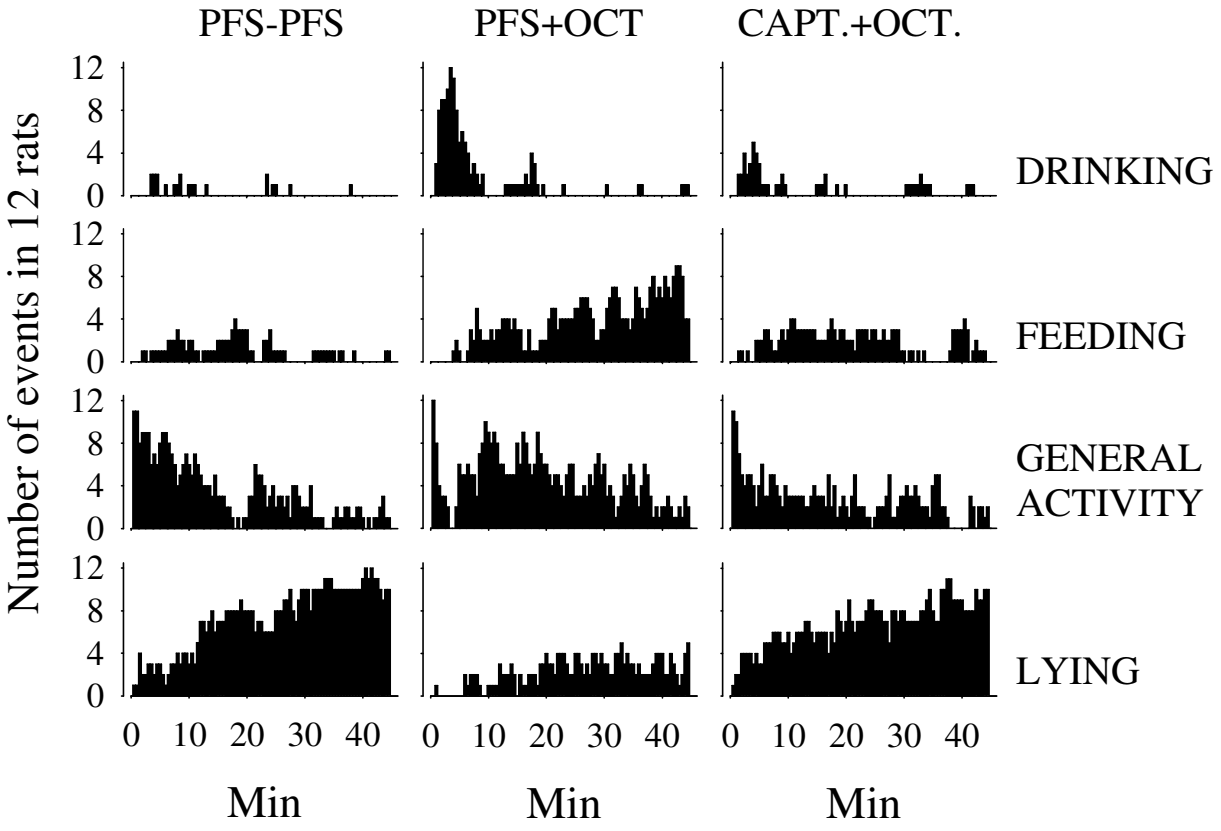

Fig. 1. *Cumulative occurrences of various behavioral responses at 30-s intervals after double icv injections of pyrogen-free physiological saline (PFS+PFS), PFS+ 0.1 μg octreotide (PFS+OCT), and 30 μg captopril + 0.1 μg OCT (CAPT+OCT) in 12 rats. There were 15 min in between the two icv injections, and behavioral scoring started 30 s after the second injection. General activity includes grooming + scratching + exploration (5).*

B) The systemically or icv injected OCT, elicited dose-dependent and prompt suppression of NREMS and GH secretion which lasted for 1-2 hours in rats. Thereafter, GH secretion recurred and the duration and, in particular, the intensity of NREMS increased and stayed above baseline for hours. This response was similar as the rebound-like sleep enhancements after SD. Because the angiotensin-like actions of OCT themselves may inhibit sleep, it was important to determine whether blockage of angiotensin production interferes with the sleep responses. Pretreatment with captopril did not alter the effects of OCT on sleep, i.e. OCT continued to elicit sleep suppression followed by enhancements in sleep intensity *(fig. 2.)*.

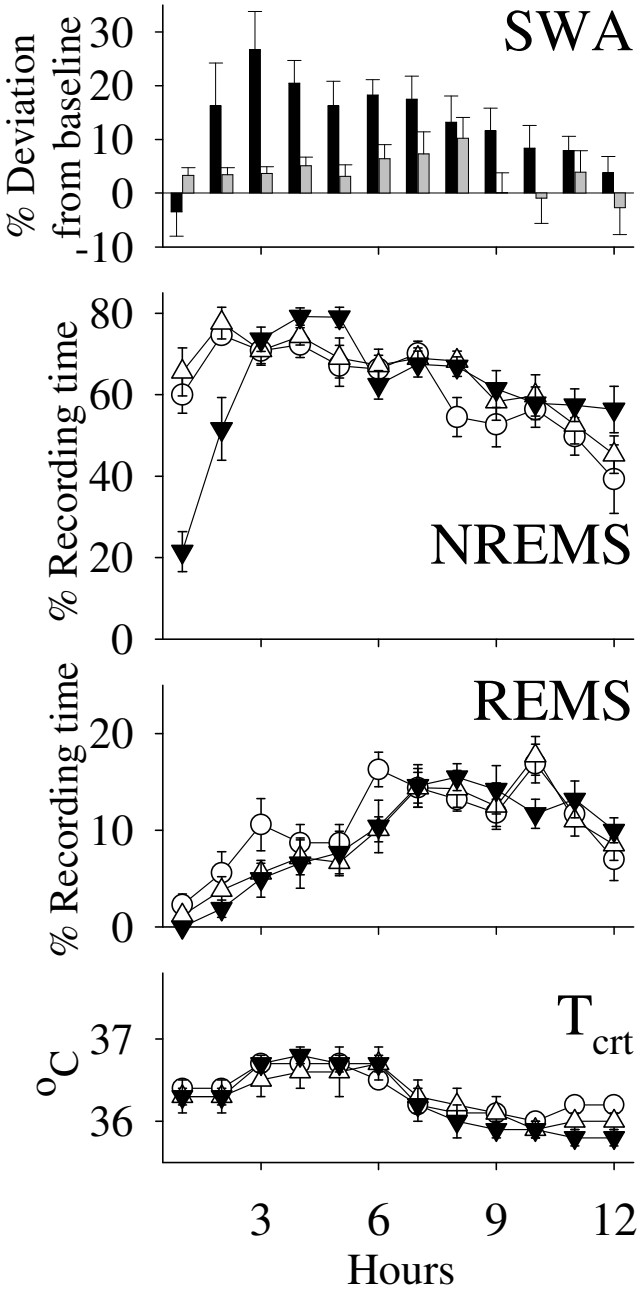

Fig. 2. *Effects of icv captopril (30 μg) on the octretide (OCT, 0.1 μg)-induced sleep alterations during the 12-h light period. The rats (n=7) received two icv injections of pyrogen-free physiological saline (PFS + PFS, open circle), captopril + PFS (open triangle), and captopril + OCT (closed triangle) with 15 min in between the two injections on three different days. NREMS: non-REM sleep, REMS: REM sleep, T_{crt}: cortical brain temperature, and SWA: EEG slow wave activity (power density in the 0.25-4 Hz range) during NREMS; for SWA, the deviations from baseline (PFS+PFS) are shown (columns) (5).*

The results suggested that the angiotensin-like and sleep suppressive actions of somatostatinergic stimulation are independent effects.

5. Neuroanatomical considerations

The cell bodies of the GHRHergic neurons regulating GH secretion reside predominantly in the hypothalamic arcuate nucleus (ARC) (12; 49). These neurons project to the median eminence. Extraarcuate GHRH-immunoreactive neurons have been described in the parvocellular portion of the paraventricular nucleus (PVN), and around the ventral rim of the ventromedial nucleus (VMN). The extraarcuate GHRH neurons innervate basal forebrain structures, particularly the AH/MPO. Terminals of extraarcuate GHRH neurons are also detected in other areas, which are implicated in sleep regulation, such as the suprachiasmatic nucleus, lateral hypothalamus, the PVN, and nucleus premamillaris. Extraarcuate GHRH neurons provide less fibers to the median eminence. However, ARC GHRH neurons also participate in the intrahypothalamic GHRHergic network, and thus they also contribute to sleep regulation (47). The ARC is destroyed in rats treated with monosodium glutamate as neonates. These rats become dwarfs, and they spend less time in NREMS than their normal littermates (38).

Somatostatin is a ubiquitous inhibitory neurotransmitter in the brain including the hypothalamus (21). Thus, somatostatin-containing interneurons are found throughout the hypothalamus. Somatostatinergic fibers projecting to the median eminence originate from the periventricular nucleus. These periventricular somatostatinergic neurons also inhibit GHRH neurons in the ARC (25). In addition, the ARC has its own intranuclear somatostatinergic network which controls intraARC GHRH neurons (7).

Angiotensin II (or III) is implicated in the dipsogenic circuit at multiple action sites (reviewed in (22). Systemic angiotensin and angiotensin in the cerebrospinal fluid elicits drinking by acting on the circumventricular organs, the organum vasculosum laminae terminalis (OVLT), and the subfornical organ (SFO). The pathways originating from these structures are also angiotensinergic and innervate structures along the lamina terminalis and around third

ventricle including the PVN. Acetylcholine is another neurotransmitter involved in the regulation of water balance. The dipsogenic cholinergic circuit is less known.

6. Aims

The general hypothesis of our experiments was that the angiotensin-like and the sleep suppressive effects of OCT are independent actions, which are mediated by separate structures. The aims included characterization of the angiotensin-like actions of OCT, study of OCT effects on sleep in the Mt-rGH mouse, and localization of the structures mediating the drinking and sleep responses to OCT:

1. Effects of OCT on drinking in rat, dose-response relationship
2. Pharmacological characterization of OCT-induced drinking in rats
3. Effects of icv OCT on plasma vasopressin concentrations in rats
4. Effects of icv OCT on blood pressure in rats
5. Characterization of sleep regulation in the MT-rGH mice (spontaneous sleep, response to SD)
6. Effects of ip OCT on sleep in the MT-rGH mice
7. Localization of the cerebral structures mediating drinking and sleep to OCT in rats

The methods and results are described and discussed in details in the papers attached, therefore, only a summary of these papers is provided herein.

METHODS

1. Animals

Male Sprague-Dawley rats (300-350 g), male MT-rGH transgenic mice, and normal mice were used. The transgenic mice were shipped from the McMaster University, Hamilton, Ontario, to the Washington State University, Pullman, WA, USA, and the mice were recorded here, in Dr. Krueger's sleep laboratory.

2. Anesthesia

The rats and mice were anesthetized by means of ketamine-xylazine (87 and 13 mg/kg, respectively).

3. Implantation for sleep recording

The rats and mice received stainless steel EEG electrodes in the skull over the frontal and parietal cortices, and over the cerebellum. The rats were also implanted with a thermistor to record cortical brain temperature (T_{crt}). EMG electrodes were implanted into the nucheal muscles in the mice.

4. Cannula implantation

The icv cannula was inserted into the left lateral ventricle. The location of the cannula was determined by the gravity method during implantation, and by means of Trypan blue staining after the experiments. In case of the cannulas implanted into the hypothalamus, a horseradish peroxidase – H_2O_2 reaction was visualized by means diaminobenzidine, and the histological

sections were stained with cresyl violet. Instead of lowering vertically, some of these cannulas were inserted at an angle to avoid penetration of the lateral ventricle.

5. Water intake

Water consumption was determined by weighing the water bottles before and 10 min postinjection.

6. Determination of vasopressin

Vasopressin was determined by means of radioimmunoassay by Janos Gardi at the Endocrine Unit of our University.

7. Measurements of blood pressure

Rats were implanted with aortic catheter. The catheter was connected to pressure transducers, and the signal from the transducers was collected by computers. The blood pressure was measured in freely moving rats.

8. Sleep recording

The rats or mice were connected to the recording cable. The recording cables were attached to commutators. The motor activity was assessed by means of recording potentials generated in electromagnetic transducers activated by movements of the cables in the rats. The digitized signals of the EEG, T_{crt}, EMG and motor activity were collected by computers. The states of vigilance were scored in 8-s (rats) or 10-s (mice) epochs from signals restored on the computer screen. Power values were calculated by fast-Fourier transformation for consecutive

8-s or 10-s epochs in the frequency range of 0.25-20.0 Hz for 0.25-Hz bands and were integrated for 0.5-Hz bins. The spectra were also displayed on the screen. The states of vigilance were determined over 8-s or 10-s epochs by the usual criteria as NREMS [high amplitude slow waves in the EEG, lack of body movements, declining cortical temperature after entry, predominant power in the delta range (0.5 to 4.0 Hz)]; REMS [highly regular theta activity in the EEG with corresponding high theta power in the spectrum, general lack of body movements with occasional twitches, flat EMG, a rapid rise in brain temperature at onset]; and wakefulness [less regular theta activity and significant theta power, higher delta power than during REMS, frequent body movements, and a gradual increase in brain temperature after arousal]. The percentage of the time spent in each state of vigilance in consecutive 1-h periods and for the 12-h light and 12-h dark periods was determined. The power values for the 0.25-4 Hz delta range during NREMS were integrated and used to characterize sleep intensity (1) in each recording hour. T_{crt} was averaged for 1-h periods.

9. Statistics

In general, ANOVA was used which was followed by the Student-Newman-Keuls test to identify the group or treatment differing from the rest.

RESULTS

1. Effects of OCT on water intake in rat: dose-response relationship (Paper I)

Both systemic and icv administration of OCT elicited drinking. The response was analyzed after icv injection of OCT. Drinking occurred in 1 min, peaked in min 4-5, and then subsided and ceased 10 min postinjection. The response was dose-dependent. Injections through a misplaced cannula not entering the lateral ventricle failed to elicit drinking.

2. Pharmacological characterization of OCT-induced drinking (Paper I)

The drinking response to icv injected 0.1 μg OCT was studied after pretreatments with substances as follows: icv 30 μg captopril 15 min prior to OCT (angiotensin convertase inhibitor); icv 10 μg saralasin 10 min prior to OCT (AT1/AT2 receptor blocker); icv 100 μg losartan 5 min before OCT (AT1 receptor blocker); icv 10 μg atropine sulfate 15 min before OCT, and 1 mg/kg subcutaneous naloxone 20 min before OCT (opiate receptor blocker). The control group received physiological saline or vehicle.

Significant suppressions of OCT-induced drinking were observed after each drug interfering with the angiotensinergic system (captopril, saralasin, and losartan), and after atropine. In contrast, naloxone failed to alter the drinking response to OCT.

3. Effects of icv OCT on plasma vasopressin concentrations in rats (Paper I)

Icv injected 0.1 μg OCT elicited 13-fold increases in plasma concentrations of vasopressin 5-min postinjection. Pretreatment with either captopril or atropine blocked the vasopressin response to OCT.

4. Effects of icv OCT on blood pressure in rats (Paper I)

Icv injected 0.1 μg OCT induced approximately 10-mmHg rise in mean blood pressure in freely moving rats. Blood pressure rose promptly in association with drinking. This initial response was followed by a second increase between 12 and 16 min postinjection. Both captopril and atropine pretreatments prevented the blood pressure changes after OCT.

5. Characterization of sleep regulation in the MT-rGH mouse (spontaneous sleep, response to SD) (Paper II)

Although the sleep response to OCT in the MT-rGH mouse is of primary interest for the current topic we will also discuss spontaneous sleep in these mice because sleep and the somatotropic axis is the major research area of our laboratory, and because the results obtained in these mice are somewhat unexpected.

The diurnal rhythm of sleep-wake activity was normal in the MT-rGH mice. They did not display alterations in the delta power or slow wave activity during NREMS. The MT-rGH mice, however, spent modestly and consistently more time in NREMS than the controls during the light period. The increase in NREMS resulted from an increase in the mean duration of the longer NREMS episodes. REMS time was greatly increased in the MT-rGH mice, it was almost doubled in the second portion of light period. These mice had more REMS bouts than the control mice did.

In contrast to the differences in spontaneous sleep, the sleep responses to SD were normal in the MT-rGH mice.

6. Effects of ip OCT on sleep in the MT-rGH mouse (Paper II)

The sleep response to OCT significantly differed in the MT-rGH mice from the response in the controls. In the control mice, injection of OCT was followed by a large suppression of NREMS in hour 1, and then EEG slow wave activity (delta power) during NREMS enhanced significantly starting in hour 2 postinjection. In the MT-rGH mice, the OCT-induced reduction of NREMS in hour 1 was significantly smaller than in the controls, and the EEG slow wave response, reflecting NREMS intensity, was practically absent.

7. Localization of the cerebral structures mediating sleep suppression and drinking to OCT in rats (Paper III)

7.a Drinking

Out of 86 rats, OCT microinjection elicited drinking in 17 rats. The injection sites resided in the SFO (5.6 ± 0.4 ml; n=4), and in the PVN (2.9 ± 0.3 ml; n=10). Drinking was observed after 2 injections in the bed nucleus of the stria terminalis but in these cases OCT might have leaked into the ventricular system because injections into the same structure through a cannula, which did not penetrate the ventricle, failed to elicit drinking. Finally, drinking was obtained in 1 rat after OCT microinjection into the dorsomedial nucleus.

7.b Sleep

There were 28 rats which responded with changes in sleep to OCT. The rats were grouped with respect to the structure where OCT was injected.

AH/MPO

In rats (n=8), which received OCT into the AH/MPO, NREMS decreased in hour 1 and stayed below baseline for 5 hours. Thereafter EEG slow wave activity tended to increase.

ARC

The rats (n=7) injected with OCT in the ARC responded with NREMS suppression in hour 1, and then NREMS returned to baseline. Increases in EEG slow wave activity were observed after hour 4.

PVN

Sleep was also determined in the group, which drank water after OCT injection into the PVN. OCT failed to alter sleep in these rats.

Lateral preoptic region / lateral hypothalamus (LPO/LH)

There were 11 rats in which OCT microinjection, instead of decreasing, increased NREMS in hour 1. EEG slow wave activity also enhanced in hour 1. The microinjections resided in the far lateral sites of the LH and LPO in these rats. It was also characteristic of these rats that the baseline value after administration of vehicle or physiological saline was relatively low.

DISCUSSION

The Discussion below attempts to focus to general issues to reach broad conclusions; detailed discussion of the results is provided with each paper.

1. Effects of OCT on water intake, vasopressin secretion and blood pressure

Drinking elicited by somatostatinergic stimulation is an original observation in our laboratory while the somatostatin-induced vasopressin secretion and rise in blood pressure were previously reported (9; 44). Somatostatin stimulates water intake in water depleted sheep (56). Drinking completed the two previously described somatostatin actions, vasopressin secretion and the increase in blood pressure, to match the response triad characteristic of both angiotensin and acetylcholine. The change in pressure is most likely results from vasopressin (9; 44). Pharmacological analysis of the OCT-induced responses suggests that both angiotensin and acetylcholine are required for these actions. The results also provide evidence that the angiotensin-like effects are mediated by AT1 receptors whereas the acteylcholine-like effects are mediated by muscarinic receptors.

That angiotensin and acetylcholine are both necessary for the OCT-elicited responses might not be obvious because angiotensin and acetylcholine are proposed to be parallel pathways in the central water homeostasis (19; 26). In addition, the fact that OCT or somatostatin stimulates a function is also unusual for somatostatin is an inhibitory neurotransmitter. Therefore, we suggest that somatostatin acts via disinhibition, i.e. removal of a tonic inhibition from the activation of water homeostasis. The inhibition is hypothesized to control both the angiotensinergic and acetylcholinergic pathways simultaneously, and the release of only one of these mechanisms does not seem to be enough for a response. Our laboratory provided evidence for the stimulation of angiotensin release by OCT. By determining hypothalamic angiotensin II contents, significant depletion was observed 10 and 60 min after icv injection of OCT (17). We also propose that a GABAergic mechanism keeps the

angiotensinergic and cholinergic mechanisms suppressed, and thus OCT or somatostatin acts by inhibiting this GABAergic inhibition *(fig. 3.)*.

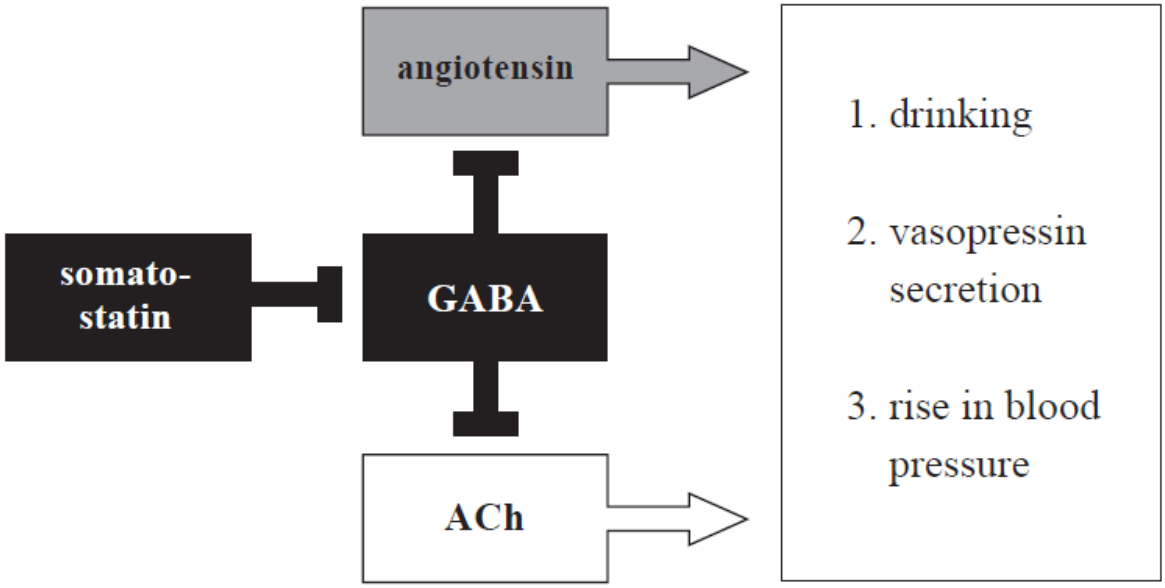

Fig. 3. *Proposed model of somatostatinergic control of water homeostasis.*

GABAergic inhibition of drinking has been reported (55), and there are also findings showing that somatostatin and GABA may interact and are often co-localized in neurons (29; 50; 53). Apart from this, however, we did only the first steps to localize the site of somatostatin action on water homeostasis. The localization experiments focused on the intracerebral angiotensinergic system because this system is much better known than the cholinergic pathways participating in drinking. Nevertheless, both the PVN and the supraoptic nucleus receive cholinergic innervation, which is likely to stimulate vasopressin secretion and perhaps drinking (reviewed in (22). As mentioned in the Introduction, angiotensin is involved at multiple action sites in the dipsogenic circuit. First, both the SFO and the OVLT are stimulated by angiotensin II. Second, the angiotensin-responsive neurons are themselves, at least in part, angiotensinergic, and the locally released angiotensin recruits further active neurons. Third, the descending pathways from the circumventricular organs are also angiotensinergic. It is noted that angiotensin III might be the major active angiotensin species produced in the brain (57). Angiotensin III is the C-terminal heptapeptide in angiotensin II.

Our experiments with microinjections show that somatostatin is capable of activating the angiotensinergic system in both the SFO and the PVN. Further experiments may explore the OVLT, and in particular, the median preoptic nucleus (MnPO), which seems to be a central nucleus receiving innervation from both the SFO and OVLT in the dipsogenic circuit.

Finally, the fact that somatostatin controls both angiotensinergic and cholinergic pathways may make somatostatin a fundamental participant in the regulation of water homeostasis. In spite of this, we are aware of only one report, which demonstrates that the vasopressin response to blood loss is compromised after depletion of intracerebral somatostatin (10). Further experiments may clarify this issue.

2. The role of somatostatin in sleep regulation

OCT caused prompt sleep suppression in rats in our previous experiments (4; 5), and this action was consistent with the first description of the effects of somatostatin on sleep (18; 45). Later, however, stimulation of REMS was reported in response to somatostatin (13), which we could not replicate apart from a slight increase in REMS after systemic OCT (5). A brain stem action site has been suggested for somatostatin-induced REMS enhancements (14), and the fact that hypothalamic OCT microinjections failed to alter REMS, may support this suggestion. Our previous experiments also revealed that the angiotensin-like actions of OCT are not involved in the sleep response to OCT. It was known that somatostatin inhibits GHRHergic neurons, and GHRH is a NREMS-promoting substance but besides these, we have had little evidence showing that GHRH mediates the sleep effects.

The high concentration of circulating GH suppresses GHRH production in the MT-rGH mice. Therefore, the importance of this model was that the sleep response to OCT could be tested in a condition with low GHRH. The effects of OCT were significantly attenuated in the MT-rGH mice thereby suggesting that normal GHRH is necessary for the OCT-induced sleep suppression and the subsequent enhancements in EEG slow wave activity. Recently, further evidence has been collected in our laboratory, which supports the importance of GHRH. OCT was tested in *lit/lit* mice which have a point mutation in the GHRH receptor gene, and the

mutation makes the receptor non-functional. OCT was completely ineffective in altering sleep in the *lit/lit* mice though it continued to elicit drinking (30). We have also determined the GHRH contents of the hypothalamus after icv OCT injections in rats (17). OCT promptly inhibited GHRH release (which appeared as an increase in GHRH contents); this effect peaked 1 h postinjection. The accumulated GHRH released thereafter. The time course of these changes corresponds to the initial sleep suppression and to the subsequent enhancements in NREMS intensity. Important findings showing that OCT modulates sleep through GHRH were obtained in the experiments with hypothalamic microinjections. The sleep response to OCT after hypothalamic microinjections was similar (ARC) to, or stronger (AH/MPO) than after icv injections. The positive hypothalamic injection sites correspond to the area where cell bodies of GHRHergic neurons reside (ARC) and to the area where GHRHergic neurons project (AH/MPO). The somatostatinergic control of GHRHergic neurons via sst2 receptors is well documented in the literature (25). GHRHergic neurons stimulate GABAergic neurons in the AH/MPO (15). Theoretically, the same GABAergic neurons can be the target of somatostatin-induced inhibition; this would be a mechanism similar to the inhibition of GH secretion in the pituitary. Alternatively, somatostatin may cause presynaptic inhibition of GHRHergic projections *(fig. 4.).* Sst2 receptor-mediated presynaptic inhibition was previously reported in the hypothalamus (25).

Instead of sleep suppression, increases in NREMS were observed after OCT microinjection in the far-lateral points of the basal forebrain. As discussed in *Paper 3*, we assume that OCT acts via inhibiting predominantly cholinergic (and some non-cholinergic, e.g. orexinergic) projection neurons which are involved in the maintenance of waking (58). This action is the function of local somatostatinergic interneurons and is independent of GHRH.

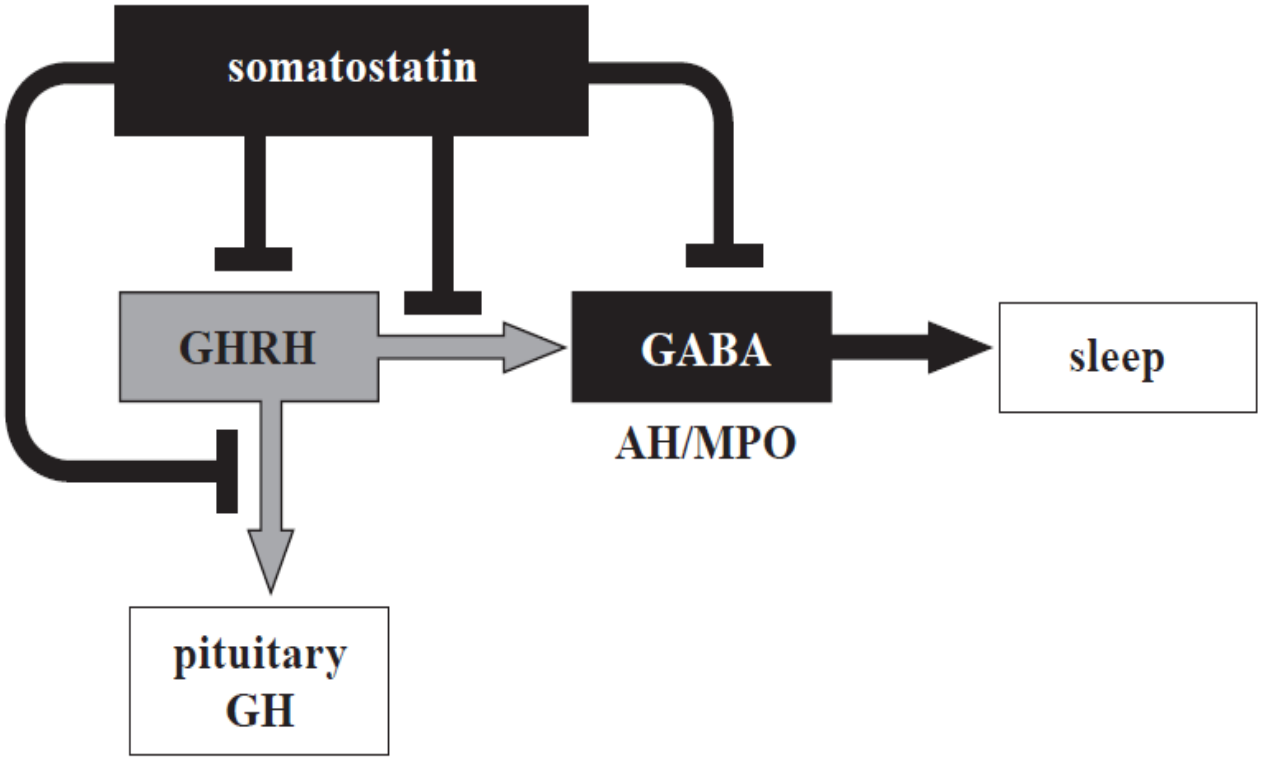

Fig. 4. *Proposed models of somatostatinergic inhibition of GHRH actions.*

3. Sleep in the MT-rGH mouse

There are a number of observations, which implicate GHRH in NREMS promotion; many of these are reviewed in the Introduction. The list includes the positive sleep responses in various species, the suppression of NREMS after GHRH inhibitors, decreases in NREMS after activation of the feedback mechanisms inhibiting GHRH, decreases in sleep in animals with chronic GHRH deficiency, the correlation between hypothalamic GHRH contents / mRNA levels and sleep, sleep reduction in rats after lesioning the rostral projections of ARC, and the demonstration of the involvement of GHRH in the sleep promoting activity of the cytokine, interleukin-1 (34). Because any defects in GHRH actions are always coupled with deficiencies downstream from GHRH, i.e. GH and IGF-I, and cause dwarfism, two findings are particularly important. First, GH replacement in the *lit/lit* mouse with a defect in GHRH

signaling normalizes REMS but leaves NREMS suppressed (30). Second, the "spontaneous dwarf rat" (SDR), which has a point mutation in the GH gene, and does not produce GH but their GHRH functions normally, displays less REMS but more than normal NREMS (34). These observations suggest that GHRH is responsible for alterations in NREMS whereas GH modulates REMS.

In fact, several other lines of evidence indicate that GH is capable of stimulating REMS; these reports are reviewed in the Discussion of *Paper 3*. Therefore, the robust increments in REMS could be anticipated in the MT-rGH mice. Whether these changes in REMS contribute the higher learning ability of these mice remains to be clarified.

The problem with the MT-rGH mice is that they also display slight increases in NREMS time though they have less GHRH and therefore they are anticipated to exhibit less NREMS. It is noted that before our experiments started it was already reported that behavioral sleep time increased in the MT-rGH mice (24). There are some papers suggesting that chronic GH excess may stimulate NREMS independently of GHRH (2). That behavioral sleep time can be normalized by increased sugar intake in the MT-rGH mice (46) is a strong argument for a metabolic cause of the enhanced NREMS. In addition, the enhancements in NREMS might be mediated by IGF-I because IGF-I seems to have a NREMS-promoting activity (32).

4. Conclusions

1. OCT, particularly after intracerebral administration, elicits dose-dependent drinking. The drinking response to somatostatinergic stimulation is an original observation in our laboratory.
2. Stimulation of drinking by OCT involves both angiotensinergic and muscarinic cholinergic mechanisms.
3. Through the same intracerebral mechanism, OCT elicits vasopressin secretion.
4. OCT also increases blood pressure via angiotensinergic and cholinergic mechanisms.
5. The MT-rGH mice, which produce excess GH, display robust increases in REMS, slight enhancements in NREMS, and normal sleep response to SD. The changes in sleep are attributed to the metabolic actions of GH.
6. The GHRH production and the sleep response to OCT are suppressed in the MT-rGH mice supporting the importance of GHRH in the effects of OCT on sleep.
7. The drinking response to OCT is elicited from the angiotensinergic circuit in the brain (SFO, PVN) whereas the sleep suppression by OCT is mediated by structures hosting the cell bodies and terminals of GHRHergic neurons (ARC, AH/MPO). In addition, OCT stimulates NREMS when injected in the far lateral sites of the basal forebrain. This effect is attributed to inhibition of cholinergic and non-cholinergic neurons promoting arousal.

The results are consistent with the hypothesis that

- The angiotensin-like effects and the sleep responses to OCT are independent.
- Sst2 somatostatin receptors may have a predominant role in eliciting the angiotensin-like effects and the sleep responses.
- Somatostatin may have an important role in the regulation of central water homeostasis
- Somatostatin contributes to sleep regulation via modulating GHRH

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APPENDIX

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