THE ROLE OF GHRELIN AND NEUROPEPTIDE Y IN THE SHARED REGULATION OF FEEDING AND AROUSAL

Éva Szentirmai, M.D.

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Éva Szentirmai, M.D.

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I. Bodosi B, Gardi J, Hajdu I, **Szentirmai É**, Obál F Jr., Krueger JM. Rhythms of ghrelin, leptin and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am J Physiol* 287: R1071-R1079, 2004.

II. **Szentirmai É**, Hajdu I, Obál F Jr., Krueger JM. Ghrelin-induced sleep responses in ad libitum fed and food-restricted rats. *Brain Res* 1088: 131-140, 2006.

III. **Szentirmai É**, Kapás L, Krueger JM. Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats. *Am J Physiol* 292(1): R575-R585, 2007.

IV. **Szentirmai É**, Krueger JM. Central administration of neuropeptide Y induces wakefulness in rats. *Am J Physiol* 291(2): R473-R480, 2006.

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3. Symposium at 52nd Congress of the German Endocrine Society (March, 2009)

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TABLE OF CONTENT

LIST of ABBREVIATIONS

ARC	arcuate nucleus
CRH	corticotropin-releasing hormone
DMH	dorsomedial hypothalamic nucleus
EEG	electroencephalogram
EMG	electromyogram
FFT	Fast-Fourier Transformation
GHS-R1a	growth hormone secretagogue receptor-1a
icv	intracerebroventricular
ip	intraperitoneal
iv	intravenous
LH	lateral hypothalamus
MPA	medial preoptic area
NPY	neuropeptide Y
NREMS	non-rapid-eye-movement sleep
PVN	paraventricular nucleus
REMS	rapid-eye-movement sleep
SCN	suprachiasmatic nucleus
SE	standard error
SNK	Student-Newman-Keuls test
SWA	slow-wave activity
TMN	tuberomammillary nucleus
VMH	ventromedial hypothalamic nucleus

SUMMARY

Sleep and wakefulness are active processes controlled by multiple neuronal circuits in the brain. The most recently identified wakefulness-promoting area of the brain resides in the perifornical area of the lateral hypothalamus (LH) where cells are producing the peptide orexin. Mounting evidence indicates the importance of orexin in the regulation of wakefulness. Orexin neurons are, however, shared by another neuronal network that also involves ghrelin and neuropeptide Y (NPY) signaling mechanisms. The role of the hypothalamic ghrelin – NPY – orexin circuit in feeding is well-established, the activation of the circuit leads to increased food intake while the inhibition results in decreased eating. Substantial evidence supports the notion that the regulation of sleep/wakefulness and feeding/metabolism are linked and coordinated by shared neuronal circuits in the brain. We hypothesize that the neuronal network, formed by ghrelin – NPY – orexin cells in the hypothalamus is one of these mechanisms. In the present experiments we studied the role of the hypothalamic ghrelin – NPY – orexin circuit in sleep by determining the diurnal rhythms of plasma and hypothalamic ghrelin levels, the dependency of these rhythms on sleep-wake activity and the sleep-modulating effects of central injections of ghrelin and NPY in rats.

We found that plasma and hypothalamic ghrelin levels display marked diurnal rhythms associated with feeding and sleep-wake activity. Food restriction to the light period reverses REMS and plasma ghrelin rhythms whereas sleep deprivation increases plasma and hypothalamic ghrelin levels. Intracerebroventricular (icv) injection of ghrelin induces dose-dependent and immediate increases in wakefulness, food intake and feeding behavior, with the concomitant suppressions of non-rapid-eye movement sleep and rapid-eye movement sleep. Microinjections of ghrelin into the LH, medial preoptic area or paraventricular nucleus mimic the effects of the icv ghrelin treatment. The effects of icv and intra-LH injections of NPY on sleep and food intake are similar to those of ghrelin's.

The first hours of the dark, behaviorally active, period in rats are characterized by increased time spent awake and increased eating activity. We named this behavioral pattern "dark onset syndrome". Central administration of ghrelin or NPY elicits all components of the dark onset syndrome. We hypothesize that the hypothalamic ghrelin – NPY - orexin circuit is a major brain center that integrates information about the energy status of the body through metabolic, circadian and visual signals. The activation of the circuit has two main parallel outputs: increased wakefulness and increased feeding activity.

INTRODUCTION

Sleep is an essential biological process, a periodical, reversible state characterized by reduced motility, stereotypic posture and decreased responsiveness to sensory stimuli. Sleep and sleep-associated pathologies affect our physical and mental well-being, productivity and safety. Even in the most primitive animals, rest-activity rhythms can be observed. Traditionally, the primary measure used to define physiological sleep-wake activity and the different physiological sleep stages in the laboratory is the electroencephalogram (EEG). In mammals and birds, three types of vigilance states can be determined by EEG, i.e., wakefulness, rapid-eye movement sleep (REMS) and non-rapid-eye movement sleep (NREMS). These states are fundamentally different in terms of regulation and each has a distinct set of associated physiological, neurological, and psychological features.

The neuronal pathways that promote arousal

For long, it was believed that sleep and wakefulness are passively occurring vigilance states; sensory inputs maintain wakefulness and the cessation of these inputs results in sleep. In 1916, however, an active role for brain in sleep-wake behavior was suggested by Baron Constantine von Economo who found that brain lesions due to viral encephalitis profoundly affected sleep-wake activity. He described that lesions at the junction of the midbrain and posterior hypothalamus resulted in hypersomnolence while lesions of the basal forebrain and anterior hypothalamus produced insomnia (Von Economo, 1930). In 1949 Moruzzi's and Magoun's landmark finding provided further evidence for a neurological basis for wakefulness and arousal. They found that electrical stimulation of the reticular formation in a sleeping cat induces immediate EEG desynchronization over the entire cerebral cortex (Moruzzi and Magoun, 1949). The reticular formation and neuronal circuits associated with the arousal response became known as the ascending reticular activating system. Today, the general consensus is that sleep and wakefulness are active processes the timing and duration of which are controlled by neuronal circuits in the brain (reviewed in Jones, 2003). The brain contains multiple wakefulness-promoting centers that reside in the brainstem, thalamus, hypothalamus and basal forebrain (Figure 1). From these structures ascending pathways projecting to the cerebral cortex to stimulate cortical activation and descending networks acting upon the spinal cord to stimulate sensory-motor responsiveness and activity arise. Various neurotransmitters and/or neuromodulators are produced and utilized to convey

information among the centers. An important characteristic of the wakefulness-promoting system of the brain is the gross redundancy. Wakefulness is not the function of a single ascending arousal pathway and none of the multiple wakefulness-promoting brain sites is necessary for the generation of arousal.

Major component of the ascending reticular activating system originates from the brainstem reticular formation and projects to the forebrain by a dorsal and a ventral pathway (see for review Jones, 1995). The dorsal pathway ascends to the thalamus, from where the thalamocortical system projects to the cerebral cortex. The ventral pathway projects through the hypothalamus and terminate in the basal forebrain. Most neurons in the reticular formation utilize the excitatory amino acid, glutamate, as neurotransmitter. In the brainstem, several neuronal groups contribute to ascending cortical projections. The pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus are two major sources of the arousal pathway. Neurons in these nuclei utilize acetylcholine (ACh) as neurotransmitter (Steriade et al., 1988). In addition, noradrenergic neurons from the locus ceruleus (LC) and serotonergic neurons from the raphe nucleus project to subcortical relay stations that are located in the thalamus, hypothalamus and basal forebrain.

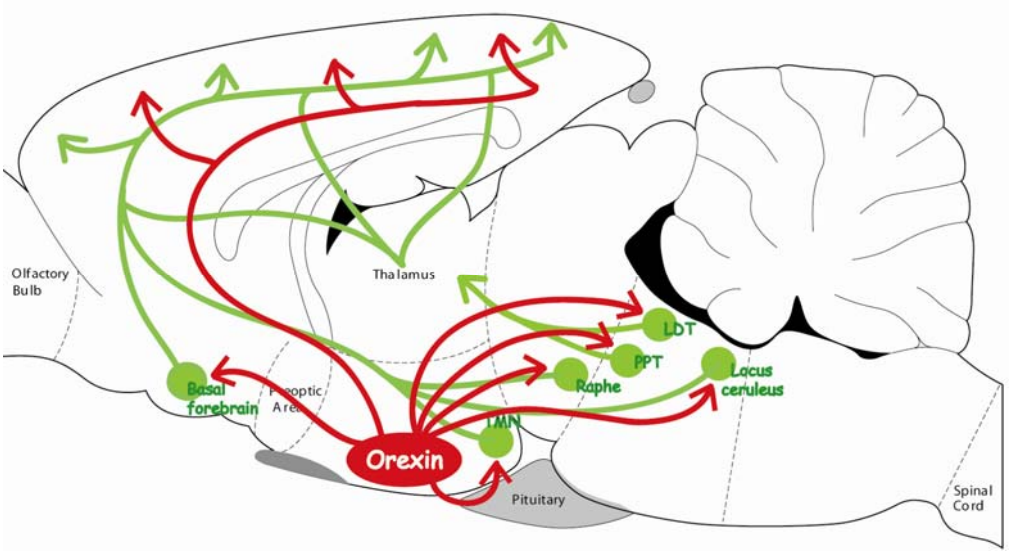

Figure 1. Wakefulness-promoting centers in the brain. Schematic sagittal drawing of the rat brain showing the major centers and pathways involved in promoting wakefulness. LDT: laterodorsal tegmental nucleus, PPT: pedunculopontine tegmental nucleus, TMN: tuberomamillary nucleus.

The thalamocortical activating system comprises multiple thalamic nuclei that receive ascending input from reticular, cholinergic, noradrenergic and serotonergic neurons (Jones

and Yang, 1985). Thalamic neurons provide widespread innervation to and prolonged activation of the cerebral cortex. Neurons in lateral posterior hypothalamus and histaminergic neurons from the tuberomamillary nucleus (TMN) project directly and activate the cerebral cortex (Panula et al., 1989). Basal forebrain cholinergic neurons receive input from all the brainstem and hypothalamic arousal centers and have widespread projections throughout the cerebral cortex (Rye et al., 1984).

The most recently discovered part of the wake-promoting system resides in the perifornical area of the lateral hypothalamus (LH) where cells are producing the peptide orexin (also called hypocretin). A growing body of evidence indicates the importance of orexinergic mechanisms in wakefulness (reviewed in Sakurai, 2002). Orexinergic neurons diffusely project and innervate the cerebral cortex and also excite other arousal centers, such as the basal forebrain, LC, TMN and raphe nucleus (Peyron et al., 1998). Lack of the orexin peptide or non-functional orexin receptors result in narcolepsy in humans, dogs and mice (Chemelli et al., 1999, Lin et al, 1999). In addition to being part of the wakefulness-promoting system, orexin-producing neurons are part of a food intake regulatory circuit in the hypothalamus (Figure 2). Orexin, as the name implies, also promotes eating (Dube et al., 1999). Hypothalamic ghrelin-, neuropeptide Y (NPY)- and orexinergic cells form a well-characterized neuronal network. Increased activity of the circuit stimulates feeding.

The hypothalamic ghrelin – NPY – orexin circuit

Synaptic circuitry through which ghrelin-, NPY- and orexin-producing cells communicate in the hypothalamus is well-characterized. Ghrelin-producing neurons are most abundantly present in the arcuate nucleus (ARC) but also found in the LH, paraventricular nucleus (PVN), the hypothalamic area adjacent to the ARC, ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH) and PVN with overlapping projections from the suprachiasmatic nucleus and the ventral lateral geniculate body of the thalamus (Cowley et al., 2003, Hou et al., 2006). Ghrelin-containing axon terminals project to the ARC and LH where they synapse with NPYergic and orexinergic neurons, respectively (Toshinai et al., 2003). In the ARC, ghrelin stimulates the production and release of NPY (Wren et al., 2002). In the LH, ghrelin stimulates orexinergic neurons directly and indirectly via NPY. NPYergic neurons originating from the ARC, synapse on orexin-positive cells in the LH which in turn project back to NPY cells (Horvath et al., 1999). Ghrelin stimulates

CRH neurons by promoting the release of NPY from axon terminals in the PVN (Cowley et al., 2003). Ghrelin-containing neurons also receive inputs from NPY-, orexin- and POMC-containing axon terminals in the ARC. Circulating ghrelin and leptin modulate the activity of the circuit stimulating or inhibiting NPY neurons in the ARC.

The role of the hypothalamic ghrelin – NPY – orexin circuit in feeding is well-established, the activation of the circuit leads to increased food intake and its suppressed activity results in decreased eating. A key component of the circuit, orexin, is shared by at least two systems, an arousal- and a food intake-promoting system. Mounting evidence suggests the existence of neuronal circuits shared by the regulation of sleep-wake activity and feeding/metabolism (Saper, 2006). The organization and localization of the hypothalamic ghrelin – NPY– orexin circuit make it a possible candidate for the mediation of arousal and metabolism. We hypothesize that the ghrelin – NPY – orexin circuit is a shared circuit by feeding and arousal.

Ghrelin

Ghrelin is the peptide product of the preproghrelin gene that also encodes obestatin (Kojima et al., 1999, Zhang et al., 2005). The major active form of ghrelin is a 28-amino acid peptide with a fatty acid chain (octanoyl group) on the third amino acid from the *N*-terminal end. The majority of circulating ghrelin is synthesized by the stomach but it is widely expressed in other tissues including the brain, the distal parts of the gastrointestinal system, thymus, gonads, and adrenal gland (Ariyasu et al., 2001). The actions of ghrelin are mediated through the growth hormone secretagogue receptor-1a (GHS-R1a) (Kojima et al., 1999). GHS-R1a mRNA is widely distributed in hypothalamic nuclei that are implicated in the regulation of feeding and/or sleep--wake activity, such as the LH, PVN, ARC, DMH, anteroventral preoptic nucleus, anterior hypothalamic area, medial preoptic area (MPA), suprachiasmatic nucleus (SCN), anterolateral hypothalamic nucleus, and the TMN (Guan et al., 1997, Mitchell et al., 2001).

Ghrelin exhibits multiple biological actions; a major focus of research is on ghrelin's effect on energy homeostasis and feeding. Ghrelin is a classic gut-brain peptide with potent feeding-promoting and growth-hormone-releasing activities. Ghrelin is the only known peripheral peptide that stimulates food intake. It promotes fat deposition (Tschöp et al.,

2000), stimulates gastrointestinal motility, gastric secretion (Masuda et al., 2000), and the activity of the hypothalamo-pituitary-adrenal axis (Wren et al., 2002). In humans, plasma ghrelin levels inversely correlate with feeding; fasting increases while eating reduces plasma ghrelin concentrations (Cummings et al., 2001). Systemic and central administration of ghrelin strongly stimulates feeding in rats (Wren et al., 2000). In humans, it enhances food intake and appetite and induces food-related images (Kojima et al., 1999, Wren et al., 2001). The orexigenic property of ghrelin has largely been attributed to its central action. GHS-R1a is expressed in the ARC predominantly by neurons co-expressing NPY (Willesen et al., 1999). Functional activation of this neuronal population by peripheral and central ghrelin has been demonstrated electrophysiologically, by induction of early gene expression and by increased expression of NPY (Kohno et al., 2003, Seoane et al., 2003). The orexigenic effects of ghrelin are markedly reduced by pretreatment with a NPY Y1 receptor antagonist (Nakazato et al., 2001). Ghrelin inhibits energy expenditure by suppressing the sympathetic outflow to the brown adipose tissue of rats (Yasuda et al., 2003). Circulating ghrelin levels negatively correlate with adipose tissue mass, i.e., it is high in anorexia and cachexia and suppressed in obesity. Ghrelin induces anxiety-like behavior and affects memory (Carlini et al., 2002). The half-life of total ghrelin is about 30 minutes in the mammalian bloodstream (Tolle et al., 2002).

NPY

NPY, a 36-amino acid peptide was discovered in 1982 (Tatemoto et al., 1982). It belongs to the pancreatic polypeptide family. NPY is one of the most abundant and widely distributed neuropeptides in the mammalian central and peripheral nervous system (Allen et al., 1983, Zukowska et al., 2003). In the periphery, it is generally found in the sympathetic nervous system and co-stored and co-released with norepinephrine. In the brain, the main source of NPY is the hypothalamus, particularly the ARC and the periventricular areas but also found in the DMH, PVN, SCN, cortex and the brain stem (Allen et al., 1983). It acts as a neurohormone and neuromodulator. NPY-synthesizing neurons also produce another orexigenic peptide, agouti-related peptide. These neurons send axons to the PVN, MPA and DMH (Bai et al., 1985). The NPY receptor family includes at least 6 subtypes from which the Y1 and Y5 are thought to mediate the food intake-promoting effect (Wolak et al., 2003). Both receptors are present in the PVN, ARC, MPA, SCN, supraoptic nucleus and the LH. Reciprocal connections between NPY/agouti-related peptide neurons in the ARC and orexin

neurons in the LH have also been identified (Horvath et al., 1999, Muroya et al., 2004). Fos expression in NPY neurons of the ARC is induced by intracerebroventricular (icv) injection of orexin (Lopez et al., 2002).

The widespread distribution of NPY is associated with a wide range of physiological activities, such as food intake (Dube et al., 1994, Levine and Morley, 1984, Stanley et al., 1985), hormone secretions (Haas and George, 1987, Leibowitz et al., 1988, Wahlestedt et al., 1987), circadian rhythms (Alberts and Ferris, 1984), thermoregulation (Jolicoeur et al., 1995), seizure (Redrobe et al., 1999), stress and blood pressure (Walker et al., 1991). NPY is one the most potent orexigenic peptides found in the brain (reviewed in Beck, 2006). When injected into the cerebral ventricles or into certain hypothalamic nuclei, it elicits robust feeding responses lasting for several hours even in satiated rats (Stanley et al., 1985). This effect is comparable to increased feeding after 36-48 h of food deprivation in normal rats. Blockade of the NPY system by NPY antibodies or gene manipulation suppresses feeding. NPY promotes food intake by reducing the latency to eat and delays satiety leading to increased meal size and time spent eating. NPY infusions also increases fat deposition and decreases brown fat thermogenesis and oxygen consumption, suggesting that NPY is also involved in the regulation of metabolism.

Hypothalamic NPY concentrations vary with the feeding state of the animal. NPY synthesis and content within the ARC and PVN is elevated in fasted animals and returns to normal levels rapidly upon refeeding. Hypothalamic NPY content shows diurnal variation (Jhanwar-Uniyal et al., 1990). NPY content in the ARC and PVN peaks one hour before dark onset and decreases one hour after lights-off. Food intake during the first few hours of darkness is associated with a peak in NPY release in the PVN and a sharp decrease in NPY mRNA. In the NPY knockout mice, food intake during the first four hours of the dark period is reduced by one third (Sindelar et al., 2005).

Several lines of evidence support the existence of a strong interaction between sleep/vigilance and feeding. Several hypothalamic areas, such as the SCN, LH and VMH have long been implicated in the regulation of both sleep and food intake (Saper, 2006). Changes in the amount and/or content of food greatly affect sleep-wake activity in rodents. For example, there is a significant correlation between meal size and the subsequent duration of sleep (Danguir and Nicolaidis, 1979). Starvation induces a marked sleep loss in rats

(Jacobs and McGinty, 1971, Dewasmes et al., 1989) while, re-feeding after food deprivation induces increased sleep in rats (Borbély, 1977, Shemyakin and Kapás, 2001). The calorie-rich "cafeteria diet" induces hyperphagia and increases the amount of sleep in rats (Danguir, 1987, Hansen et al., 1998). Intravenous (iv) administrations of nutrients affect sleep in rats differently (Danguir and Nicolaidis, 1980). The diurnal distributions of NREMS and REMS in rats are significantly altered when food access is restricted to the light period (Mouret and Bobillier, 1971, Roky et al., 1999). Feeding-related peptides and hormones also affect sleep (Kapás and Szentirmai, 2008).

Sleep and feeding are mutually exclusive behaviors. In mammals, periods of fasting are accompanied by increased wakefulness and sleep loss in order to maximize food seeking activity, feeding opportunities and therefore survival. The timing of sleep and feeding is highly species dependent. Humans consolidate waking and feeding cycles during the day, nocturnal rodents are awake and eat during the dark phase. Substantial evidence indicates that the regulation of sleep/wakefulness and feeding/metabolism are linked and coordinated by shared neuronal circuits in the brain. We hypothesize that the neuronal network, formed by ghrelin – NPY – orexin cells in the hypothalamus is one of these mechanisms. The objective of our work was to study the role of the hypothalamic ghrelin – NPY – orexin circuit in sleep. In a series of experiments, we determined the diurnal rhythm of plasma and hypothalamic ghrelin levels, the dependency of this rhythm on sleep-wake activity and the sleep-wake activity-modulating effects of central injections of ghrelin and NPY in rats.

MATERIALS and METHODS

General Methods

Animals. Male Sprague-Dawley rats, weighing 275-350 g, were used in the experiments. Water and food were available *ad libitum* except where otherwise stated. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the respective institutional committees.

Surgery. The surgeries were performed using intraperitoneal (ip) ketamine-xylazine (87 and 13 mg/kg, respectively) anesthesia. Each animal was implanted with stainless steel screw

electrodes for EEG recordings over the frontal and parietal cortices and the cerebellum and electromyographic (EMG) electrodes were implanted into the dorsal neck muscle. Stereotaxic equipment was used to implant guide cannulae for the icv and microinjections. For the icv injections a guide cannula (Plastics One, 22 G) was inserted into the left or right lateral cerebral ventricle [0.80 mm posterior from bregma, 1.4 mm lateral from midline, and 4.0 mm ventral from the surface of the skull according to the rat brain atlas by Paxinos and Watson, 2005]. For the microinjections, microinjection guide cannulae (Plastics One, 26 G) were implanted bilaterally into the LH and MPA or unilaterally into the PVN of the hypothalamus. The coordinates of the tip of the guide cannulae were: 2.1 mm posterior and 2 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for LH; 0.4 mm posterior and 1.85 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for MPA, and 1.80 mm posterior and 0.4 mm lateral to the bregma, and 7.5 mm ventral from the surface of the skull for PVN according to the rat brain atlas by Paxinos and Watson, 2005. The guide cannulae into the MPA and PVN were inserted at a 10º angle from vertical. Substances were administered by injector cannulae (30 G for icv injections and 33 G for the microinjections): both extended 0.5 mm beyond the tip of the guide. The guide cannulae and the EEG electrodes were fixed with dental cement to the skull.

Verification of cannula placements. The location of the icv cannulae was determined by the gravity method (sudden drop in pressure) during implantation and by the drinking response to icv injection of angiotensin II (200 ng in 2 µl) tested 3-4 days after surgery and also after the end of the experiments. Only data from rats responding to angiotensin II were included in the data analysis. To verify the location of the microinjection cannulae in the LH, PVN and MPA, 0.2 µl 5 % horseradish peroxidase was injected into the cannulae at the end of the experiment. Rats, anesthetized with Isoflurane, were perfused with saline and 4% paraformaldehyde. Brains were removed and kept at 4°C in paraformaldehyde until further examinations. The peroxidase–H_2O_2 reaction was visualized by diamonobenzidine in 100-µm thick neutral red-stained coronal brain sections. The spread of the injections was less than 1 mm as indicated by the enzyme reaction. The injection sites were localized with reference to the rat brain atlas (Paxinos and Watson, 2005).

Materials. Rat ghrelin, NPY and angiotensin II were purchased from Bachem Inc., Torrance, CA. All chemicals were dissolved in isotonic NaCl. Injection volumes were 2-5 µl for icv injections and 100 nl/injection side for microinjections.

Sleep-wake recordings. The rats were housed in individual sleep-recording cages. The cages were in a sound-attenuated recording room for Experiments 1 and 2. For Experiments 3 and 4, recording cages were placed in sound-attenuated, temperature controlled environmental chambers. For all experiments, rats were kept at 12:12-hour light-dark cycle with an ambient temperature of 24 ± 1°C. Animals were kept under these conditions for at least 1 week before the operation and for least 10 days of recovery after surgery during which they were connected to the recording cable and handled daily to habituate them to the experimental conditions. The recording cables were attached to commutators. The motor activity was assessed by recording potentials generated in electromagnetic transducers by cable movements (in Experiments 1-2) or by recording EMG signals (Experiment 3-4). Cables from the commutators and electromagnetic transducers were connected to amplifiers. The digitized (128 Hz sampling rate) signals of the EEG and motor activity or EMG were collected by computers. For off-line scoring, the EEG and motor activity or EMG signals were displayed on the computer screen. Power density values were calculated by fast-Fourier transformation (FFT) for consecutive 10-s epochs in the frequency range of 0.125–20.0 Hz for 0.25-Hz bands and were integrated in 0.5 Hz bins. The states of vigilance were determined for 10-s epochs by the usual criteria as NREMS [high-amplitude EEG slow waves, lack of body movement, predominant EEG power in the delta range (0.125-4.0 Hz)]; REMS (highly regular EEG theta activity with corresponding high FFT theta power, general lack of body movements with occasional twitches); and wakefulness (less regular theta activity, higher delta power than during REMS, frequent body movements). The time spent in each vigilance state was determined in 1-h time blocks. EEG power values for the 0.5-4.5-Hz delta range during NREMS were integrated and used to characterize sleep intensity, also known as EEG slow-wave activity (SWA). On the baseline day, average EEG SWA values were calculated across the 24-hour recording period for each rat to obtain a reference value for each animal. Power densities in 1-hour blocks on the baseline day and the test days were expressed as a percentage of the reference value.

Hormone measurements. In Experiment 1, trunk blood was collected and immediately centrifuged, and the plasma was stored at -80°C until the hormone assays. The hypothalamus was harvested by using the following landmarks: frontal edge of the optic chiasm, lateral sulci, caudal edge of the mammary bodies, and a depth of 2 mm from the ventral surface of the brain; tissue samples were also stored at -80° until used. The mean (± SE) mass of the hypothalamus was 42.0 ± 0.81 mg without significant differences among the groups.

Commercially available radioimmunoassay kits were used to determine ghrelin (Phoenix Pharmaceuticals, Belmont, CA) and leptin (LINCO Research, St. Charles, MO). The hormones were measured in duplicate or triplicate with a sensitivity of 4 pg/tube for ghrelin and 0.5 ng/ml for leptin. The intra- and inter-assay coefficients of variation were less than 8% and 14%, respectively. For extraction of hypothalamic samples, the frozen samples were weighed and placed in tubes containing 0.5 ml 2 M acetic acid and then boiled for 5 min. The tissues were individually homogenized by means of ultrasound. The homogenates were centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was lyophilized for the ghrelin radioimmunoassay.

Recording of feeding activity. In Experiment 1, food pellets were placed in vertical aluminum tubes hanging in one corner of the cage. A stainless steel bars kept the pellets in the opening of the tube. Potentials generated in electromagnetic transducers by the movements of the tube were recorded. The records were subjected to spectral analysis, and the total power in 8-s epochs was regarded as feeding activity. Feeding activity was integrated for 1-h periods. The total feeding activity was calculated across the 24 h and was taken as 100%. The percent fraction of feeding activity was calculated for each recording hour on the baseline and experimental days. This method of determination of feeding activity did not measure the quantity of food consumed, and it also missed food spillage and eating from the cage floor. However, the reproducibility of feeding activity was high in the three experiments, indicating that the method was suitable for the aims of these studies.

Measurement of food intake. In Experiments 2, 3 and 4 immediately after injection, animals were returned to cages containing a known amount of chow. Food pellets were reweighed at 1 hour after the injection. Results are expressed as g food intake/kg body weight ± SE.

Behavioral testing. To quantify the behavioral responses to ghrelin, the behavior of 6 rats was recorded with web cameras for 60 min after 1 μg ghrelin and isotonic NaCl injections on two different days. The recordings were scored off-line in 30-sec intervals. The dominant behavior of during a 30-sec block was classified as one of the following: drinking, feeding, grooming, exploring, and inactivity (irrespective that the rats slept or were obviously awake). For analysis the following three categories were used: inactivity, feeding and behavioral activity, which included drinking, grooming and exploring.

Statistical Analysis. Within-subject cross over design was used whenever possible, i.e., the same animals were tested under control and experimental conditions. Two-way ANOVA for repeated measures was used to analyze sleep-wake activity, feeding activity and power spectra data (factors: treatment and time effect for sleep-wake and feeding activity and treatment and frequency for power spectra). For EEG SWA analysis, those hours during which an animal did not have at least 5 min NREMS were excluded from the analysis. This resulted in occasional missing data points. Therefore, instead of repeated measure ANOVA, two-way ANOVA was performed on EEG SWA data. Data for the amount of wakefulness, NREMS and REMS, feeding activity and EEG SWA were collapsed into 1-h bins. The number and average duration of NREMS and REMS episodes and food intake during the first hour after ghrelin and NPY injections were analyzed by paired t-test. Hormone concentrations were analyzed by using one-way ANOVA. When ANOVA indicated significant variations, Student-Newman-Keuls test (SNK test) or t-test was used as post hoc test. $P < 0.05$ was considered to be significant.

Experimental Design

Experiment 1. Diurnal rhythms of sleep, feeding activity, plasma leptin, ghrelin and hypothalamic ghrelin levels rats.

Baseline sleep-wake and feeding activity were recorded for 3 days. For the test days, rats were divided into three groups (n = 7-12) and subjected to three different experimental manipulations.

Experiment 1/a. The access to the feeding tubes was continuous.

Experiment 1/b. Rats were kept on a restricted feeding schedule with *ad libitum* access to the feeder only during the light period. Animals were maintained on restricted feeding schedule for at least 3 wk before recordings.

Experiment 1/c. On the experimental day, rats were sleep deprived by gentle handling in the first 5 h of the light period. The rats stayed in their home cage, and whenever behavioral or EEG signs of sleep were observed, they were aroused by knocking on the cage or lightly touching them. After sleep deprivation, the animals remained in their home cages and were allowed to sleep *ad libitum*. Food was continuously available during the sleep deprivation and recovery periods. The animals were sacrificed by

guillotine at the following time points: 1 and 5 h after the beginning of sleep deprivation (i.e., 1 and 5 h after light onset) and 4, 8, 12, and 16 h after the end of sleep deprivation (i.e., 9, 13, 17 and 21 h after light onset). Blood and hypothalamus were collected from each animal for hormone assays. At each time point, control rats not subjected to sleep deprivation, were also sacrificed.

Experiment 2. The effects of icv administration of ghrelin on sleep in rats.

Experiment 2/a. The effects of three doses of ghrelin, 0.2 μg (n = 11), 1 μg (n = 8), and 5 μg (n = 13), injected 10-15 minutes before dark onset on sleep were tested.

Experiment 2/b. Three separate groups of rats received the same doses of ghrelin (n = 8, n = 6, n = 12, respectively) before light onset. In both Experiment 2/a and 2/b, two conditions were used: a baseline day when 2 μl of isotonic NaCl was administered and the experimental day when ghrelin was injected. The order of the baseline and experimental days was randomly chosen. Each rat was recorded from light onset or dark onset, respectively, for 12 hours after injections. Some of the rats (n = 15) were injected with more than one dose of ghrelin; in these cases the ghrelin days were separated by at least two days when no treatment was given to animals. These rats were not selected based on previous responses to ghrelin. Food intake after the light onset administration of 1 μg ghrelin was also measured using the same group of animals as for the sleep recordings but the food intake determinations were done 4 days after the sleep studies.

Experiment 2/c. Rats were allowed to eat only during the light phase. Food pellets were removed at light onset and returned 12 hours later at dark onset, each day for at least 10 days before recording. Sleep-wake activity was recorded for 12 hours on 2 consecutive days: a baseline day when isotonic NaCl was administered and an experimental day when 1 μg ghrelin was injected. One-half of the rats received NaCl on day 1 and ghrelin on day 2, whereas the order of the baseline day and experimental day was reversed for the rest of the rats.

Experiment 3. The effects of hypothalamic microinjections of ghrelin on sleep and food intake in rats.

Rats with bilateral microinjection cannulae in the LH or MPA and unilateral cannula in the PVN were used in the experiment. On the control day, the animals received 100 nl pyrogen-free isotonic NaCl; on the experimental day, they were injected with ghrelin (0.04 μg, 0.2 μg,

or 1 μg/injection site, 12, 60, and 300 pmol, respectively; dissolved in 100 nl isotonic NaCl). The order of the control and experimental days was counterbalanced. Microinjections took place over a 1-minute period and the microinjection cannulae were left in place for one additional minute. Immediately after injection, the animals were returned to their home cages. Food and water were provided *ad libitum* during the entire recording period. A pre-weighed amount of chow was placed on a plastic tray at light onset; the pellet leftovers were collected and reweighed 1 hour later. Sleep was continuously recorded for 23 hours starting from the beginning of light period.

Experiment 4. The effects of NPY on sleep and food intake in rats

Experiment 4/a. The three doses of NPY (0.4 μg, n = 8, 2 μg, n = 9, and 10 μg, n = 8) on sleep were tested. Rats received icv injection of pyrogen-free isotonic NaCl on the baseline and NPY on the experimental day 10-15 min before light onset. All injections were given in a volume of 4 μl. The order of the baseline and experimental days was randomly chosen. Some of the rats were injected with more than one dose of NPY; in these cases, at least one week separated the injections. Repeatedly injected rats were not selected based on previous responses to NPY.

In Experiment 4/b, rats (n = 8) received bilateral microinjection of 2 μg NPY in a volume of 0.2 μl into the LH on the test day and equal volumes of isotonic NaCl on the control day. Microinjections took place over a 1-min period and the microinjection cannulae were left in place for one additional minute.

RESULTS

Experiment 1/a. Diurnal rhythms of sleep, feeding activity, plasma leptin, ghrelin and hypothalamic ghrelin levels in free-feeding rats (Figure 2).

The diurnal rhythm of sleep-wake activity displayed the normal patterns of rats with more time spent in NREMS and REMS during the light phase and less during the dark. NREMS was the highest at the beginning of the light; REMS reached its maximum during the second half of light period. Thereafter, both declined steadily toward lights off and dropped markedly at dark onset.

Free-feeding rats ate mostly at night; only a small fraction of the total daily feeding activity occurred during the light period. Dark onset was associated with increased eating; 13.9 ± 1.24% of the daily feeding activity occurred during the first hour of the dark period. Feeding activity bouts recurred throughout the night.

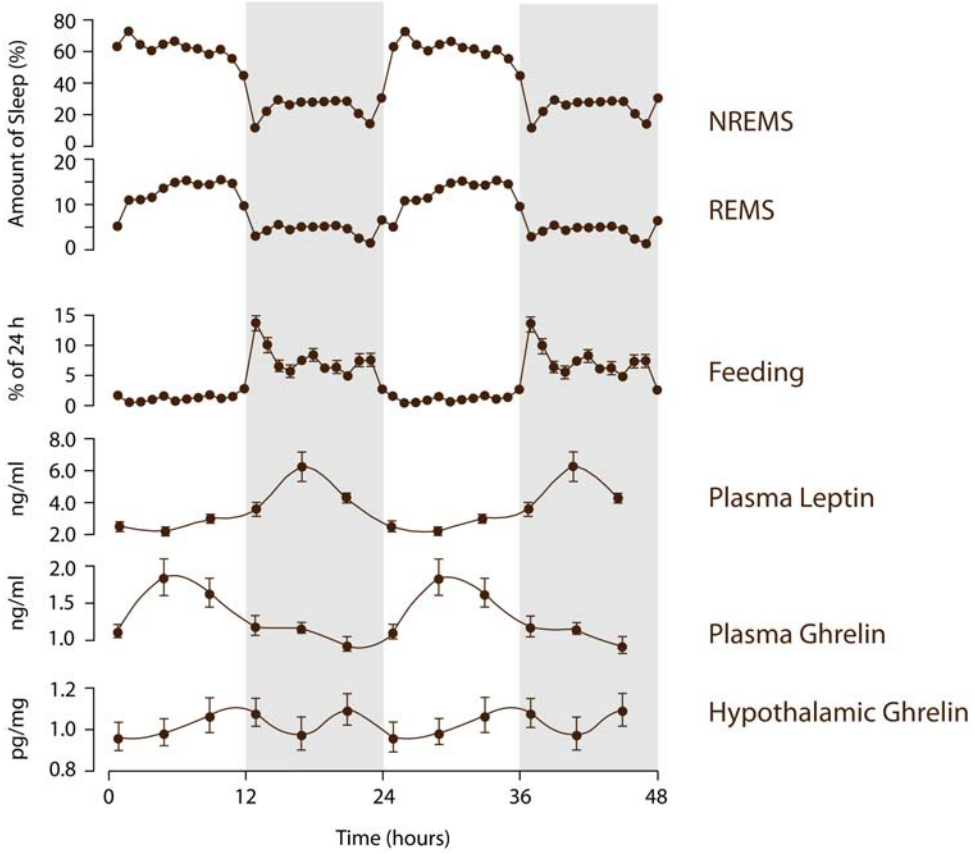

Figure 2. Diurnal rhythms of non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS), feeding activity, plasma leptin and ghrelin levels and hypothalamic ghrelin contents in free-feeding rats. Sleep and feeding activity values are the average of 3 consecutive days from 51 rats and expressed as percent of recording time in 1-hour data blocks. Plasma leptin and ghrelin levels and hypothalamic ghrelin contents were determined on day 4 of recording from groups of rats (n = 7-10) sacrificed at 4-hour intervals. The 24-hour curves are double-plotted to help visualization of the rhythms. Gray shaded area: dark period. Modified from Bodosi et al., 2004, Figure 1.

Plasma leptin [$F(5,45) = 12.3$, $p < 0.0001$] and ghrelin [$F(5,44) = 5.1$, $p < 0.001$] displayed distinct diurnal rhythms with their peak values occurring at opposite times of the day (Figure 2 and Attachment 1, Table 1). The leptin maximum followed the dark onset-elicited eating peak and occurred 5 hour after dark onset. The ghrelin peak preceded the eating peak and occurred 5 hour after light onset when leptin dropped to its diurnal trough value. Hypothalamic ghrelin showed modest oscillations with a rise after the plasma ghrelin peak

and a second increase toward the end of the dark period. These changes in hypothalamic ghrelin were not significant.

Experiment 1/b. Diurnal rhythms of sleep-wake activity, plasma leptin and ghrelin and hypothalamic ghrelin levels in feeding-restricted rats (Figure 3).

Restriction of feeding to the light period significantly altered the sleep-wake activity of rats. NREMS decreased by 11% during the light period [treatment effect: $F(1,100) = 179.0$, $p < 0.001$] and increased by 10% during the dark [treatment effect: $F(1,100) = 102.1$, $p < 0.001$]. The normal diurnal rhythm with more NREMS during the day was maintained. NREMS during the day was reduced only in the first and last hours of the light when feeding activity was increased [group × time interaction: $F(11,1100) = 36.0$, $p < 0.001$]. In contrast to NREMS, restricted feeding fundamentally altered the diurnal rhythm of REMS (See also Attachment 1, Table 1). REMS decreased throughout the light period [treatment effect: $F(1,100) = 203.9$, $p < 0.001$] and increased in the dark period [treatment effect: $F(1,100) = 291.5$, $p < 0.001$], resulting in more REMS at night than during the day. The daily, 24-hour total amount of NREMS and REMS did not change under restricted feeding schedule.

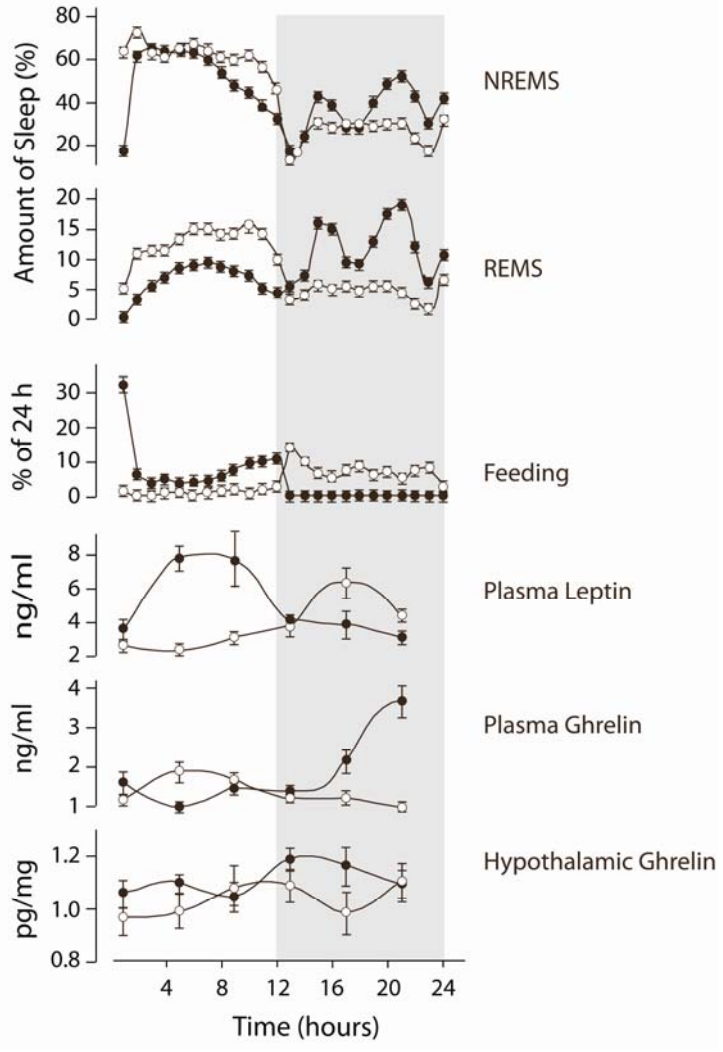

Figure 3. The effects of restricted feeding on the amount of NREMS and REMS, feeding activity, plasma leptin and ghrelin levels and hypothalamic ghrelin content. Data are obtained from 55 rats. Open symbols: baseline day, solid symbols: restricted feeding day. See legend for Figure 2 for details. Modified from Bodosi et al., 2004, Figure 2.

When food was available for 12 hours during the day, feeding activity was the highest at the beginning of the light period. Feeding activity in the first hour of the light period was $31.8 \pm 2.2\%$ of the total daily activity, and this value was significantly higher (*t*-test, $p < 0.001$) than the feeding activity of rats on *ad libitum* feeding in the first hour of the dark. Feeding activity decreased after the first hour of the light period but remained significantly elevated compared to the baseline conditions for the rest of the light period.

Restricted feeding had significant effect on plasma ghrelin [comparison with free-feeding group, group effect (independent): $F(1,93) = 22.5$, $p < 0.001$] and leptin rhythms [group effect: $F(1,94) = 9.7$, $p < 0.002$]. The diurnal rhythms of these hormones reversed in such a way that they maintained their relationship with respect to one another and to feeding activity. The ghrelin peak continued to precede the major feeding peak, which was now in the first hour of the light, and thus the ghrelin maximum was observed at the end of the dark period. The leptin peak followed the major feeding activity peak, and it occurred during the day between hours 5 and 9 after light onset. The concentrations calculated for 24 hours increased significantly for both leptin and ghrelin (Attachment 1, Table 1). The amplitude of leptin peak during restricted feeding did not differ significantly from the leptin peak on the normal cycle. The nocturnal ghrelin peak in the rats on restricted feeding was significantly higher than the diurnal ghrelin peak in the rats on normal feeding rhythm. Ghrelin content of the hypothalamus displayed slight oscillations, which did not reach the level of statistical significance. However, comparisons of hypothalamic ghrelin contents revealed significant differences between free-feeding and food restricted rats [group effect: $F(1,85) = 5.1$, $p < 0.03$], and when calculated for 24 hours, the mean hypothalamic ghrelin content was significantly higher in rats on restricted feeding than in the rats on the normal feeding schedule (Attachment 1, Table 1).

Experiment 1/c. Rhythms of plasma leptin, ghrelin and hypothalamic ghrelin levels after 5 hours of sleep deprivation (Figure 4).

Five hours of sleep deprivation was followed by significant increases in the duration of both NREMS and REMS (See Attachment 1, Figure 3 and Results for details). NREMS time increased immediately after sleep deprivation and remained elevated during the dark period. Increases in REMS occurred towards the end of the light and during the dark period.

Feeding activity was slightly but significantly stimulated during sleep deprivation [treatment × time interaction: $F(20,452) = 3.3$, $p < 0.001$] (Figure 4). During the 5 hours of sleep deprivation, 11.1 ± 2.9% of the 24-hour feeding activity occurred. There was a tendency towards decreased feeding activity during the recovery period subsequent to sleep deprivation (hours 6–12 of the light period: 5.8 ± 1.98% after sleep deprivation and 11.8 ± 1.98% on the baseline day, t-test, $p < 0.05$), and the feeding activity peak after dark onset was also significantly suppressed.

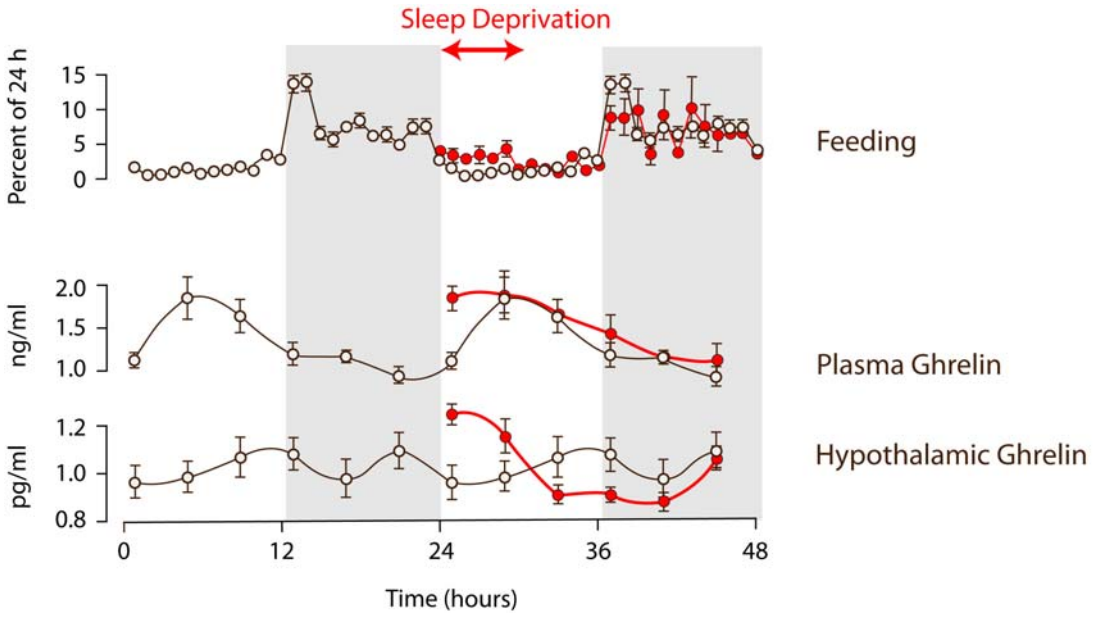

Figure 4. The effects of 5 hours of sleep deprivation on feeding activity, plasma ghrelin levels and hypothalamic ghrelin contents. Open symbols: baseline day, solid red symbols: sleep deprivation day. Data are obtained from 47 rats. Red arrow represents the 5 hours of sleep deprivation period started at light onset. Baseline values are double-plotted. Modified from Bodosi et al., 2004, Figure 3.

Sleep deprivation did not alter plasma concentration of leptin (Attachment 1, Figure 3). In contrast, plasma ghrelin increased significantly in the first hour of sleep deprivation (*t*-test, $p < 0.001$). Thereafter, significant differences were not observed in plasma ghrelin between the sleep deprivation and the baseline days. Hypothalamic ghrelin was highly responsive to sleep deprivation, displaying statistically significant biphasic variations [$F(5,39) = 11.1$, $p < 0.001$]. Ghrelin contents of the hypothalamus increased significantly during sleep deprivation and dropped below baseline after sleep deprivation. These variations in hypothalamic ghrelin levels were significantly different between the free-feeding and sleep-deprived rats [time × treatment interaction: $F(5,75) = 6.4$, $p < 0.001$].

Experiment 2/a. The effects of dark onset administration of ghrelin on sleep in rats (Figure 5).

Dark onset injection of 0.2 μg ghrelin did not change the amounts of wakefulness, NREMS or REMS. One μg ghrelin induced significant decreases in NREMS and REMS in hours 1 and 2 [treatment effect, NREMS: $F(1,7) = 6.0$, $p < 0.05$, REMS: $F(1,7) = 13.7$, $p < 0.05$]. In hours 3-12 after injection, sleep returned to baseline. Five μg ghrelin had a biphasic effect on NREMS. In the first 2 hours after injection NREMS decreased while NREMS was increased during the rest of the recording period [treatment effect, hours 1 and 2: $F(1,12) = 6.1$, $p < 0.05$, hours 3-12: $F(1,12) = 7.4$, $p < 0.05$]. Similar tendencies in REMS were observed but the changes only reached the level of significance in hours 1 and 2 [treatment effect, $F(1,12) = 9.8$, $p < 0.05$].

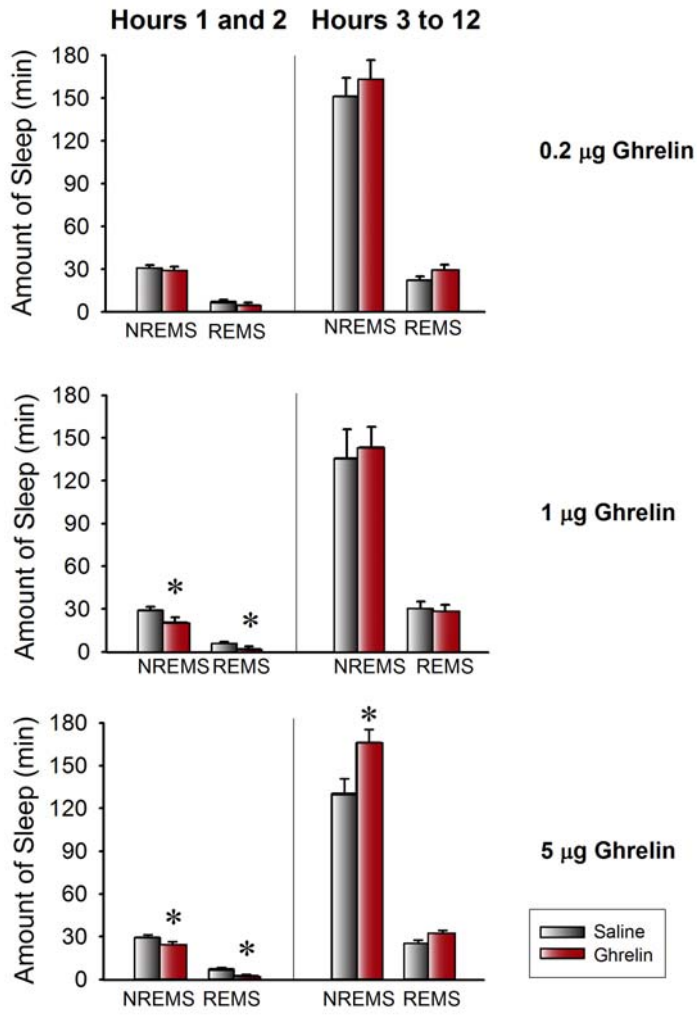

Figure 5. The effects of intracerebroventricular injection of 3 doses of ghrelin on NREMS and REMS. Sleep is expressed in minutes, as time spent in NREMS and REMS during the 2- or 10-hour period. Asterisk denote significant differences between control and test day ($p < 0.05$, paired *t* test). Modified from Szentirmai et al., 2006, Figure 1.

EEG delta power during NREMS, a measure of NREMS intensity, showed dose-dependent decreases in response to ghrelin injected at dark onset [treatment effect, $F(2,78) = 4.1$, $p < 0.05$] (see Attachment 2, Figure 2 and Results for details). There was no change in EEG SWA following injection of 0.2 μg ghrelin as compared to baseline. One μg and 5 μg ghrelin caused significant decreases in EEG SWA [treatment effect, 1 μg ghrelin injection: $F(1,65) = 18.2$, $p < 0.05$, 5 μg ghrelin injection $F(1,51) = 9.2$, $p < 0.05$].

Experiments 2/b and 2/c. The effects of light onset administration of ghrelin on sleep, activity and food intake in free-feeding and feeding restricted rats (Figures 6 and 7).

Ghrelin induced significant dose-dependent changes in wakefulness, NREMS and REMS in hours 1 and 2 after injection [treatment effect, NREMS: $F(2,46) = 3.8$, $p < 0.05$, REMS: $F(2,46) = 5.2$, $p < 0.05$]. There was a tendency towards decreased NREMS and increased wakefulness in the first 2 hours after 0.2 μg injections, but these changes did not reach statistical significance. This dose did not alter REMS. One μg ghrelin had a biphasic effect on wakefulness and NREMS. Wakefulness was significantly increased in hours 1 and 2 after injection, simultaneously, NREMS and REMS were suppressed [treatment effect, NREMS: $F(1,5) = 43.3$, $p < 0.05$, REMS: $F(1,5) = 15.4$, $p < 0.05$]. During hours 3 to 12, NREMS was elevated. One μg ghrelin increased wakefulness in feeding restricted rats, i.e., rats with no access to food after ghrelin injection, and induced significant decreases in NREMS in hour 1 [treatment effect: $F(1,7) = 6.4$, $p < 0.05$]. In the following hours, NREMS did not differ from baseline values. REMS did not change in the first 2 hours but was significantly decreased

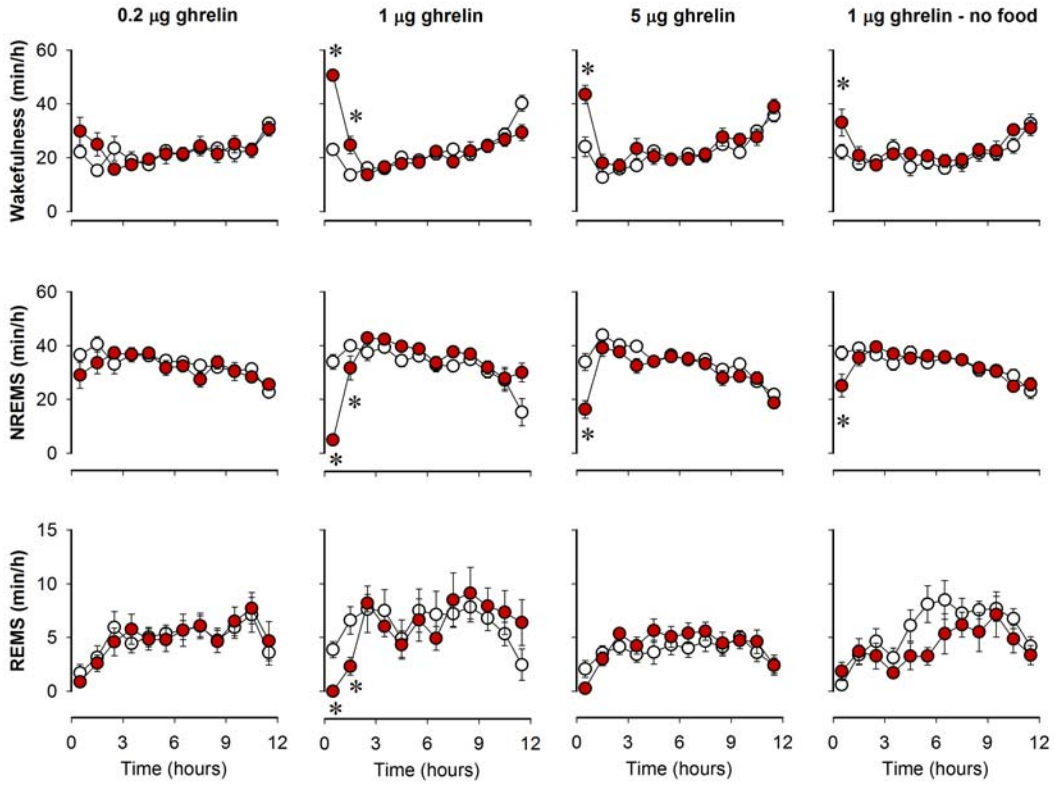

Figure 6. The effects of icv injection of 3 doses of ghrelin on wakefulness, NREMS and REMS in free-feeding and 1μg ghrelin in feeding restricted rats. Open symbols: baseline day, solid red symbols: ghrelin day. Time 0: time of injections. Asterisks denote significant differences between control and ghrelin treatment ($p < 0.05$, SNK-test). Data are expressed in minutes, as time spent in wakefulness, NREMS and REMS during the 1-hour period. Modified from Szentirmai et al., 2006, Figure 4.

from hours 3 to 12 [treatment effect: $F(1,6) = 10.8$, $p < 0.05$]. The 1 µg ghrelin-induced NREMS decrease in hours 1 and 2 was significantly higher in free-feeding rats than in feeding restricted animals [group factor: $F(1,24) = 8.3$, $p < 0.05$]. EEG SWA did not show significant changes between the baseline and the experimental day following 0.2 µg and 5 µg ghrelin injections (See Attachment 2, Figure 4 and Results for details). One µg ghrelin induced significant increase in EEG SWA [treatment effect $F(1,60) = 9.8$, $p < 0.05$] between hour 1 and 8.

Sleep suppression after ghrelin administration was accompanied by behavioral activation. Rats were restless throughout the 60 minute observation period; their behavior included increased locomotor activity, eating, drinking, grooming and exploration. The first bout of eating was already observed in 10 minutes after the injection and eating continued throughout the first hour of the light period. During this time food intake was significantly higher than after the control treatment. Food and water intake did not differ in the two groups across the 24-hour post-injection period (Attachment 2, Figure 6).

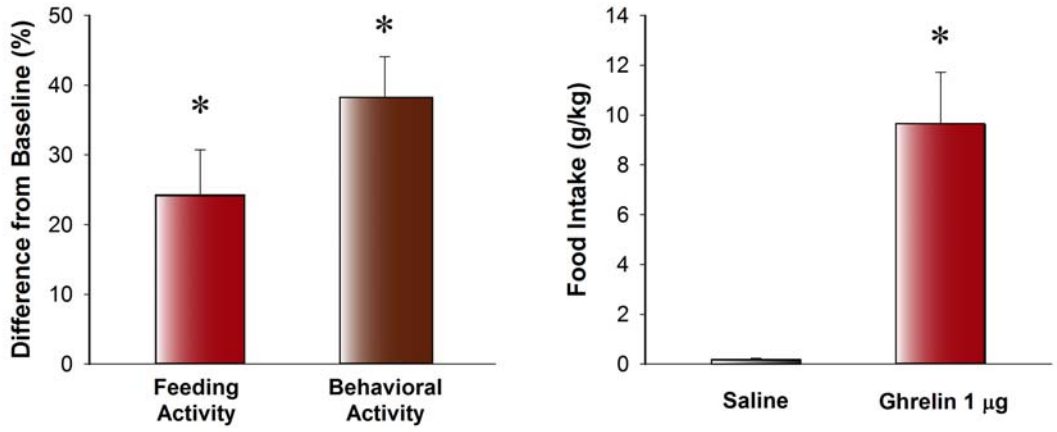

Figure 7. The effects of icv administration of 1 µg ghrelin on feeding and behavioral activity (left panel) and on 1-h food intake (right panel) in rats. Food intake is expressed as g consumed food/kg body weight. Asterisks denote significant differences between control and experimental day ($p < 0.05$, paired *t* test). Modified from Szentirmai et al., 2006, Figures 5 and 6.

Experiment 3/a. The effects of ghrelin microinjection into the LH on sleep-wake activity, EEG and food intake in rats (Figures 8 and 11).

The lowest dose of ghrelin, 0.04 µg, did not have any significant effect on the amount of wakefulness, NREMS or REMS and did not affect EEG SWA (Figure 8). Detailed analysis of the EEG power (See Attachment 3, Figure 2 and Results for details) revealed a significant

increase in the 6-8 Hz frequency range during wakefulness. There was no significant change in the food intake of the rats in response to 0.04 μg ghrelin (Figure 11).

Administration of 0.2 μg ghrelin significantly increased the time spent in wakefulness and decreased the time in NREMS and REMS [treatment effect for wake: $F(1,7) = 16.2$; $p < 0.05$; for NREMS: $F(1,7) = 13.4$, $p < 0.05$; for REMS: $F(1,7) = 27.5$, $p < 0.05$]. The effects on wakefulness and NREMS were confined to the first two hours of the recording period, while REMS changes were significant in hours 2 and 3 [$p < 0.05$, Student Newman Keuls test (SNK test)]. There was a significant effect on EEG SWA in the first 3 hours as indicated by ANOVA [treatment x time interaction: $F(2,38) = 3.6$, $p < 0.05$], but post hoc analysis did not show significance in any single hour. In hours 4 and 5 after injection EEG SWA was significantly elevated [treatment x time interaction: $F(6,98) = 2.6$, $p < 0.05$]. EEG power (Attachment 3, Figure 2 and Results for details) showed a significant increase during wake and REMS and a decrease during NREMS. Injection of 0.2 μg ghrelin significantly increased the 1-hour food intake of the rats

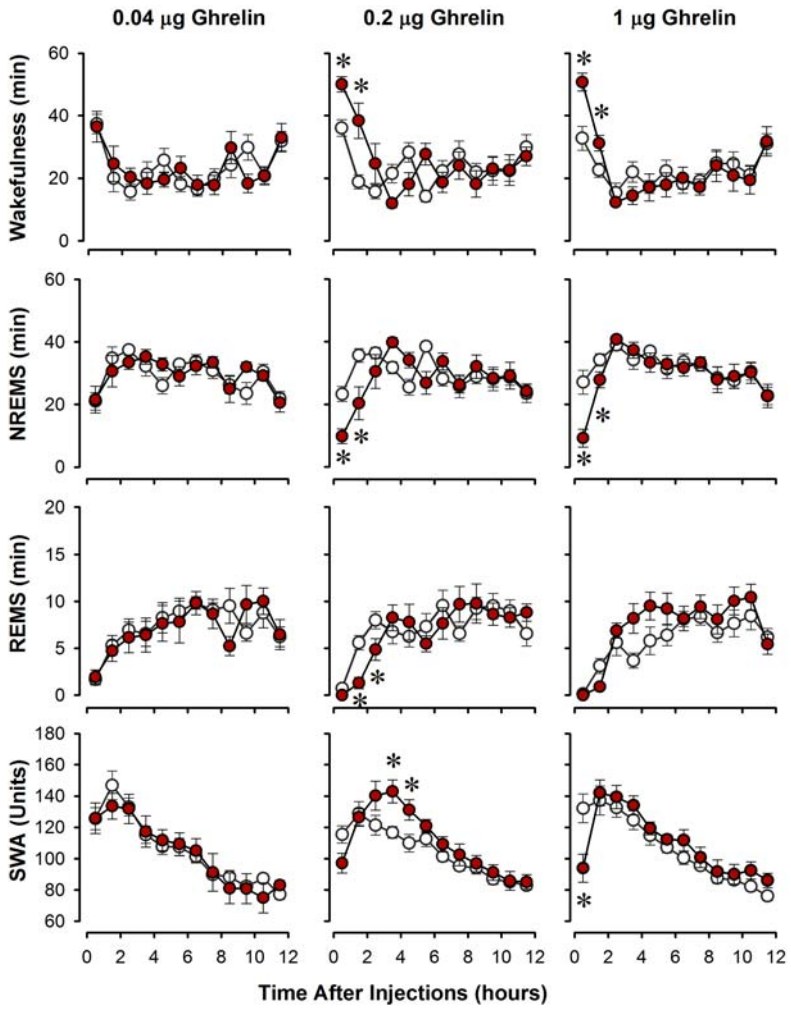

Figure 8. The effects of lateral hypothalamic (LH) administration of three doses of ghrelin on wakefulness, NREMS, REMS and electroencephalographic (EEG) slow-wave activity (SWA). Open symbols: baseline day, solid red symbols: ghrelin day. Time 0: time of injections. Data are expressed in minutes, as time spent in wakefulness, NREMS and REMS during the 1-hour period. Asterisks denote significant differences between baseline and experimental days ($p < 0.05$, SNK-test). Modified from Szentirmai et al., 2007, Figure 1.

from a baseline of 0.65 ± 0.56 g/kg BW to 6.36 ± 1.16 g/kg BW after ghrelin treatment (Figure 11).

The effects of 1 µg ghrelin injection on sleep were similar to those of the middle dose. Following 1 µg ghrelin injection wakefulness increased and NREMS decreased [treatment effect for wake: $F(1,7) = 8.5$, $p < 0.05$; for NREMS: $F(1,7) = 9.9$, $p < 0.05$]. Post hoc analysis showed significant changes in wake and NREMS in the first two hours after ghrelin treatment. EEG SWA was significantly attenuated in the first hour following ghrelin injection [treatment x time interaction: $F(2,36) = 3.9$, $p < 0.05$]. Detailed analysis of the EEG power (Attachment 3, Figure 2) showed a significant decrease during NREMS and REMS. One µg ghrelin significantly stimulated the 1-hour food intake of the rats (0.25 ± 0.14 g/kg BW after control treatment vs. 7.02 ± 1.75 g/kg BW after ghrelin treatment, Figure 11).

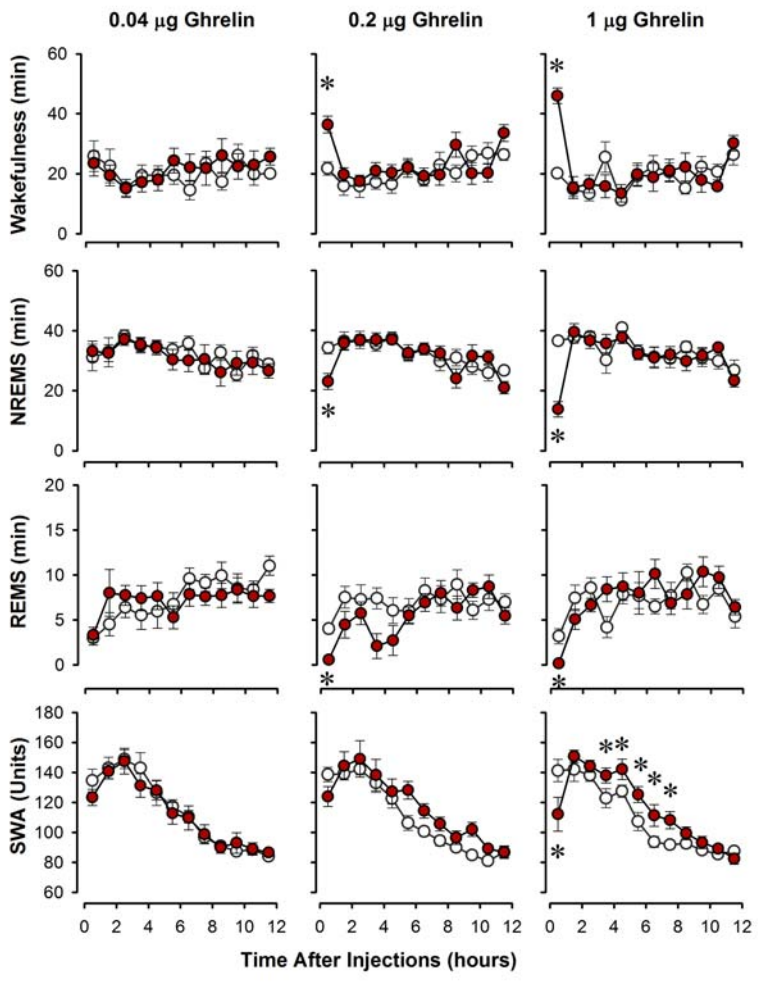

Figure 9. The effects of 3 doses of ghrelin microinjection into the medial preoptic area (MPA) on wakefulness, NREMS, REMS and EEG SWA. See legend to Figure 8 for details. Modified from Szentirmai et al., 2007, Figure 1.

Experiment 3/b. The effects of ghrelin microinjection into the MPA of the hypothalamus on sleep-wake activity, EEG and food intake in rats (Figures 10 and 12).

The lowest dose of ghrelin, 0.04 µg, did not induce any statistically significant change in the time spent in wakefulness, NREMS or REMS and did not alter EEG SWA or food intake (Figures 9 and 11). There was a significant increase in the EEG power (Attachment 3,

Figure 4) during wake [treatment x time inter-action: $F(31,186) = 2.0$, $p < 0.05$] and a significant decrease during NREMS [treatment x time inter-action: $F(31,186) = 3.6$, $p < 0.05$].

The middle dose of ghrelin, 0.2 μg, induced a significant increase in time spent awake at the expense of both NREMS and REMS (Figure 9), as indicated by ANOVA [treatment effect for wake: $F(1,6) = 26.6$; $p < 0.05$; for NREMS: $F(1,6) = 12.9$; $p < 0.05$; for REMS: $F(1,6) = 15.9$; $p < 0.05$]. Post hoc analysis showed the effects to be confined to the first hour of the recording period. EEG SWA slightly but significantly increased beginning from the fourth hour [treatment effect: $F(1,6) = 9.9$, $p < 0.05$]. EEG power (Attachment 3, Figure 4) during wake was increased [treatment x time interaction: $F(31, 186) = 1.6$, $p < 0.05$], while it decreased during NREMS [treatment x time interaction: $F(31,186) = 2.0$, $p < 0.05$]. Injection of 0.2 μg ghrelin was followed by a significant increase (5.01 ± 0.53 g/kg BW vs. 0.45 ± 0.23 g/kg BW after saline injection) in food intake (Figure 11).

The highest dose of ghrelin induced a statistically significant increase in time spent in wake at the expense of NREMS and REMS [treatment effect for wake: $F(1,6) = 244.6$; $p < 0.05$; for NREMS: $F(1,6) = 101.6$; $p < 0.05$; for REMS: $F(1,6) = 28.4$; $p < 0.05$]. The effects were confined to the first hour of the recording period. The NREMS changes in the first hour were accompanied by a significant decrease in EEG SWA [treatment x time interaction: $F(2,34) = 4.7$, $p < 0.05$]. The initial decrease in EEG SWA was followed by an increase beginning from the fourth hour [treatment x time interaction: $F(6,84) = 3.4$, $p < 0.05$]. The EEG power (Attachment 3, Figure 4) during wake and sleep was also affected. One μg ghrelin induced a decrease in EEG power during wake, NREMS and REMS. Food intake was significantly stimulated by 1 μg ghrelin injection (0.74 ± 0.34 g/kg BW on the control day and 7.44 ± 1.26 g/kg BW on the treatment day, Figure 11).

Experiment 3/c. The effects of ghrelin microinjection into the PVN of the hypothalamus on sleep-wake activity, EEG and food intake in rats (Figures 10 and 11).

The lowest dose of ghrelin did not induce any significant change in wakefulness, NREMS, REMS, EEG SWA or food intake when microinjected into the PVN (Figures 10 and 11).
Injection of 0.2 μg ghrelin in the PVN did not change the time spent awake, in NREMS or REMS and there was no significant effect on EEG SWA. Detailed analysis of EEG power

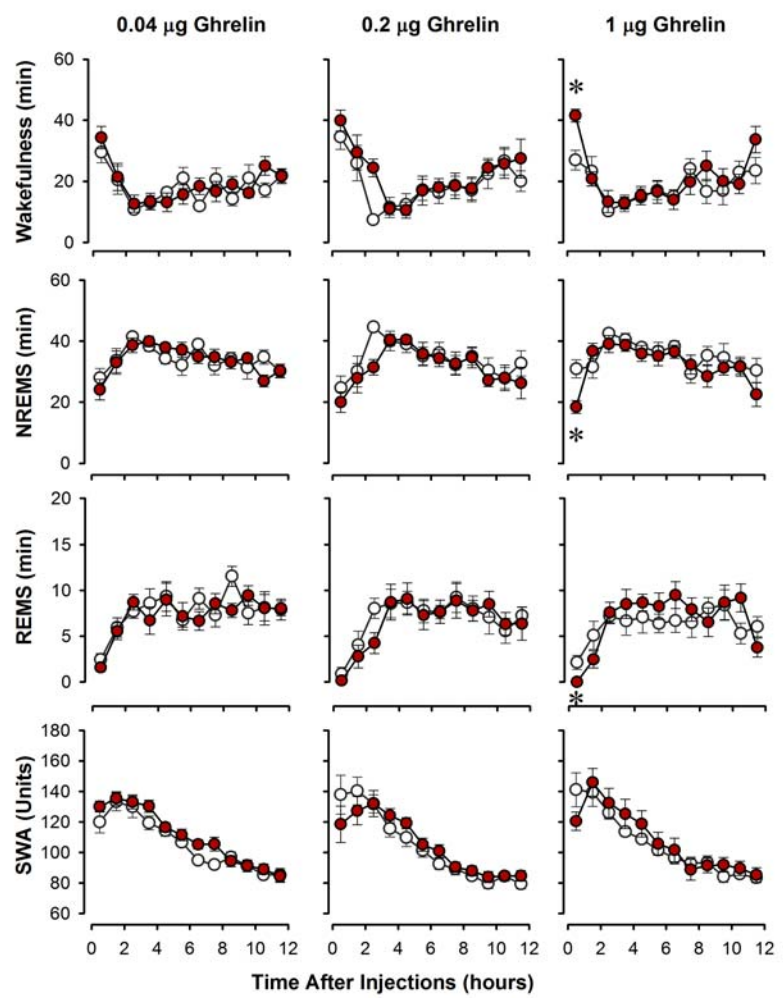

Figure 10. The effects of 3 doses of ghrelin microinjection into the paraventricular nucleus (PVN) on wakefulness, NREMS, REMS and EEG SWA. See legend to Figure 8 for details. Modified from Szentirmai et al., 2007, Figure 5.

(Attachment 3, Figure 6) revealed a significant suppression during NREMS. Food intake was significantly increased from a baseline of 0.82 ± 0.35 g/kg BW to 4.52± 1.14 g/kg BW in response to 0.2 µg ghrelin injection.

One µg ghrelin induced a statistically significant increase in time spent awake in the first hour after injection [treatment x time interaction: $F(2,14) = 3.8$; $p < 0.05$], which was accompanied by a significant decrease in NREMS [treatment x time interaction: $F(2,14) = 4.5$, $p < 0.05$]. EEG SWA did not change in response to 1 µg ghrelin injection. There was a significant increase in EEG power (Attachment 3, Figure 6) during wakefulness [treatment x

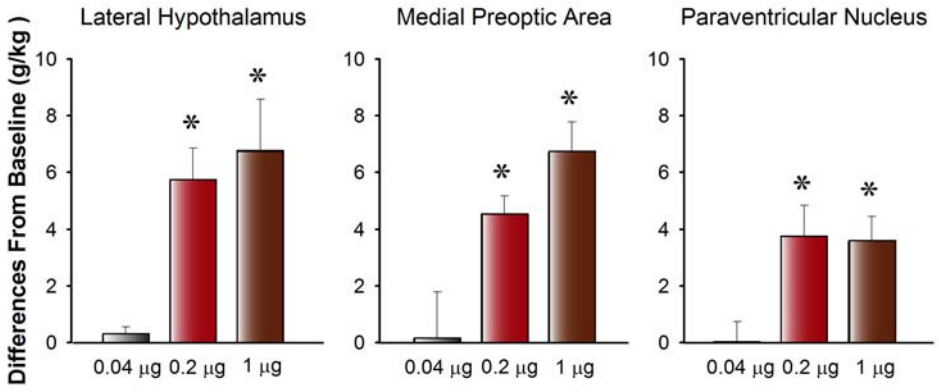

Figure 11. The effects of 3 doses of ghrelin microinjected into the LH, MPA or PVN on food intake. Data are expressed as gram food intake per kg body weight ± SE and plotted as differences from baseline. *: Significant differences from baseline ($p < 0.05$, paired *t*-test). Modified from Szentirmai et al., 2007.

time inter-action: $F(31,186) = 1.9$, $p < 0.05$]. Injection of 1 µg ghrelin into the PVN of the rats induced a significant, about 4-fold increase, in food intake (Figure 11).

Experiment 4/a. The effects of icv administration of NPY on sleep-wake activity and food intake in rats (Figure 12).

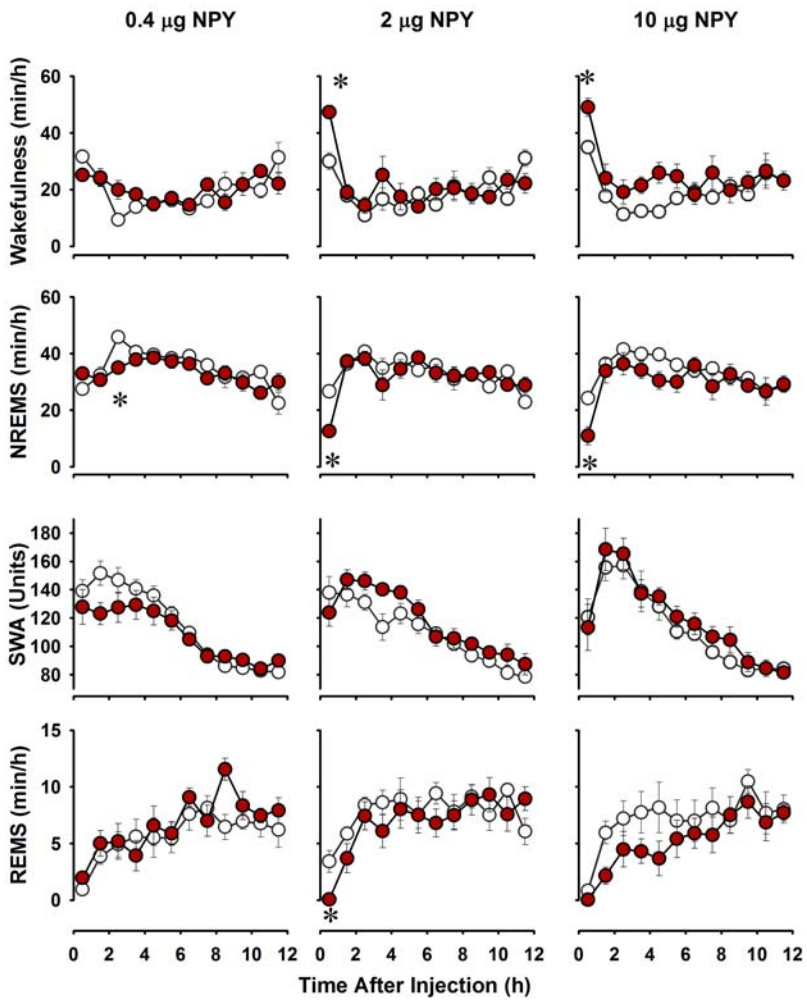

Figure 12. The effects of 3 doses of icv injection of neuropeptide Y (NPY) on wakefulness, NREMS, REMS and EEG SWA. Open symbols: baseline day, solid red symbols: NPY day. Time 0: time of injections. Data are expressed in minutes, as time spent in wakefulness, NREMS and REMS during the 1-hour period. Asterisks denote significant differences between baseline and experimental days ($p < 0.05$, SNK-test). Modified from Szentirmai and Krueger, 2006, Figure 2.

The lowest dose, 0.4 µg NPY had statistically significant effect on NREMS as indicated by ANOVA (Attachment 4, Table 1) which was confined to the third hour after the injection ($p < 0.05$, SNK-test); the biological significance of this isolated difference in NREMS between the baseline and experimental day is questionable. There was no significant effect on the total episode number and episode duration of NREMS and REMS (Table 1) and on EEG SWA after the 0.4 µg dose of NPY (Figure 12). The detailed analysis of EEG power spectrum in the first three hours revealed significant decreases in the NREMS power spectrum in the 0.5-4.5 Hz frequency band; wake and REMS EEG were not affected (Attachment 4, Figure 3). There was no significant change in food intake in response to 0.4 µg NPY (Attachment 4, Figure 4).

Administration of 2 μg NPY induced significant increase in wakefulness and decrease in both NREMS and REMS. In first hour after injection NREMS decreased from a baseline of 26.6 ± 2.2 min to 12.6 ± 2.3 min after NPY treatment. REMS virtually disappeared in hour 1 after NPY injection. The reduced time spent in sleep may have resulted from a significant decrease in the average duration of NREMS episodes and a significant decrease in the number of REMS episodes (Table 1). There was a tendency toward decreased NREMS episode number, but statistical analyses did not show significant differences. The EEG SWA was not altered by 2 μg NPY. Detailed analysis of the EEG showed a significant increase in EEG power spectrum during wake and REMS (Attachment 4, Figure 3). Food intake in the first hour after NPY injection increased significantly compared to baseline (Attachment 4, Figure 4).

Table 1. The total number of NREMS and REMS episodes and the average NREMS and REMS episode durations in the first hour after icv and LH administration of NPY and isotonic NaCl injections. NPY: neuropeptide Y, NREMS: non-rapid-eye-movement sleep, REMS: rapid-eye-movement sleep, LH: lateral hypothalamus. When, due to the small number of REMS episodes, average episode duration could not be calculated in a statistically sound way, data are not shown (N/A: not available). *: significant difference between baseline and treatment, $p < 0.05$.

	NREMS Episode Number	Average NREMS Episode Duration (min)	REMS Episode Number	Average REMS Episode Duration (min)
Baseline	6.8 ± 0.8	4.4 ± 0.5	0.6 ± 0.3	1.6 ± 0.2
0.4 μg NPY	8.4 ± 1.1	4.4 ± 0.6	1.3 ± 0.4	1.9 ± 0.4
Baseline	7.2 ± 0.7	4.0 ± 0.5	1.5 ± 0.4	2.3 ± 0.3
2 μg NPY	5.2 ± 1.1	2.5 ± 0.4*	0.1 ± 0.1*	N/A
Baseline	8.1 ± 0.6	3.1 ± 0.3	0.4 ± 0.3	N/A
10 μg NPY	4.4 ± 0.9 *	2.6 ± 0.8	0.0 ± 0.0	N/A
Baseline	6.9 ± 0.9	4.5 ± 0.8	0.5 ± 0.3	N/A
LH 2 μg NPY	3.8 ± 1.3 *	2.4 ± 0.7	0.2 ± 0.2	N/A

Similarly to the middle dose of NPY, 10 µg injection of NPY was followed by a significant increase in wakefulness and decrease in both NREMS and REMS amount (Attachment 4, Table 1). Post hoc analyses showed significant suppression in NREMS in hour 1. The NREMS decrease may be due to the significant decrease in the number of NREMS episodes; the changes in average NREMS episode duration were not significant (Table 1). In hour 1, on the baseline day, rats had already minimal amount of REMS, and on the NPY day they had no REMS at all. Injection of 10 µg NPY did not change the EEG SWA. The EEG power spectra did not showed any significant difference in any vigilance state (Attachment 4, Figure 3). The highest dose of NPY significantly increased the food intake in the first hour after injection (Attachment 4, Figure 4).

Experiment 4/b. The effects of LH administration of NPY on sleep-wake activity and food intake in rats (Figure 13).

The effects of NPY microinjection into the LH on sleep-wake activity and feeding were similar to those observed after icv treatment. Wakefulness was significantly elevated in the first hour after the injections. The amounts of NREMS and REMS were significantly decreased (Attachment 4, Table 1). NREMS episode number significantly decreased in hour 1 and there was a tendency toward decrease in average NREMS episode duration, as well (Table 1). EEG SWA increased in response to the injection starting from hour 3, however, post hoc analyses did not show significance in any particular hour. Food intake was significantly enhanced by LH injection of NPY (Attachment 4, Figure 4).

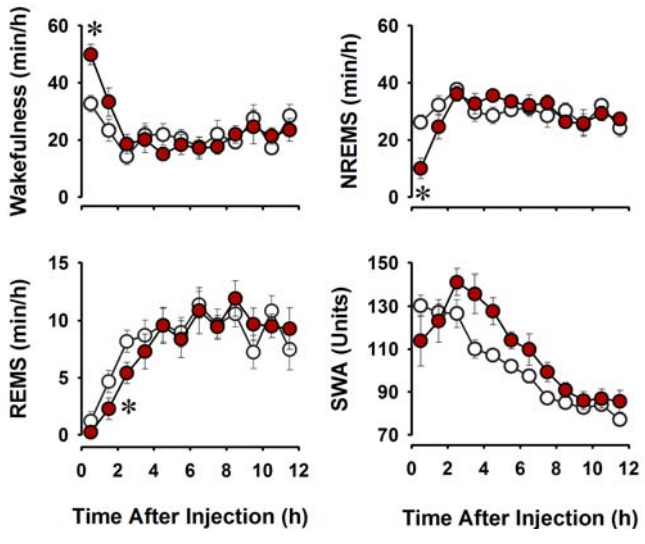

Figure 13. The effects of 2 µg NPY microinjection into the LH on wakefulness, NREMS, REMS and EEG SWA in rats. See legends to Figure 12 for details. Modified from Szentirmai and Krueger, 2006, Figure 2.

DISCUSSION

The major findings of the series of experiments presented here are the following.

1) Plasma ghrelin and leptin levels and hypothalamic ghrelin content display marked diurnal rhythm associated with feeding and sleep-wake activity. In free feeding rats, plasma and hypothalamic ghrelin contents reach their highest levels before the onset of the dark phase preceding the peak in feeding activity. During the dark, ghrelin levels gradually decrease and stay low for the rest of the dark and beginning of the light period. Diurnal rhythm of plasma leptin shows opposite pattern of ghrelin's.

2) Food restriction to the light period reverses REMS, plasma ghrelin and leptin rhythms and sleep deprivation increases plasma and hypothalamic ghrelin levels.

3) Intracerebroventricular injection of ghrelin induces dose-dependent and immediate increases in wakefulness, food intake and feeding behavior, with the concomitant suppression of NREMS and REMS.

4) Microinjections of ghrelin into the LH, MPA and PVN mimic the effects of the icv ghrelin treatment. Intra-LH microinjections have the most robust and long-lasting effect among the hypothalamic nuclei tested.

5) The effects of icv and intra-LH injections of NPY on sleep are similar to those of ghrelin's.

Our findings are in agreement with previous studies reporting that icv and microinjections of ghrelin into the LH, MPA and PVN increase food intake (Kojima et al., 1999, Wren et al., 2001). In our experiments, rats started to eat quickly after ghrelin injection and continued to eat throughout the first hour after injection. The feeding-inducing activity of ghrelin was sufficiently strong that it was able to stimulate food intake at the beginning of the light period when rats are usually satiated and sleep pressure is the highest.

Our studies are the first to test the effects of centrally administered ghrelin on vigilance. Icv and hypothalamic microinjections of ghrelin at light onset induced consistent, robust and dose-dependent increases in wakefulness and suppression of NREMS and REMS in rats. This is in agreement with the hypothesis that ghrelin may serve as a signaling molecule in central arousal systems. Previously, the effects of systemically administered ghrelin were tested on sleep. In rats, iv injection of ghrelin during the light period suppresses NREMS and REMS (Tolle et al., 2003). These effects are similar to those we observed after

central injections. In humans, the effects of systemically administered ghrelin are less clear; increased and decreased sleep and no effect on sleep have been reported. In young healthy male subjects, iv bolus injections of ghrelin in the late evening increase slow-wave sleep during the first part and decrease REMS in the middle of the night (Weikel et al., 2003). The increase in slow-wave sleep is associated with an increase in the power of delta wave activity. No effects of ghrelin on sleep in young men were observed when it was injected in the early morning (Kluge et al., 2007a). In elderly men, ghrelin suppresses stage 1 sleep and REMS and increases stage 2 and slow-wave sleep (Kluge et al., 2010). Ghrelin does not have any effect on sleep in young and elderly women (Kluge et al, 2007b). MK-677, an orally active growth hormone secretagogue, increases the duration of stage 4 sleep by 50% and REMS by 20% in young male subjects and enhances REMS in elderly subjects after subchronic administration (Copinschi et al., 1997). A single iv bolus injection of growth-hormone-releasing peptide-2, given after the third REM period during the night, has no effect on sleep (Moreno-Reyes et al., 1998). Pulsatile administration of growth-hormone-releasing peptide-6 to young men induced a modest increase in stage 2 sleep, without any change in slow-wave sleep and SWA (Frieboes et al., 1999). Hexarelin, the most potent known agonist of the GHS-R in terms of growth hormone release, decreases stage 4 sleep and suppresses EEG delta power during NREMS, but does not affect REMS or sleep continuity (Frieboes et al., 2004). In one study, ip administration of ghrelin at dark onset increased NREMS in mice (Obal et al., 2003). The difference between our findings of consistent wake-promoting effects of ghrelin in rats and the reported somnogenic effects in humans and mice may reflect true species specificity in the effect or may be due to other differences in the experimental conditions, such as the route of administration or the timing of the treatment.

In any case, our findings that ghrelin induces wakefulness are consistent with previous studies showing prompt increases in wakefulness at the expense of both NREMS and REMS after intravenous injections of ghrelin in rats. When we injected ghrelin at light onset, the sleep suppressive effect was robust, sleep almost completely disappeared in the first hour of the light period in response to 1 μg ghrelin. Sleep decreases after dark onset injections were considerably attenuated, but still significant in the first 2-hour time block. The attenuated wakefulness-promoting activities are most likely due to a ceiling effect. Under baseline conditions, rats sleep minimal amounts right after dark onset; this can hardly be suppressed further by any experimental manipulations. Similar to our findings with icv

injections, systemic injection of ghrelin during the dark period to rats when sleep propensities are low, failed to suppress sleep further (Tolle et al., 2003).

Ghrelin-induced changes in sleep duration after icv injections had a biphasic pattern. The immediate effect is a prominent dose-dependent increase in wakefulness which is followed by increases in NREMS during hours 3-12 post-injection in response to 1 μg (given at light onset) and the 5 μg dose (given at dark onset) in freely-feeding rats. We posit that the primary effect of ghrelin is to stimulate wakefulness. Since the half-life of ghrelin is about 30 minutes (Tolle et al., 2003), it is unlikely that the delayed sleep increases are due to the presence of exogenous ghrelin in biologically relevant quantities. It is possible, that these increases in NREMS are secondary to sleep loss and/or increased eating in the first hour(s). Sleep loss leads to subsequent homeostatic increases in sleep (Borbély, 1982) while eating is known to elicit postprandial increases in sleep (Danguir and Nicolaidis, 1979). The finding, that the secondary increases in NREMS are absent in rats that were not allowed to eat after ghrelin treatment strongly suggests that increased feeding could be, at least in part, responsible for the delayed sleep responses.

REMS was also altered by icv injection of ghrelin. Higher doses of ghrelin given light onset or dark onset induced a prominent decrease in REMS immediately after injections in agreement with previous observations reporting suppressed REMS after ghrelin treatment. During the day, injection of 1 μg ghrelin suppressed REMS in the first two hours in freely fed rats, whereas in food restricted animals the same treatment decreased REMS during the second part of the light period. A possible explanation for the lack of immediate REMS effects of ghrelin in food restricted rats is that there was already a tendency towards decreased REMS on the baseline day in this group and it is likely that ghrelin could not elicit further decreases in REMS. The mechanisms responsible for the long-lasting REMS suppressions in the second half of the recording period in feeding restricted rats remain to be clarified. It is possible that the REMS changes are related to changes in body temperature that accompany fasting, since REMS is sensitive to changes in body temperature (Kent et al., 1988).

The mechanism of the wakefulness-promoting effects of ghrelin in rats is unknown. We directly addressed one possibility in the present study. Since rats cannot eat and sleep at the same time, increased eating activity after ghrelin treatment could be responsible for

increases in wakefulness in the first two hours. The wake-promoting effects of ghrelin, however, did not disappear in rats which had no access to food. This strongly suggests that the wake-promoting effects of ghrelin are not simply due to the stimulation of the eating behavior but other activating mechanisms are also involved. Ghrelin's food intake-promoting effect is mediated by a central action involving primarily the NPY-signaling pathway in the ARC of the hypothalamus (Kohno et al., 2003, Seoane et al., 2003). We hypothesized that ghrelin's wakefulness-promoting effect is also mediated by central mechanisms.

The increased sensitivity/high responsiveness of hypothalamic ghrelin levels to sleep deprivation supports this notion. Ghrelin contents of the hypothalamus were significantly increased during the extended period of forced wakefulness and dropped below normal levels during recovery sleep. Plasma ghrelin level was also elevated during sleep deprivation and returned to normal during the rebound sleep. A strong stimulus for ghrelin secretion is fasting (Kojima et al., 1999). In our sleep deprivation experiments, however, the animals were not fasted; feeding activity was even increased during the sleep deprivation period.

In response to restricted feeding, rhythm of plasma leptin levels shifted but remained coupled to the feeding activity. Similar to our finding, restricted food availability to 4 hours of the light period resulted in the abolishment of the nocturnal leptin peak and led to the appearance of a diurnal leptin secretion 4 hours after the initiation of feeding in the rat (Xu et al., 1999). Further, a 12-hour time zone shift (reversal of day/night) also reversed the timing of the peaks and minimum values of plasma leptin in humans (Schoeller et al., 1997). Our results with restricted feeding suggest that the diurnal rhythm of ghrelin secretion is as strongly coupled to feeding as the diurnal rhythm of leptin.

The light-dark reversal in the diurnal rhythms of ghrelin and leptin by restricted feeding is mirrored by similar fundamental changes in the diurnal distribution of REMS. Comparable changes in REMS were previously reported (Roky et al., 1999). The mechanism through which restricted feeding modulates REMS is not clear, but it has been suggested that changes in REMS might be related to body temperature, another parameter markedly influenced by restricted feeding. Restricted feeding had less impact on NREMS, but the circadian amplitude of NREMS distribution was attenuated. It is possible that NREMS time decreased in the light phase due to increased eating behavior, and this loss was compensated with more sleep at night.

GHS-R1a is the only known receptor for ghrelin. Ghrelin receptors have been found in various hypothalamic structures implicated in sleep-wake regulation. For example, GHS-R1a mRNA, detected by in situ hybridization and RNase protection assays, is found in several hypothalamic nuclei, including the anteroventral preoptic nucleus, anterior hypothalamic area, SCN, anterolateral hypothalamic nucleus, ARC, PVN, and TMN (Guan et al., 1997, Mitchell et al., 2001). To test possible hypothalamic sites that may mediate ghrelin's wake-promoting action we characterized sleep and food intake after intrahypothalamic ghrelin microinjections.

Ghrelin-induced increase in wakefulness was most marked when the peptide was microinjected into the LH. The effects of icv injection of 1 μg ghrelin were similar to those seen after LH microinjection of 0.2 μg; increasing the icv dose to 5 μg did not result in a further enhancement of the wake-promoting activity. A similar ceiling-like phenomenon is seen after LH microinjection. The classic view that the LH is a "wake-center" originates from von Economo's observation of excessive sleepiness in patients with the damage of this region (Von Economo, 1930). In the last decade this view gained a new momentum with the discovery of the orexinergic system. Orexinergic mechanisms play a central role in the maintenance of wakefulness. Orexin-producing neurons are located in the LH and diffusely project to the cerebral cortex as well as they innervate forebrain and brainstem structures that are implicated in arousal (Peyron et al., 1998). Orexins stimulate wakefulness when injected icv or into the lateral POA, PVN, TMN or locus ceruleus (Bourgin et al., 2000, Huang et al., 2001, Methippara et al., 2000, Sato-Suzuki et al., 2002). Orexinergic neurons discharge during active wakefulness and they are silent in slow-wave sleep (Lee et al., 2005). Narcolepsy is linked to the lack of orexin and/or orexin receptors (Chemelli et al., 1999, Lin et al, 1999). There is a close relationship between ghrelin- and orexin-producing cells in the LH (Figure 14). Neurons in the LH express GHS-R1a and ghrelin fibers make direct synaptic contact with orexin-producing neurons in this area (Mitchell et al., 2001, Toshinai et al., 2003). A growing body of evidence indicates that ghrelin, in fact, stimulates orexinergic neurons in the LH. Icv or local application of ghrelin into the LH of rats induces feeding (Lawrence et al., 2002, Olszewski et al., 2003b, Toshinai et al., 2003, Yamanaka et al., 2000). Orexinergic mechanisms may contribute to the feeding effects of ghrelin since pretreatment with orexin antibodies attenuates ghrelin-induced eating and the effect of ghrelin was significantly reduced in orexin-deficient mice (Toshinai et al., 2003). We hypothesize that

the wake-promoting effects of ghrelin in the LH may also involve the activation of orexinergic mechanisms.

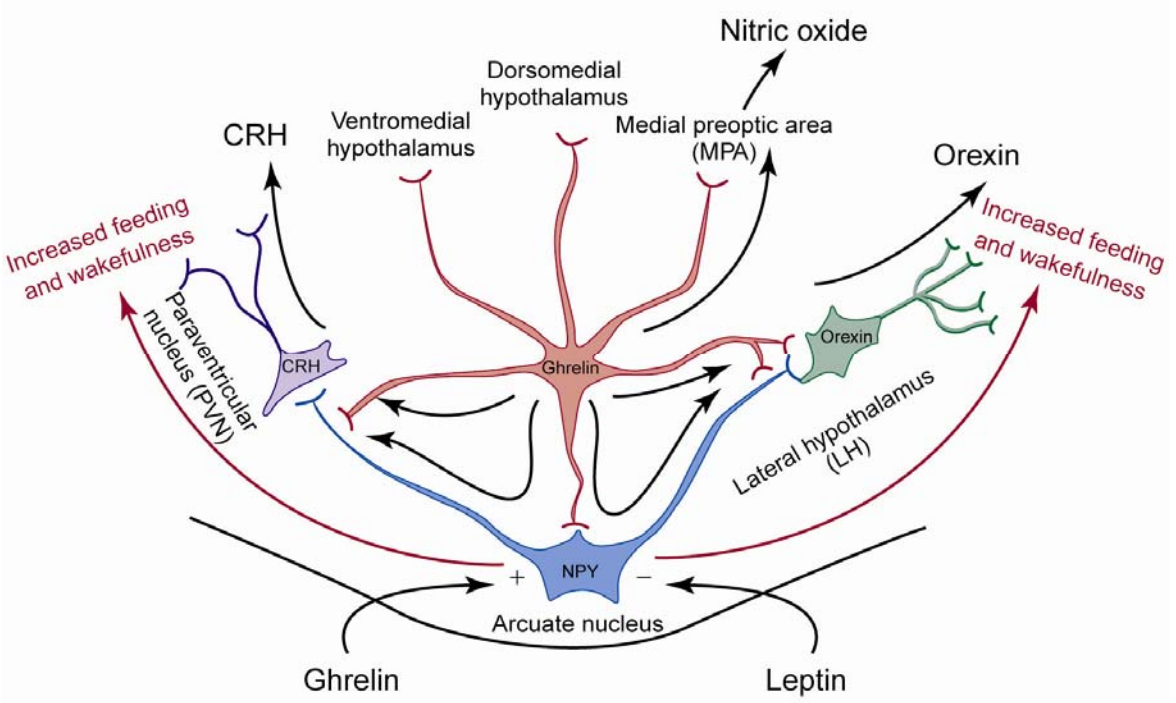

Figure 14. Schematic drawing of the hypothalamic ghrelin-NPY–orexin circuit with possible pathways mediating the wakefulness-promoting effects of ghrelin and NPY. Hypothalamic ghrelin-, orexin-, and NPYergic neurons form a well-characterized circuit that is implicated in the regulation of food intake and sleep. Circulating ghrelin and leptin can modulate the activity of the circuit through NPY in the arcuate nucleus. Ghrelin promotes wakefulness when injected into the LH, PVN, or MPA. Ghrelin administration and ghrelinergic neurons activate orexinergic cells in the LH and NPY-containing cells in the ARC. In the PVN, ghrelin facilitates CRH release indirectly through the stimulation of NPY-ergic neurons. Ghrelin microinjection into the LH may promote wakefulness through the stimulation of orexin release. In the PVN, ghrelin indirectly facilitates CRH release that leads to increased wakefulness. In the MPA, ghrelin's wakefulness-promoting effect might be mediated by nitric oxide (NO). Several actions of ghrelin are NO-dependent, and NO-ergic mechanisms are implicated in sleep regulation.

Ghrelin injection into the MPA also increased the amount of wakefulness. The importance of the MPA in the hypothalamic sleep-regulating system is well-documented. Lesion of the preoptic area suppresses sleep (McGinty and Sterman, 1968), electrical or thermal stimulation of the MPA increases sleep and sleep deprivation induces c-fos expression in the MPA (Roberts and Robinson, 1969). Intra-MPA microinjection of

adenosine agonist (Ticho and Radulovacki, 1991), tumor necrosis factor alpha (Kubota et al., 2002) and growth hormone-releasing hormone (Zhang et al., 1999) enhances sleep, while prostaglandin E2 (Matsumura et al., 1988) and octreotide (Hajdu et al., 2003) induce arousal. Similar to ghrelin, microinjection of orexin-A into the MPA increases time spent awake (Espana et al., 2001). GHS-R1a has been detected in the MPA. Feeding is increased after local ghrelin application to this area (Wren et al., 2001). The latter finding was confirmed in our present study. It is possible that ghrelin's wakefulness- and feeding-promoting effects are mediated through nitric oxide (NO) in the MPA (Figure 14). NO-producing mechanisms are implicated in the regulation of sleep (Kapás et al., 1994) and feeding (Morley et al., 1995). Microinjection of a NO-donor into the MPA increases arousal (Ribeiro and Kapás, 1999) and the feeding-stimulatory actions of ghrelin are NO-dependent (Gaskin et al., 2003).

The PVN plays important role in arousal, autonomic, and behavioral responses to stressors (Pfaff et al., 2005). It has reciprocal connections with arousal centers, such as LC and raphe nuclei (Cederbaum et al., 1978). Lesion of the PVN decreases REMS sleep and abolishes the circadian sleep-waking cycles (Piepenbrock et al., 1985). Microinjections of orexin or histamine into the PVN elicit arousal responses (Sato-Suzuki et al., 2002). The PVN is also one of the major targets for ghrelin to induce feeding; injections of ghrelin induce c-fos expression in the PVN and stimulate eating (Olszewski et al., 2003a, Wren et al., 2001). Similar to prior findings, we did not observe a clear dose-response relationship on feeding after PVN injections of ghrelin. Ghrelin injection into the PVN also enhanced wakefulness and suppressed NREMS, but this region appears to be the least sensitive among the three sites for sleep effects. Ghrelin did not reduce REMS, which may be due to the already short REMS on the baseline day at the beginning of the light period. It is possible that ghrelin's actions in the PVN are mediated, in part, through the activation of the HPA axis. Ghrelin facilitates CRH-release in the PVN through stimulating GABA-release from NPY neurons (Cowley et al., 2003) (Figure 14). CRH inhibits sleep (Ehlers et al., 1986). Recent findings suggest, that there is indeed a functional interaction in regulating digestive functions within the PVN between ghrelin and NPY and ghrelin and CRH. Intra-PVN injection of ghrelin stimulates colonic motor function, an effect that is inhibited by local pretreatment with a NPY1- or CRH receptor antagonist. Activation of the PVN may also contribute to the wake-promoting effects of the intra LH-injected ghrelin since the LH-infusion of ghrelin activates neurons in the PVN as shown by enhanced c-Fos IR (Olszewski et al., 2003a).

Previous studies concerning NPY's sleep-modulating effect did not yield consistent findings. In one study, visual inspection of the EEG suggested that NPY induces a reduction in desynchronized EEG activity and, an increase in synchronized and mixed activity in rats (Zini et al., 1984). In rats, icv injection of NPY three hours after light onset failed to change the amount of time spent is slow-wave sleep (Ehlers et al., 1997). The differences in the results between previous studies and current one may be due to the different time of injection. In humans, repeated intravenous bolus injections of NPY during the dark period promoted NREMS and had no effect on sleep EEG spectra in normal young male subjects (Antonijevic et al., 2000). The same research group carried out a more recent study in older male and female patients with depression. NPY infusion caused the shortening of NREMS and REMS latencies, but did not affect the time spent in stage 2 sleep, slow wave sleep, REMS or total sleep time (Held et al., 2006). There was no significant difference in the responsiveness to NPY between the depressed and control groups. In our experiments, when NPY was injected at light onset, the sleep suppressive effects were robust; both NREMS and REMS significantly decreased in the first hour of the light period, REMS practically disappeared. The decrease in NREMS amount in the first hour after the injection is clearly reflected in the decreased total number of NREMS episodes; nevertheless, there was a tendency towards decreased average duration of NREMS episodes, as well. After NPY injection REMS completely disappeared in the first hour of the light period, however the amount of REMS on the baseline day was also relatively low.

The mechanism through which NPY promotes wakefulness is unknown. NPY-immunoreactive cell bodies are present in the ARC, PVN, SCN, DMH and LH; nuclei implicated in feeding and sleep-wake regulation. NPY Y1 and Y5 receptors, which are mainly involved in the food intake stimulatory activity of NPY, are also present in these hypothalamic nuclei (Wolak et al., 2003). NPY-containing axon terminals innervate orexinergic neurons in the LH. Icv injection of NPY increases c-fos immunoreactivity in the ARC, PVN and in the orexinergic neurons of the LH (Li et al., 1994). It is also possible that NPY's stimulatory action on orexinergic cells in the LH mediates the wake-promoting effect of NPY. This notion is supported by our observation that NREMS decreased in response to LH injection of NPY. A reciprocal relationship exists between NPY and orexinergic neurons. Icv administration of orexins stimulates NPY expression in the ARC (Lopez et al., 2002). Orexinergic neuron terminals originating from the LH form synapses on NPY-immunoreactive cells in the ARC and also have close contact with NPYergic cells in the

PVN (Horvath et al., 1999). Orexin receptor immunoreactivity is present on NPY neurons in the ARC (Backberg et al., 2002). The feeding stimulatory effect of orexin may be mediated, at least partly, by NPY, since orexin-induced feeding is inhibited by pretreatment with NPY receptor antagonists (Yamanaka et al., 2000). Conversely, orexin antiserum significantly attenuates the feeding response to NPY (Niimi et al., 2001). In addition to orexinergic neurons, NPY activates other mechanisms in the hypothalamus known to be involved in promoting arousal. For example, NPY stimulates CRH release and increases CRH gene expression in the PVN (Suda et al., 1993). CRH is known to inhibit sleep (Ehlers et al., 1986). Therefore, CRH is another candidate for mediating the wakefulness stimulating effect of NPY.

CONCLUSIONS AND PERSPECTIVES

In summary, the sleep-suppressing and food-intake-promoting activities of central NPY, ghrelin, and orexin in rats are strikingly similar (Figure 15). The first hours of the dark, behaviorally active, period in rats are characterized by increased time spent awake, increased duration of the individual wake episodes, and increased eating activity. We named this behavioral pattern "dark onset syndrome". Central administration of ghrelin or NPY elicits all components of the dark onset syndrome. We posit that the increased feeding activity and the stimulation of wakefulness are two parallel outputs of the activation of the same hypothalamic orexin-ghrelin- NPY circuit.

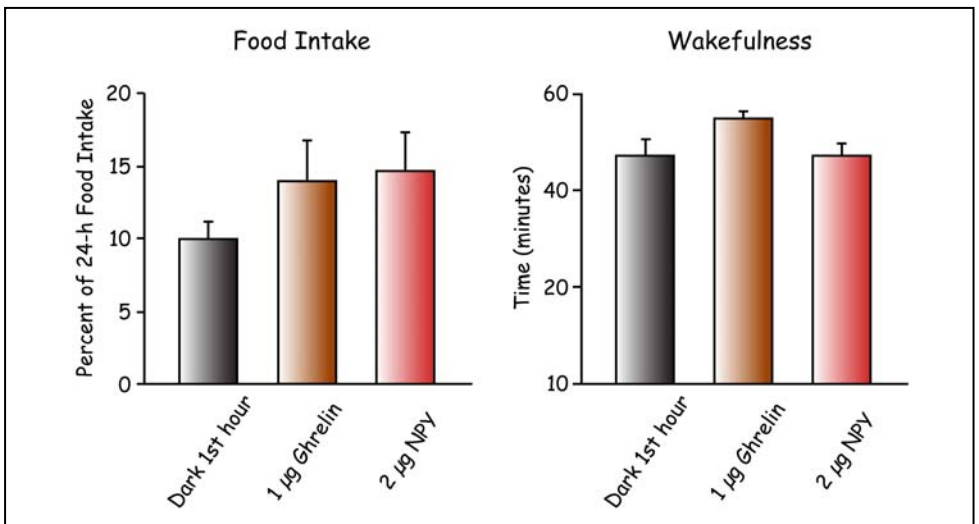

Figure 15. Food intake and the amount of wakefulness during the first hour of the dark phase under normal conditions and in the first hour of light after icv administration of ghrelin and NPY in rats. Central injections of ghrelin and NPY stimulates eating and wakefulness in the light period. These changes are characteristic of and spontaneously occurring during the first hour of the dark phase.

Food intake occurs at environmentally advantageous times and in response to homeostatic needs. The concept that the hypothalamus plays a crucial role in the maintenance of energy balance was founded by seminal works showing that electrolytic lesion of the VMH causes marked hyperphagia and obesity in rats whereas a lesion in the LH produces the opposite. The use of genetically modified animals and the development of modern molecular biological techniques have expanded our knowledge about the mechanisms that regulate food intake and energy balance. However, relatively little attention has been given to a major influence on energy balance, to the temporal organization of feeding throughout the daily cycle. While feeding and sleep are mutually exclusive behaviors, wakefulness, with increased sensory awareness and motor activity, is a prerequisite for successful feeding. The diurnal distribution of wakefulness and feeding are highly species dependent. Humans consolidate waking and feeding cycles during the daytime. Nocturnal rodents, such as rats and mice, are awake and feed primarily at night (reviewed in Strubbe and Van Dijk, 2002). From an evolutionary perspective, during shortages of food availability, central mechanisms promoting wakefulness, therefore feeding opportunities during the appropriate circadian phase are crucial for survival. Mounting evidence supports the idea that mechanisms responsible for feeding behavior and the control of sleep-wake activity are coordinated by partly overlapping hypothalamic neuronal systems. These systems integrate information about the energy status of the body through hunger, adiposity, and satiety signals and metabolic and neural signals. We hypothesize that the hypothalamic ghrelin – NPY - orexin circuit is a major the integrative center (Figure 16). It receives and integrates metabolic, circadian, and visual signals. The activation of the circuit has two main parallel outputs: increased wakefulness and increased feeding activity.

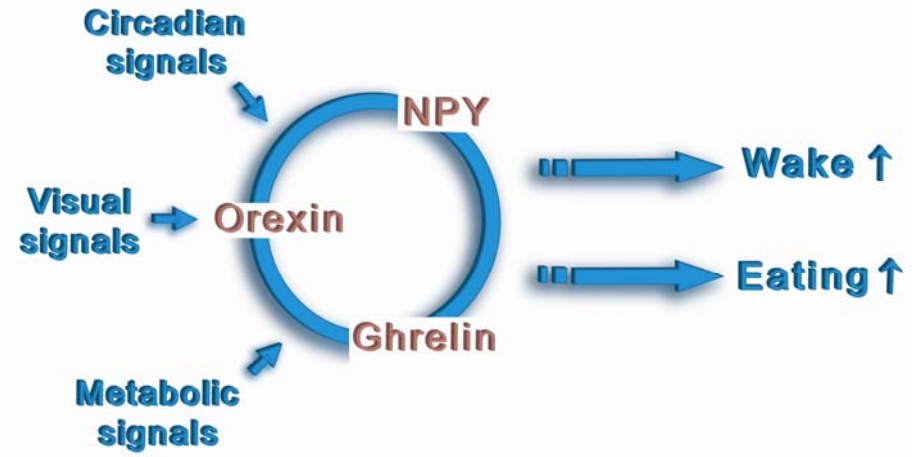

Figure 16. Schematic drawing of the inputs to and outputs of the hypothalamic network formed by ghrelin, orexin and NPY neurons.

Studying the interaction between sleep, feeding and energy balance is a relatively novel but timely direction in sleep research. Because of the co-dependency between sleep-wakefulness and feeding, stressors that primarily affect one very often affect the other. Indeed, disturbances of sleep, appetite, and metabolism are well-described symptoms of obesity, metabolic syndrome, anorexia nervosa and depression (Van Cauter et al., 2007).

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ATTACHMENT

I.

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Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation

B. Bodosi,[1] J. Gardi,[2] I. Hajdu,[1] E. Szentirmai,[1] F. Obal, Jr.,[1] and J. M. Krueger[3]

[1]*Department of Physiology and* [2]*Endocrine Unit, University of Szeged, A. Szent-Györgyi Medical Center, 6720 Szeged, Hungary; and* [3]*Department of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, Washington 99164-6520*

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Bodosi, B., J. Gardi, I. Hajdu, E. Szentirmai, F. Obal, Jr., and J. M. Krueger. Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am J Physiol Regul Integr Comp Physiol* 287: R1071–R1079, 2004; doi:10.1152/ajpregu.00294.2004.—To determine the relationships among plasma ghrelin and leptin concentrations and hypothalamic ghrelin contents, and sleep, cortical brain temperature (T_{crt}), and feeding, we determined these parameters in rats in three experimental conditions: in free-feeding rats with normal diurnal rhythms, in rats with feeding restricted to the 12-h light period (RF), and in rats subjected to 5-h of sleep deprivation (SD) at the beginning of the light cycle. Plasma ghrelin and leptin displayed diurnal rhythms with the ghrelin peak preceding and the leptin peak following the major daily feeding peak in *hour 1* after dark onset. RF reversed the diurnal rhythm of these hormones and the rhythm of rapid-eye-movement sleep (REMS) and significantly altered the rhythm of T_{crt}. In contrast, the duration and intensity of non-REMS (NREMS) were hardly responsive to RF. SD failed to change leptin concentrations, but it promptly stimulated plasma ghrelin and induced eating. SD elicited biphasic variations in the hypothalamic ghrelin contents. SD increased plasma corticosterone, but corticosterone did not seem to influence either leptin or ghrelin. The results suggest a strong relationship between feeding and the diurnal rhythm of leptin and that feeding also fundamentally modulates the diurnal rhythm of ghrelin. The variations in hypothalamic ghrelin contents might be associated with sleep-wake activity in rats, but, unlike the previous observations in humans, obvious links could not be detected between sleep and the diurnal rhythms of plasma concentrations of either ghrelin or leptin in the rat.

hypothalamic ghrelin; corticosterone; brain temperature; electroencephalogram delta power; sleep duration

GHRELIN AND LEPTIN are humoral feedback signals from the periphery to the hypothalamic neuronal network regulating energy homeostasis. Neuropeptide Y (NPY)-containing neurons in the arcuate nucleus are major targets of both hormones (reviewed in Ref. 24). Ghrelin and growth hormone (GH) secretagogues (GHSs), which are synthetic ligands of the GHS receptor that bind ghrelin, stimulate these neurons (14, 27), promote NPY expression (45, 47) and release (59), and elicit food intake (47, 60). Leptin inhibits NPY neurons (27) and NPY expression (2, 47, 56) and suppresses feeding (47, 56). Ghrelin promotes deposition of fat (55), whereas leptin stimulates energy expenditure (22) mediated by sympathetic activity-induced stimulation of uncoupling proteins in brown fat tissue and perhaps elsewhere (reviewed in Ref. 50). Leptin is released from white adipose tissue as a function of insulin-dependent glucose metabolism in fat cells (reviewed in Ref. 20). Ghrelin is produced predominantly by endocrine cells in the oxyntic gastric mucosa (12, 28). Ghrelin is also a neurotransmitter in the hypothalamic arcuate nucleus (28, 31).

Both leptin (53, 57) and ghrelin (and GHSs) (13, 46, 60) stimulate the somatotropic system. This ghrelin or GHS action requires intact functioning of the hypothalamic growth hormone (GH)-releasing hormone (GHRH) system (32, 38, 40, 52). Ghrelin/GHSs stimulate GHRH neurons (14) and elicit GHRH release (59). GHRH is also involved in the mediation of leptin's action on GH secretion. Leptin enhances GHRH mRNA expression (9) and elicits GHRH release (57). Several lines of evidence demonstrate that GHRH is part of a hypothalamic network promoting physiological non-rapid eye movement sleep (NREMS) (39). Through GHRH, ghrelin and leptin may modulate sleep. In fact, both leptin and ghrelin have been linked to sleep regulation. For example, systemic leptin decreases rapid eye movement sleep (REMS) and stimulates deep NREMS in rats (49). Two GHSs (GHRP-6 and MK-677) are reported to promote sleep (10, 18) and one (GHRP-2) was inactive (33) in human subjects. Ghrelin itself also enhances NREMS in humans (58) and mice (38) although it increased wakefulness in rats due to stimulation of feeding (54). Another line of experiments implicating ghrelin and leptin in sleep regulation suggests that secretions of ghrelin and leptin are in part related to sleep because sleep deprivation (SD) alters plasma concentrations of these hormones (16, 35), or shifts in the sleep period are followed by shifts in hormone secretions (48). These observations were all obtained in human subjects.

The aim of our experiments was to study whether variations in plasma leptin and ghrelin concentrations and hypothalamic ghrelin contents are related to sleep-wake activity in the rat. First, diurnal rhythms of ghrelin and leptin were determined in rats with normal diurnal feeding (free feeding) and sleep-wake rhythms. Then, hormone rhythms and sleep-wake rhythms were compared in rats on a restricted feeding (RF) schedule when food was available only during the 12-h light period. It was hypothesized that the normal relationship between sleep and hormone secretions is maintained in the RF condition if there are links between these events. Finally, SD was used to disturb sleep, and the changes in plasma hormone concentra-

Address for reprint requests and other correspondence: J. M. Krueger, Dept. of VCAPP, Washington State Univ., Pullman, WA 99164-6520 (E-mail: krueger@vetmed.wsu.edu).

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tions and hypothalamic ghrelin were studied during SD and during recovery after SD.

METHODS

Animals. Male Sprague-Dawley rats weighing 300–330 g were used. There were 47–55 rats in each of the three experiments. Institutional guidelines for the care and use of research animals were followed and approved by the institutional committees. The experiments are consistent with the "Guiding Principles for Research Involving Animals and Human Beings" issued by the American Physiological Society (4).

Surgery. The surgeries were carried out under ketamine-xylazine (87 and 13 mg/kg, respectively) anesthesia. Each animal was implanted with stainless steel screws as electrodes for electroencephalographic (EEG) recording over the frontal and parietal cortexes and the cerebellum. A thermistor placed over the parietal cortex was used to record changes in cortical brain temperature (T_{crt}).

Sleep-wake recording. The rats were housed in individual Plexiglas cages. The cages were placed in recording chambers with a 12:12-h light-dark cycle and with an ambient temperature regulated at 26°C. Water was continuously available. The rats were kept in conditions identical to those in the recording chambers for at least 1 mo before the operation. After surgery, the rats were provided at least 10 days of recovery during which they 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. The digitized (64-Hz sampling rate) signals of the EEG, T_{crt}, and motor activity were collected by computers in an adjacent room. The states of vigilance were scored in 8-s epochs from signals restored on the computer screen. Power density values were calculated by fast-Fourier transformation for consecutive 8-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 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 (0.5–4.0 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, and 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- to 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.

Recording of feeding activity. Food pellets were placed in vertical aluminum tubes hanging in one corner of the cage; stainless steel bars kept the pellet in the opening of the tube. Movements of the tube generated potentials in electromagnetic transducers, and these potentials were recorded together with the EEG signals, T_{crt}, and motor activity. The records were subjected to spectral analysis, and the total power in 8-s epochs was regarded as feeding activity. Feeding activity was integrated for 1-h periods. To standardize the different values obtained in the various feeders, the total feeding activity in 24 h was taken as 100%, and the percent fraction of feeding activity was calculated for each recording hour. It is noted that this method of determination of feeding activity does not measure the quantity of food consumed, and it also misses food spillage and eating from the cage floor. However, the reproducibility of feeding activity was high in the three experiments, indicating that the method was suitable for the aims of these studies.

Experimental protocol. Sleep-wake activity, T_{crt}, and feeding activity were recorded for 3 days in each rat. On *day 4*, groups of rats (n = 7–12) were killed by means of a guillotine at 4-h intervals at the following time points: light period at 1, 5, and 9 h after light onset; dark period at 13, 17, and 21 h after light onset, i.e., 1, 5, and 9 h after dark onset. The trunk blood was collected and immediately centrifuged, and the plasma was stored at −80°C until the hormone assays. The hypothalamus was harvested by using the following landmarks, i.e., frontal edge of the optic chiasm, lateral sulci, caudal edge of the mammary bodies, and a depth of 2 mm, and was also stored at −80° until used. The mean (±SE) mass of the hypothalamus was 42.0 ± 0.81 mg without significant differences among the groups.

In *experiment 1* with free-feeding rats, the access to the feeding tubes was continuous. In *experiment 2* with RF, the feeders were removed at dark onset and returned at light onset. These rats were on RF for 3 wk before recording. Rats in an identical experiment learned to adapt in 1 wk to RF so that the daily amount of food and their body weight remained normal (43). The amounts of daily-consumed food were determined in eight free-feeding rats (26.4 ± 0.73 g) and in eight RF rats (24.6 ± 0.59 g) without significant differences between them (Student's *t*-test) in our experiments. In *experiment 3*, the rats were sleep deprived by means of gentle handling. The rats stayed in their home cage, and whenever behavioral or EEG signs of sleep were observed, they were aroused by knocking on the cage or lightly touching them. The feeding tubes were continuously available. The SD started at light onset and lasted for 5 h. Because the groups of rats were killed at 4-h intervals, feeding activity could not be standardized as described above for 24-h on *day 4*. Feeding values were obtained on *day 4* in all rats until the time of their death. To make the percent distribution feeding calculation, a 24-h value is needed. To obtain those values for *day 4* in those rats killed before the end of *day 4*, the mean hourly feeding activity for the previous 3 recording days for each rat were used for the missing hours (after death). The calculation provided the fraction of feeding activity during and after SD assuming that the total 24-h feeding activity remained unaltered. The last group of rats was killed in *hour 21*, and the pattern of feeding activity in this group supported that this calculation was correct.

Hormone measurements. Commercially available radioimmunoassay kits were used to determine ghrelin (Phoenix Pharmaceuticals, Belmont, CA) and leptin (LINCO Research, St. Charles, MO), whereas corticosterone was measured by means of enzyme immunoassay (Diagnostic Systems Laboratories, Webster, TX). According to the supplier, the antiserum to rat ghrelin displayed 100% cross-reactivity with human and canine ghrelin, human and rat ghrelin-(des-octanoyl-Ser3), and ghrelin-(17–28) (human, rat). There was a 3% cross-reaction between the antiserum and the COOH-terminal ghrelin hexapeptide (human, rat). The antiserum to ghrelin did not recognize some other ghrelin fragments, human secretin, vasoactive intestinal peptide (human, rat, porcine), human and rat prolactin-releasing peptide-31, human and rat galanin, human and rat GHRH, NPY (human, rat), orexin A (human, rat, mouse), and human orexin B. The antiserum to rat leptin does not recognize glucagon, somatostatin, and insulin. The antiserum to rat corticosterone displayed 5.4% cross-reactivity with dehydroxycorticosterone. The hormones were measured in duplicate or triplicate with a sensitivity of 4 pg/tube for ghrelin, 0.5 ng/ml for leptin, and 4 ng/ml for corticosterone. The intra- and interassay coefficients of variation were less than 8% and 14%, respectively.

For extraction of hypothalamic samples, the frozen samples were weighed and placed in tubes containing 0.5 ml 2 M acetic acid and then boiled for 5 min. The tissues were individually homogenized by means of ultrasound. The homogenates were centrifuged at 15,000 *g* for 20 min at 4°C. The supernatant was lyophilized for the ghrelin radioimmunoassay.

Statistics. Two-way ANOVA for repeated measures was used to compare sleep-wake activity, T_{crt}, and feeding among groups/experiments. Hormone concentrations were analyzed by means of one-way

ANOVA. When ANOVA indicated significant variations, the different group or hour was identified by means of the Student-Newman-Keuls test. Although each experiment included 47–55 rats, the sample sizes varied slightly in the individual tests because a few records and samples were lost or omitted for technical reasons. In particular, the sample sizes dropped significantly in the SD experiment because the repeated-measures ANOVA on *day 4* accepted only the last survivor group of rats. Student *t*-test was used to compare mean values in the light and dark periods. An α-level of $P < 0.05$ was considered to be significant in all tests.

RESULTS

Experiment 1: normal rhythm in free-feeding rats. Free-feeding rats ate mostly at night; only a small fraction of the total daily feeding activity was observed in the light period (Table 1, Fig. 1). Dark onset was rapidly followed by vigorous eating, and 13.9 ± 1.24% of the daily feeding activity occurred during the first hour of the dark period. Feeding activity bouts recurred throughout the night.

The diurnal rhythm of sleep-wake activity displayed the normal patterns of rats (Fig. 1, Table 1). NREMS peaked at the beginning of the light cycle. Thereafter it declined steadily toward lights off. NREMS time dropped markedly at dark onset and stayed low during the dark period. As characterized by EEG delta power during NREMS, maximum NREMS intensity occurred at the beginning of the rest period. Delta power then decreased throughout the day. In the dark period, the depth of consecutive sleep bouts increased and delta power during NREMS gradually increased to reach the subsequent morning peak. REMS also displayed a diurnal rhythm with high and low amounts during the light and the dark period, respectively. REMS time was maximum in the second portion of the light period.

Table 1. *Mean (± SE) values of non-REM and REM sleep time, cortical brain temperature, feeding activity, plasma leptin and ghrelin concentrations, and hypothalamic ghrelin contents in 24-h, in the 12-h light period, and in the 12-h dark period in rats free-feeding on normal diurnal rhythm and in rats with food restricted to the 12-h light period*

	Free Feeding	Restricted Feeding
NREMS, %Rec time		
Total	43.5±0.42	43.0±0.44
Light	61.4±0.54	50.3±0.62*
Dark	25.7±0.78	35.6±0.63*
REMS, %Rec time		
Total	8.4±0.19	8.9±0.15
Light	12.4±0.37	6.2±0.25*
Dark	4.5±0.27	11.5±0.31*
T_{crt}, °C		
24 h	37.6±0.08	37.7±0.08
Light	37.2±0.09	37.6±0.09*
Dark	38.1±0.08	37.7±0.09*
Feeding Activity, %24 h		
Light	13.5±1.67	100
Dark	86.5±1.62	0
Leptin, ng/ml		
24 h	3.5±0.24	5.3±0.47*
Light	2.6±0.15	6.6±0.71*
Dark	4.6±0.38	3.7±0.33
Peak	6.2±0.91	7.8±0.72
Plasma ghrelin, pg/ml		
24 h	1,333.3±77.58	1,735.4±142.23*
Light	1,549.0±123.54	1,247.7±91.96*
Dark	1,099.6±64.60	2,365.3±252.96*
Peak	1,857.7±257.53	3,649.8±401.12*
Hypothalamic ghrelin, pg/mg		
24 h	1.04±0.027	1.10±0.018*
Light	1.01±0.038	1.07±0.020
Dark	1.07±0.038	1.15±0.029

Values are means ± SE. REM, rapid eye movement; REMS, REM sleep; NREMS, non-REM sleep; T_{crt}, cortical brain temperature; %Rec time, % recording time. * Significant difference (Student *t*-test) between free-feeding rats and rats on restricted feeding.

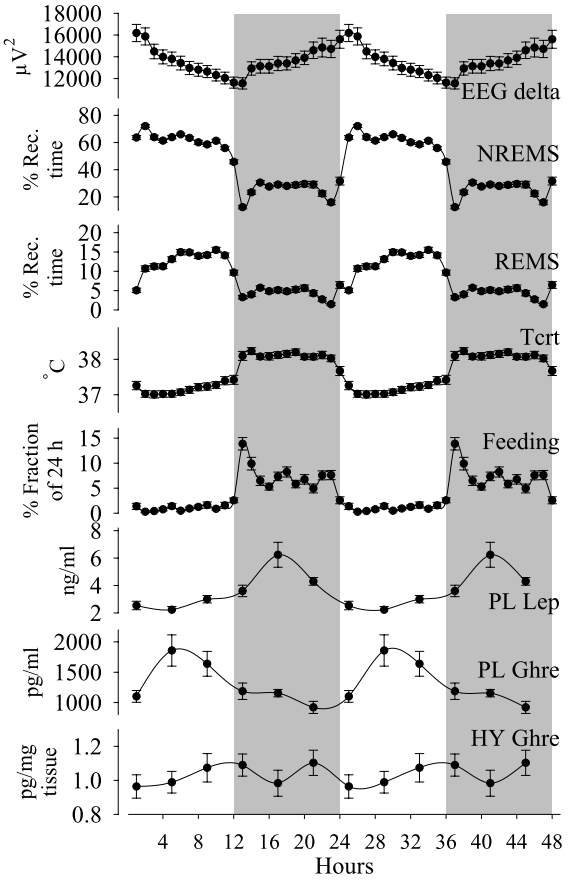

Fig. 1. Diurnal rhythms (hourly mean ± SE) of EEG delta power during non-rapid eye movement sleep (NREMS), time spent in NREMS and rapid eye movement sleep (REMS), cortical brain temperature (T_{crt}), and feeding activity were determined on 3 days and averaged for 24 h in 51 rats. Diurnal rhythms of plasma (PL) leptin (Lep) and ghrelin (Ghre) concentrations, and hypothalamic (HY) ghrelin contents were determined on *day 4* of recording in groups of rats (n = 7–10) killed at 4-h intervals. The 24-h curves are double-plotted to promote visualization of the rhythms. *Time 0*, light onset. Gray columns, dark period. %Rec time, %recording time; %Distrib/24 h, %distribution/24 h.

T_{crt} displayed 0.9°C difference between day and night [$F(23,1027) = 143.652$, $P < 0.001$] with the mean T_{crt} at night being significantly ($P < 0.001$) higher than during the light period (Fig. 1, Table 1).

Plasma leptin [$F(5,45) = 12.3$, $P < 0.0001$] and ghrelin [$F(5,44) = 5.05$, $P = 0.001$] displayed distinct diurnal rhythms with their peak values occurring at opposite times of the day (Fig. 1, Table 1). The leptin maximum followed the dark onset-elicited eating peak and occurred at 17 h (5 h after dark onset) at night. The ghrelin peak preceded the eating peak and occurred 5 h after light onset when leptin dropped to its diurnal trough value. Ghrelin was still relatively high in *hour 9* of the light period.

The ghrelin content of the hypothalamus was low (Table 1). Hypothalamic ghrelin showed modest oscillations with a rise after the plasma ghrelin peak and a second increase toward the end of the dark period (Fig. 1). However, these changes in hypothalamic ghrelin did not reach the level of statistical significance.

Plasma corticosterone was close or below detection limit in most rats in *hours 1* and *5* of the light cycle and started to rise toward the end of the light period [$F(2,22) = 25.099$, $P < 0.001$] (see Fig. 4). The value in *hour 9* differed significantly from corticosterone in *hour 1* or *5*.

Experiment 2: restricted feeding. When food was available only in the light cycle, the rats produced vigorous eating in *hour 1* of the light period (Fig. 2). The fraction of the daily feeding activity in *hour 1* of the light period was 31.8 ± 2.24%, and this value was significantly higher (*t*-test, $P < 0.001$) than the feeding activity of rats on the normal cycle in *hour 1* of the dark period. After *hour 1*, feeding initially dropped to low levels and then tended to increase toward the end of the light period in the RF rats.

RF altered the various sleep parameters differentially. Total NREMS did not change (Table 1).

NREMS time decreased by 11% in the light period [treatment factor: $F(1,100) = 179.012$, $P < 0.001$] and increased by 10% in the dark period [treatment factor: $F(1,100) = 102.147$, $P < 0.001$]. However, the diurnal rhythm with more NREMS during the day was clearly maintained (Table 1, Fig. 2). Reductions in NREMS during the day occurred only in *hour 1* and in the last hours when the rats spent more time with eating [group × time interaction: $F(11,1100) = 35.984$, $P < 0.001$]. In between, NREMS stayed at baseline values. At night, sleep bouts consisted of more NREMS epochs than in free-feeding rats [time × treatment interaction, $F(11,1100) = 11.333$, $P < 0.001$]. The daily rhythm EEG delta power during NREMS was particularly resistant to restricted feeding (Fig. 2). There were only three hourly values that differed from baseline [time × treatment interaction for 24-h recording: $F(23,2263) = 22.584$, $P < 0.001$]: *hour 1* of the light period when the rats slept little, and the first and the last hours of the dark period. In contrast to NREMS, RF fundamentally altered the diurnal rhythm of REMS (Fig. 2, Table 1). REMS decreased throughout the light period [treatment factor: $F(1,100) = 203.910$, $P < 0.001$] and increased in the dark period [treatment factor: $F(1,100) = 291.540$, $P < 0.001$], resulting in more REMS at night than during the day. These changes in REMS occurred without alterations of the total REMS time in 24 h.

T_{crt} was also responsive to RF. Mean T_{crt} increased in the light cycle and decreased in the dark cycle, and thereby the mean 24-h T_{crt} did not change although the diurnal difference disappeared between the means calculated for the light and the dark periods (Table 1). However, T_{crt} did vary [treatment × time interaction: $F(23,1191) = 30.207$, $P < 0.001$]: it peaked at the beginning of the dark period, and then T_{crt} dropped rapidly to low levels during the dark period followed by a slow increase in the subsequent light cycle (Fig. 2). The courses of T_{crt} differed between free-feeding and RF rats [time × treatment interaction: $F(23,2254) = 48.690$, $P < 0.001$].

RF had a significant impact on plasma ghrelin [comparison with free-feeding, group factor: $F(1,93) = 22.497$, $P < 0.001$; time factor: $F(5,93) = 7.360$, $P < 0.001$; time × group interaction: $F(5,93) = 21.896$, $P = 0.001$] and leptin [group factor: $F(1,94) = 9.722$, $P = 0.002$; time factor: $F(5,94) = 2.896$, $P = 0.02$; time × group interaction: $F(5,94) = 8.986$, $P < 0.001$]. The diurnal rhythm of these hormones reversed in such a way that they maintained their relationship with respect to one another and to feeding activity. The ghrelin peak continued to precede the major feeding peak, which was now in *hour 1* after lights-on, and thus the ghrelin maximum was observed at 21 h toward the end of the dark period. The leptin peak followed the major feeding activity peak, and it occurred

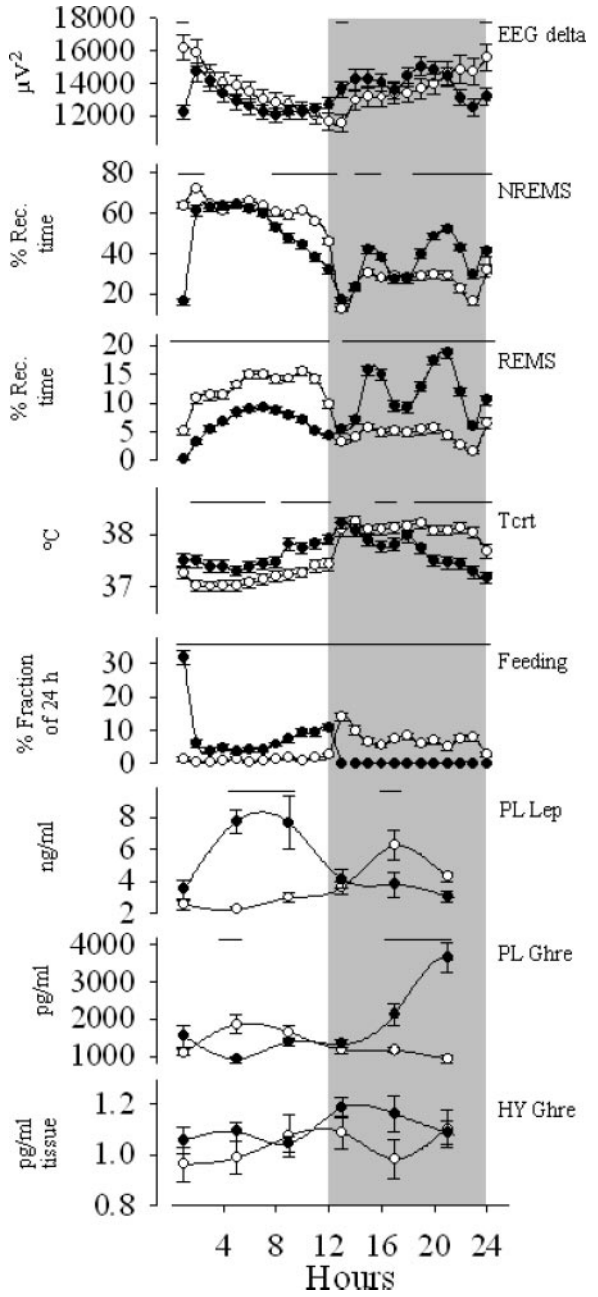

Fig. 2. Effects of feeding restricted to the light cycle on the diurnal rhythms in 55 rats (RF rats). Filled symbols, RF rats; open symbols, values obtained in the free-feeding rats shown in Fig. 1, plotted over the 24-h values of the RF rats. See legends of Fig. 1 for abbreviations. Horizontal lines above the curves: significant differences between free-feeding rats and RF rats (Student-Newman-Keuls test after ANOVA). Gray column, dark period.

during the day sometime between *hours 5* and *9* after light onset. The concentrations calculated for 24 h increased significantly for both leptin and ghrelin (Table 1). Although the leptin peak during RF did not differ significantly from the leptin peak on the normal cycle (Table 1), the diurnal rise in leptin obviously lasted longer than the nocturnal increase (Fig. 2). The nocturnal ghrelin peak in the rats on RF was almost double that of, and significantly higher than, the diurnal ghrelin peak in the rats on normal feeding rhythm (Table 1, Fig. 2).

Ghrelin content of the hypothalamus displayed slight oscillations (Fig. 2), which did not reach the level of statistical significance. However, comparisons of hypothalamic ghrelin contents revealed significant differences between free-feeding and RF rats [group factor: $F(1,85) = 5.149$, $P = <0.03$], and calculated for 24 h, the mean hypothalamic ghrelin content was significantly higher ($P = 0.03$) in the rats on RF than in the rats on the normal feeding schedule (Table 1).

Mean concentrations of corticosterone in the plasma of rats on RF slightly exceeded corticosterone in rats on the normal feeding at each time point during the light cycle, but significant differences were not detected (Fig. 4). Like in free-feeding rats, corticosterone concentration increased as the day progressed [$F(2,25) = 17.8$, $P < 0.001$] with the value in *hour 9* being significantly higher than corticosterone in the morning or in *hour 5*.

Experiment 3: sleep deprivation. Baseline values of sleep, T_{crt}, and feeding activity in the SD group did not differ from the values in the rats on the normal feeding cycle (Fig. 3). SD for 5 h was followed by significant albeit modest increases in the duration of both NREMS [treatment factor: $F(1,21) = 94.126$, $P < 0.001$; treatment × time interaction: $F(6,126) = 2.363$, $P = 0.03$] and REMS [treatment × time interaction: $F(6,126) = 6.570$, $P < 0.001$]. NREMS time increased immediately after SD and in the dark period [treatment factor: $F(1,6) = 29.842$, $P < 0.002$]. Enhancements of REMS were delayed to the end of the light period and into the dark period [$F(1,6) = 21.873$, $P < 0.005$]. Delta power during NREMS was the parameter most affected by SD [calculated for 21 h, treatment factor: $F(1,6) = 12.107$, $P = 0.013$; treatment × time interaction: $F(15,90) = 11.723$, $P < 0.001$]. The depth of NREMS increased promptly after SD. Thereafter, delta power declined and tended to decrease below baseline in the dark period.

T_{crt} rose significantly during SD and tended to decrease below baseline during recovery sleep [treatment × time interaction: $F(15,90) = 3.665$, $P < 0.001$] (Fig. 3).

Feeding activity was slightly but significantly stimulated during SD [treatment × time interaction: $F(20,452) = 3.291$, $P < 0.001$] (Fig. 3). When calculated for individual hours, feeding activity increased only in *hour 5*, but determined for the entire SD period, 11.1 ± 2.87% of the 24-h feeding activity occurred during SD, which was significantly higher (*t*-test, $P < 0.01$) than the corresponding value without SD (2.8 ± 1.22%). The rats tended to display less feeding activity during the recovery period subsequent to SD (*hours 6–12* of the light period: 5.8 ± 1.98% after SD and 11.8 ± 1.98% on the baseline day, *t*-test, $P < 0.05$), and the feeding activity peak after dark onset also decreased significantly.

SD failed to alter plasma concentration of leptin (Fig. 3). In contrast, plasma ghrelin increased significantly in *hour 1* of SD (*t*-test, $P < 0.001$). Thereafter, significant differences were not observed in plasma ghrelin between the SD and the baseline days.

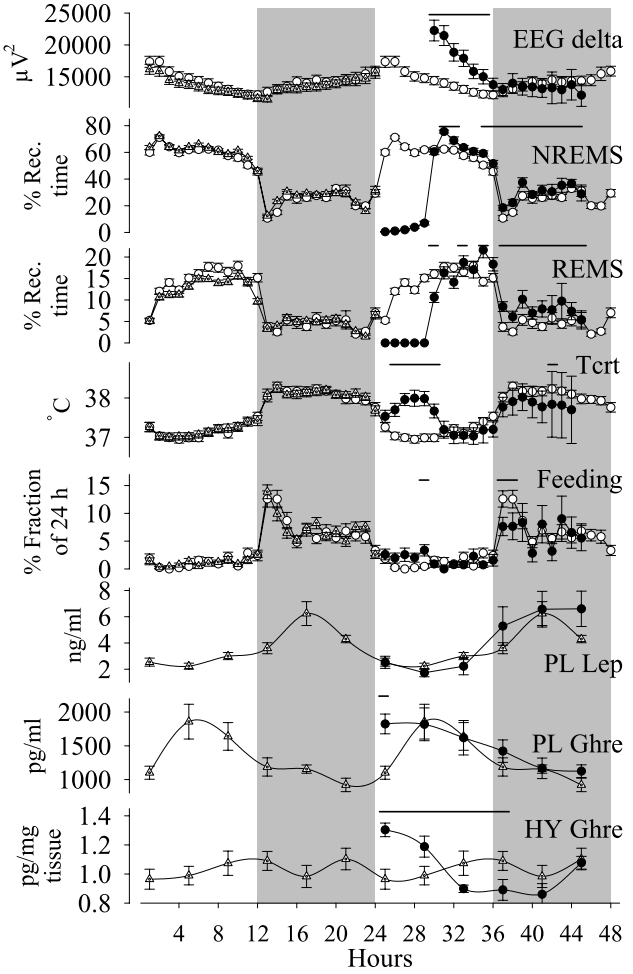

Fig. 3. Sleep, T_{crt}, feeding, and hormone responses to sleep deprivation (SD) in 47 rats. Open circle, baseline values (means of 3 days of recording) in the SD group double-plotted for 48 h. Triangle with dot, free-feeding rats depicted in Fig. 1 shown superimposed over the first 24 h. Filled circle, values during and after SD. SD started at light onset and lasted for 5 h. See legends of Fig. 1 for abbreviations. Horizontal lines above the curves: significant differences between free-feeding rats and RF rats (Student-Newman-Keuls test following ANOVA). Gray columns, dark period.

Hypothalamic ghrelin was highly responsive to SD, displaying statistically significant biphasic variations [$F(5,39) = 11.073$, $P < 0.001$]. Ghrelin contents of the hypothalamus increased significantly during SD and dropped below baseline after SD. These variations in the courses of ghrelin were significant between the free-feeding rats and sleep-deprived rats [time × treatment interaction: $F(5,75) = 6.411$, $P < 0.001$].

Plasma corticosterone concentration (Fig. 4) in the SD rats was significantly higher than in the other groups [treatment factor: $F(2,66) = 15.670$, $P < 0.001$, time factor: $F(2,4) = 8.792$, $P < 0.001$; treatment × time interaction: $F(4,66) = 15.012$, $P < 0.001$]. The difference was significant in *hour 1*. Corticosterone declined thereafter, it did not differ from corticosterone in the other groups in *hour 5*, and it tended to decrease below baseline during post-SD recovery, in *hour 9*.

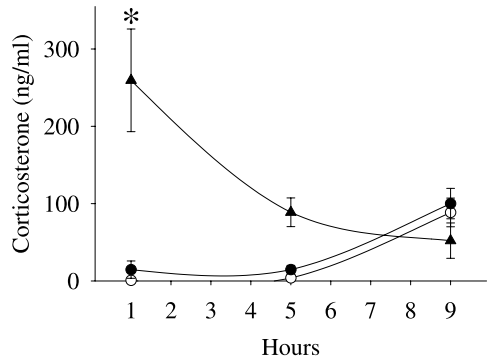

Fig. 4. Plasma corticosterone concentrations (mean ± SE) 1, 5, and 9 h after light onset in free-feeding rats (○), in RF rats (●), and in SD rats (▲; sleep deprivation: *hours 1–5*). *Significant difference between the SD rats and the other groups (Student-Newman-Keuls test after ANOVA).

DISCUSSION

The major current findings are that strong links between sleep and plasma ghrelin or leptin are not detected, whereas the intimate relationships between feeding and these hormones are confirmed in the rat. Rats do not eat or they consume little food during the light cycle (reviewed in Ref. 50). The onset of the dark phase is a potent stimulus for ingestive behavior, and rats generally have one large meal soon after lights go off. The last feeding bout before light onset is also special because it is based on learning the time of light onset. Our free-feeding rats displayed normal diurnal feeding patterns. Marked diurnal rhythms in plasma leptin and ghrelin were observed. The single nocturnal peak of plasma leptin after feeding corresponds to the dark phase-associated leptin peak described previously in free-feeding rats (61). Mice also exhibit a nocturnal rise in plasma leptin (3). The diurnal rhythm of ghrelin was opposite to that of leptin with its peak occurring in the light period. We found no signs of a previously reported second rise in plasma ghrelin before light onset in the rat (36). The highest values of ghrelin and the lowest values of leptin occurred at the same time, and this is in agreement with the suggestion that withdrawal of leptin tone may promote secretion of ghrelin (24). Concentrations of both leptin and ghrelin exhibit ultradian variations in the plasma (6, 54). These changes could not be detected with the 4-h sampling rate used in our experiments. The ultradian pulse variations of ghrelin correlate with feeding episodes (54); fasting increases the frequency and amplitude of ghrelin pulses and diminishes leptin pulse amplitudes (6). Ghrelin secretion is usually observed immediately before feeding, and plasma ghrelin levels fall within 1 h after a meal in humans (11) and sheep (51). It may seem, therefore, that the hormonal rhythms observed in our experiments are more consistent with a circadian regulation than a feeding-induced rhythm.

Although we are not aware of reports demonstrating that a circadian oscillator controls variations in plasma ghrelin, there is evidence showing that the suprachiasmatic nucleus is capable of regulating the diurnal rhythm of leptin. Lesion of the suprachiasmatic nucleus eliminates the diurnal rhythm of plasma leptin in the rat (25), and the nocturnal rise in plasma leptin is maintained in human subjects on continuous enteral nutrition, i.e., without specific feeding signals (48). Nevertheless, several observations suggest that meal timing might be more important than the circadian regulation for the diurnal rhythm of leptin. Plasma leptin increases regularly within 5 h after each meal in humans (17). More importantly, leptin secretion shifts with feeding. Restricted food availability to 4 h of the light period resulted in the abolishment of the nocturnal leptin peak, and the appearance of a diurnal leptin secretion 4 h after the initiation of feeding in the rat in a previous study (61), which is a finding fully confirmed in our experiments. Day/night reversal also reversed the timing of the peaks and minimum values of plasma leptin in humans (44). Our results with RF suggest that the diurnal rhythm of ghrelin secretion is as strongly coupled to feeding as the diurnal rhythm of leptin. Finally, glucocorticoids have been proposed as a stimulator of leptin secretion (8, 17, 37). The diurnal rhythm of corticosterone in rats is such that the peak occurs around dark onset, and then corticosterone concentration declines and reaches a trough after light onset followed by gradual increases in the light period (5). Food restriction to 2 (29, 30) or 4 h (21, 61) of the light period shifts the corticosterone peak to the onset of the eating period. A corticosterone peak is, however, not detected when food availability is extended to 6 h or more in this condition; the rats are able to consume the amount of food normally ingested in 24 h (21). In agreement with this, corticosterone was unaltered in our RF studies. Dissociation between the diurnal rhythms of cortisol and leptin was also observed in human subjects when the leptin rhythm was altered by meal timing (44). That corticosterone might not be fundamental in modulating the diurnal rhythm of leptin is shown by the finding that adrenalectomy and corticosterone replacement do not alter the leptin rhythm (25).

Entrainment of the diurnal rhythms of ghrelin and leptin by RF is in sharp contrast with the resistance of the diurnal rhythm of NREMS to the same condition. That RF has little impact on NREMS in rats was previously reported (43). The RF condition is also a model of the subtle sleep alterations experiencing by Muslims during the diurnal fasting in the lunar month of Ramadan (41, 42). The homeostatic and circadian aspects of NREMS regulation are described by the two-process model (7). The homeostatic process determines sleep propensity as the function of previous wakefulness or neuronal use. The activity of homeostatic process is characterized by the intensity of NREMS described by EEG delta power during NREMS (1). This function was practically unaltered by RF. The circadian control of NREMS was also markedly resistant to RF. NREMS time decreased only when the rats were occupied with eating in the light phase, and this modest loss was compensated with more sleep at night.

Unlike NREMS, REMS was fundamentally altered in RF condition. The responsiveness of REMS to RF was also previously reported (34, 43). The mechanism through which RF modulates REMS is not clear, but it has been suggested that changes in REMS might be related to changes in T_{crt} or body temperature, another parameter markedly influenced by RF. Alterations in T_{crt} can be linked to metabolic activity determined by feeding: higher metabolism results in higher T_{crt} during the light period in rats on RF, whereas T_{crt} drops at night when the rats have to fast. REMS duration is inversely proportional to body temperature in humans (15), and lowering body temperature promotes REMS in pontine cats (23). Diurnal leptin secretion may also contribute to REMS suppression in RF conditions because leptin is posited to decrease REMS

(49). Theoretically, hypersecretion of glucocorticoids may also be implicated in the RF-induced sleep alterations (42), but this is unlikely considering the lack of corticosterone response to RF in our experiments.

The dissociation of the NREMS response and the plasma hormonal response to RF suggests that the links between sleep regulation and leptin are relatively weak. Results from the SD experiments provide serious challenge to such putative links: thus, if there are links between sleep and leptin, then one might anticipate alterations in leptin secretion during SD, during recovery sleep, or both. SD failed to alter plasma leptin concentrations in our experiments. This finding is in agreement with reports in human subjects (44, 48). Another paper reported a reduced amplitude of plasma leptin during SD in humans (35). However, direct comparisons between humans and rats are difficult because in the rat, the rest phase is associated with the diurnal trough of leptin, and thus suppressions of leptin levels would be hard to detect. SD stimulated corticosterone secretion, which apparently did not influence leptin secretion. In contrast to leptin, plasma ghrelin responded to SD with an increase in *hour 1*. This response is the opposite of that observed in human subjects where the nocturnal rise in plasma ghrelin was blunted during SD (16). The increase in ghrelin secretion in *hour 1* of SD may signal hunger and eating in the rat, and in fact, our rats did consume food during SD.

Interestingly, hypothalamic ghrelin, exhibiting biphasic changes during and after SD, was much more responsive to SD than plasma ghrelin. Various functions display biphasic changes in association with SD; the list includes the amounts of NREMS and REMS, which decrease during SD and increase during recovery, and T_{crt} and feeding activity, which increase during SD and decrease during recovery. The problem with linking alterations in hypothalamic ghrelin to feeding activity is that rapid rises in peptide contents generally indicate accumulation, i.e., inhibition of ghrelin release, which is not consistent with increased feeding during SD, and decreases in peptide contents suggest enhanced release, which is not anticipated during recovery sleep when feeding decreases. GH secretion via stimulating GHRH neurons in the arcuate nucleus is another well-documented action of GHSs, including ghrelin. GH secretion correlates with the observed changes in intrahypothalamic ghrelin because GH secretion is inhibited during SD and tends to enhance during recovery in the rat (26). GHRH neurons also promote NREMS. Therefore, SD-induced changes in hypothalamic ghrelin may reflect the first sign of sleep-related alterations in ghrelin in our experiments.

Previous findings suggesting that leptin and ghrelin secretions have sleep-associated components were predominantly obtained in human subjects. A comparison of the observations between humans and rats, however, shows significant differences in the relationship between sleep and these hormones. Both humans (17, 35, 44, 48) and rats exhibit a nocturnal peak in leptin, but this rise is opposite in terms of the rest phase of the day in the two species. In addition to the diurnal rhythm, a sleep-associated increase in leptin secretion is also suggested in humans (48) that is obviously missing in rodents. A negative correlation was reported between plasma leptin rhythm and rectal temperature in humans (48) that does not occur in rats. The diurnal rhythm of ghrelin is in phase with that of leptin in humans (11), i.e., both leptin and ghrelin rise at night, whereas the rhythms of ghrelin and leptin are shifted by 180° in rats. Thus, at least ghrelin peaks in the rest phase in both humans and rats. However, plasma ghrelin concentration increases in sleeping subjects during the early part of the night, and there is a positive correlation between plasma ghrelin and the peak of GH secretion during the first hours of sleep, suggesting that sleep-associated processes modulate nocturnal ghrelin secretion (16). In contrast, the peak of the ghrelin rhythm is in the middle of the rest phase in the rat, and by this time the NREMS-promoting GHRH activity is over (19), and correlation is not observed between ghrelin pulses in the plasma and GH secretion (54). Although the differences in the relationship between sleep and plasma leptin and ghrelin rhythms do not support a universal role of these hormones in sleep regulation, sleep responses can be elicited by exogenous administration of leptin and ghrelin (38, 49, 58). These responses might represent pharmacological actions, but it is also possible that endogenous leptin and ghrelin contribute to sleep promotion in special conditions. For example, ghrelin released before feeding may stimulate postprandial sleep. Further experiments may determine the significance of intrahypothalamic ghrelin in sleep regulation.

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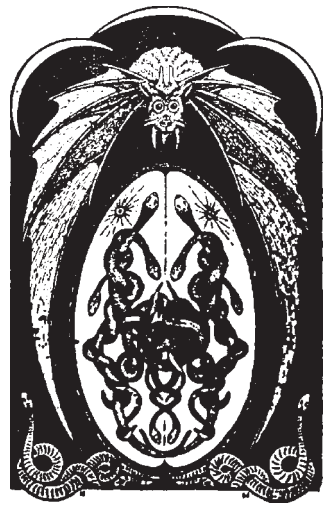

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Research Report

Ghrelin-induced sleep responses in ad libitum fed and food-restricted rats

E. Szentirmai[a,b], *I. Hajdu*[a], *F. Obal Jr.*[a], *James M. Krueger*[b,*]

[a]*Department of Physiology, A. Szent-Györgyi Medical and Pharmaceutical Center, University of Szeged, Szeged, Hungary*
[b]*Neuroscience Program, Department of VCAPP, College of Veterinary Medicine, Washington State University, PO Box 646520, Pullman, WA 99164-6520, USA*

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ABSTRACT

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor and a well-characterized food intake regulatory peptide. Hypothalamic ghrelin-, neuropeptide Y (NPY)-, and orexin-containing neurons form a feeding regulatory circuit. Orexins and NPY are also implicated in sleep–wake regulation. Sleep responses and motor activity after central administration of 0.2, 1, or 5 μg ghrelin in free-feeding rats as well as in feeding-restricted rats (1 μg dose) were determined. Food and water intake and behavioral responses after the light onset injection of saline or 1 μg ghrelin were also recorded. Light onset injection of ghrelin suppressed non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) for 2 h. In the first hour, ghrelin induced increases in behavioral activity including feeding, exploring, and grooming and stimulated food and water intake. Ghrelin administration at dark onset also elicited NREMS and REMS suppression in hours 1 and 2, but the effect was not as marked as that, which occurred in the light period. In hours 3–12, a secondary NREMS increase was observed after some doses of ghrelin. In the feeding-restricted rats, ghrelin suppressed NREMS in hours 1 and 2 and REMS in hours 3–12. Data are consistent with the notion that ghrelin has a role in the integration of feeding, metabolism, and sleep regulation.

1. Introduction

Ghrelin, the natural ligand of the growth hormone (GH) secretagogue (GHS) receptor (GHS-R), is produced by the endocrine cells of the stomach (Kojima et al., 1999) and is synthesized in various brain structures (Cowley et al., 2003). It stimulates food intake (Wren et al., 2000), gastrointestinal motility (Masuda et al., 2000), and it promotes deposition of fat (Tschop et al., 2000). Plasma ghrelin concentrations inversely correlate with feeding (Bodosi et al., 2004), and circulating ghrelin may transmit hunger signals to the central nervous system (Cummings et al., 2001). The most clearly documented target site of circulating ghrelin to stimulate feeding is the arcuate nucleus (ARC) (Bagnasco et al., 2003). The ARC, implicated in the central control of meal initiation (Kalra et al., 1999), is also affected by neuropeptide Y (NPY) and agouti gene-related peptide (AgRP) (Broberger et al., 1998), two appetite-stimulating peptides (Konturek et al., 2004). Ghrelin stimulates food intake by stimulating NPY/AgRP neurons in the hypothalamus (Kohno et al., 2003; Seoane et al., 2003) to promote the production and release of NPY and AgRP (Wren et al., 2002).

* *Corresponding author*. Fax: +1 509 335 4650.
E-mail address: Krueger@vetmed.wsu.edu (J.M. Krueger).

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Ghrelin reaches the ARC via blood and, in addition, it is also synthesized within the hypothalamus. Ghrelin-immunoreactive cell bodies are found in the internuclear space between the ARC, paraventricular nucleus (PVN), dorsomedial and ventromedial nuclei and in the lateral hypothalamus (LH) (Cowley et al., 2003). Central administration of ghrelin induces increased c-fos protein expression in orexinergic (Lawrence et al., 2002; Olszewski et al., 2003) and NPYergic (Nakazato et al., 2001) neurons and also stimulates electrophysiological activity of these cells (Kohno et al., 2003; Yamanaka et al., 2003). Pretreatment with orexin (Toshinai et al., 2003) or NPY antibodies (Nakazato et al., 2001) attenuates ghrelin-induced feeding, suggesting the role of these peptides in the feeding effects of ghrelin. GHS-R mRNA is expressed in several hypothalamic nuclei including the LH and PVN (Guan et al., 1997; Mitchell et al., 2001). In the ARC and ventromedial nucleus, the receptor is expressed on NPYergic (Kohno et al., 2003) and growth hormone-releasing hormone (GHRH)-containing neurons (Tannenbaum et al., 1998), while in the LH, the receptor is present on orexinergic cells (Mitchell et al., 2001). The role of orexins in maintaining wakefulness is well documented (Sakurai, 2002), and central administration of NPY also induces wakefulness in rats (Szentirmai and Krueger, in press(b)).

In addition to participating in the hypothalamic food-intake regulatory circuit, ghrelin also affects the somatotropic axis. Ghrelin stimulates GHRH neurons and elicits GHRH release (Wren et al., 2002). Stimulation of GH secretion via ARC GHRH neurons and direct stimulation of pituitary GHS-Rs to release GH are well-documented actions of ghrelin both in rats (Date et al., 2000; Kojima et al., 1999; Seoane et al., 2000) and humans (Arvat et al., 2001). In addition, some (Tannenbaum et al., 2003; Tolle et al., 2001) but not all (Wren et al., 2002) reports suggest that ghrelin may also inhibit somatostatin secretion. Somatotropic axis hormones such as GHRH, GH, insulin-like growth factor-1 (IGF-1), and somatostatin have the capacity to modify sleep (Obal and Krueger, 2001). For example, both peripheral and central administration of GHRH enhances the duration and the intensity of non-rapid-eye-movement sleep (NREMS) in several species (Ehlers et al., 1986; Nistico et al., 1987; Obal et al., 1988) including humans (Kerkhofs et al., 1993; Steiger et al., 1992). Hypothalamic GHRH mRNA levels (Bredow et al., 1996) and GHRH peptide (Gardi et al., 1999) content exhibit sleep-related variations with a maximum GHRH release and synthesis when the duration and the intensity of NREMS are the highest. Sleep deprivation increases hypothalamic GHRH mRNA levels and decreases somatostatin mRNA levels (Zhang et al., 1998). Sleep loss is also associated with enhanced GHRH release and increased sleep propensity (Gardi et al., 1999). Mutant dwarf rats (dw/dw) with a defective GHRH receptor signaling mechanism have less spontaneous NREMS (Obal et al., 2001). Somatostatin inhibits hypothalamic GHRH synthesis and release, and it reduces NREMS (Rezek et al., 1976).

Ghrelin may participate in sleep regulation by activating orexinergic and NPYergic neurons, which are implicated in maintaining wakefulness and stimulating GHRH, which has well-documented sleep-promoting effects. Previously, the effects of systemically administered ghrelin were studied on sleep. Systemic injection of ghrelin enhances (Weikel et al., 2003) while hexarelin, a more potent ghrelin analogue, suppresses sleep in humans (Frieboes et al., 2004). Intraperitoneal (i.p.) injection of ghrelin promotes NREMS in mice (Obal et al., 2003), while its intravenous (i.v.) administration decreases NREMS and REMS in rats (Tolle et al., 2002). Rat hypothalamic ghrelin content displays diurnal oscillations, and it increases during sleep deprivation and in response to feeding restriction (Bodosi et al., 2004).

The aim of our experiments was to study sleep responses to centrally injected ghrelin in rats. We report that ghrelin inhibits sleep while simultaneously stimulates food and water intake. The sleep-inhibitory actions also occur in rats lacking access to food. After the ghrelin-induced sleep suppression, secondarily increases in NREMS were observed after some doses of ghrelin.

2. Results

2.1. *Experiment 1: dark onset injection of ghrelin in free-feeding rats*

During the dark period, the effect of ghrelin on sleep was dose-independent. Injection of 0.2 μg ghrelin did not change NREMS or REMS. One microgram ghrelin induced significant decreases in NREMS and REMS in post-injection hours 1 and 2 [two-way ANOVA, treatment effect, NREMS: $F(1,7) = 6.035$, $P < 0.05$, REMS: $F(1,7) = 13.728$, $P < 0.05$]. In hours 3–12 after injection, sleep returned to baseline. Five micrograms of ghrelin had a biphasic effect on NREMS. In the first post-injection hour, NREMS decreased and during the rest of the recording period NREMS increased [two-way ANOVA, treatment effect, hours 1 and 2: $F(1,12) = 6.084$, $P < 0.05$, hours 3–12: $F(1,12) = 7.369$, $P < 0.05$]. Similar tendencies in REMS were observed, but the changes only reached the level of significance in hours 1 and 2 [two-way ANOVA, treatment effect, $F(1,12) = 9.804$, $P < 0.05$] (Figs. 1 and 2).

EEG delta power during NREMS, a measure of NREMS intensity, showed dose-dependent decreases in response to ghrelin injected at dark onset [ANOVA, treatment effect, $F(2,78) = 4.111$, $P < 0.05$]. There were differences between the effect of 0.2 μg and 1 μg ghrelin injections and between the 1 μg and 5 μg ghrelin injections. There was no change in EEG SWA following injection of 0.2 μg ghrelin as compared to baseline. One microgram and five micrograms of ghrelin caused significant decreases in EEG SWA [ANOVA, treatment effect, 1 μg ghrelin injection: $F(1,65) = 18.225$, $P < 0.05$, 5 μg ghrelin injection $F(1,51) = 9.159$, $P < 0.05$]. Post hoc analyses did not show significant changes in EEG SWA between either time block (1–2 and 3–12 h post-injection) after the 1 or 5 μg dose of ghrelin.

2.2. *Experiment 2: light onset injection of ghrelin in free-feeding rats*

The amount of NREMS was high at the beginning of the light period and declined toward dark onset after icv PFS

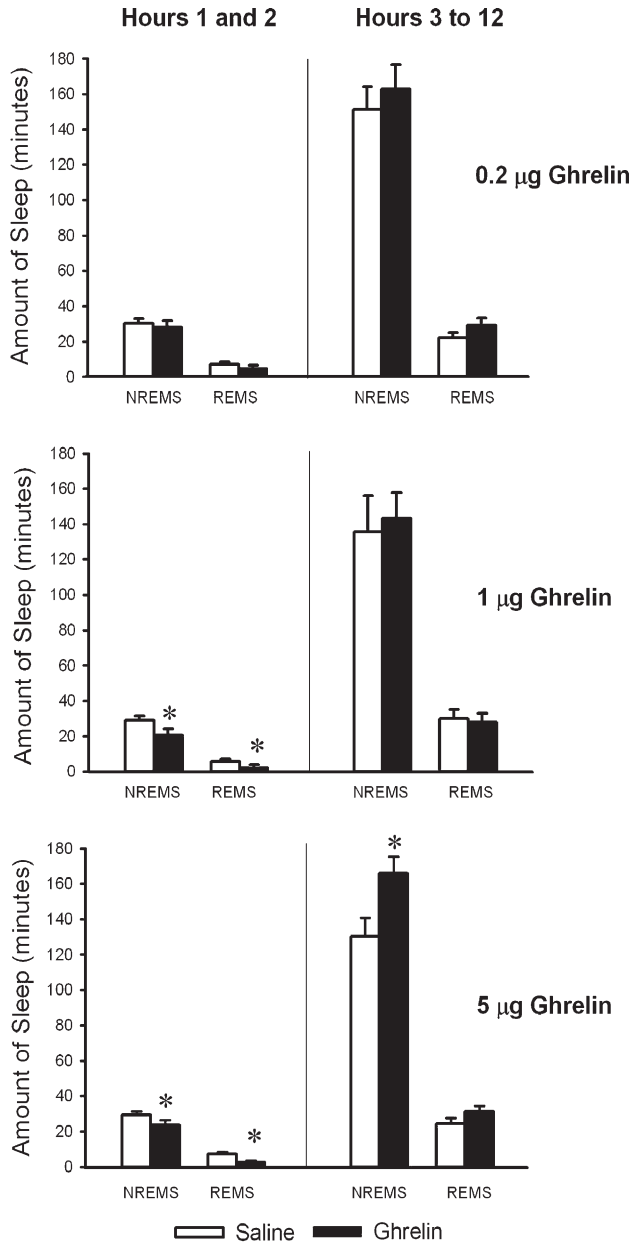

Fig. 1 – The effects of intracerebroventricular (icv) administration of ghrelin and pyrogen-free physiological saline (PFS) on sleep at dark onset. White bars: PFS injection, black bars: ghrelin injection. Error bars: standard error. Asterisks denote significant differences between baseline day and experimental day ($P < 0.05$, paired *t* test).

in each group of rats (Figs. 3 and 4). In the light phase, ghrelin treatment induced a significant dose-response relationship on both NREMS and REMS across post-injection hours 1 and 2 [two-way ANOVA, treatment effect, NREMS: $F(2,46) = 3.752$, $P < 0.05$, REMS: $F(2,46) = 5.245$, $P < 0.05$]. There were differences between the effects of 0.2 μg and 1 μg ghrelin on NREMS and between the responses to 0.2 μg and 1 μg and between 1 μg and 5 μg ghrelin on REMS. The 0.2 μg dose of ghrelin decreased NREMS in the first 2 h, but these changes did not reach the level of statistical significance. This dose failed to alter REMS. One microgram of ghrelin had a biphasic effect on both NREMS and REMS. NREMS and REMS were suppressed in post-injection hours 1 and 2 [two-way ANOVA, treatment effect, NREMS: $F(1,5) = 43.319$, $P < 0.05$, REMS: $F(1,5) = 15.352$, $P < 0.05$]. Post hoc analyses showed that these effects on NREMS and REMS were significant in hours 1 and 2 (SNK test, $P < 0.05$). This sleep suppression was accompanied by behavioral activation. Rats were restless throughout the 60-min observation period; their behavior included increased locomotor activity, eating, drinking, grooming and exploration (Fig. 5). The first bout of eating was already observed in 10 min after the injection, and eating continued throughout the first hour of the light period. During this time food intake was significantly higher than after the control treatment (Fig. 6). Food and water intake did not differ in the two groups across the 24-h post-injection period. From hour 3, during the rest of the light period, NREMS increased significantly above baseline [two-way ANOVA, treatment effect: $F(1,5) = 37.559$, $P < 0.05$]. Post hoc analyses showed that the effect on NREMS was significant in hour 12 (SNK test, $P < 0.05$). A tendency for REMS to increase was also observed during the last hours of the light period, but these changes were not significant. After injection of 5 μg ghrelin NREMS decreased significantly in hours 1 and 2 [two-way ANOVA, treatment effect: $F(1,11) = 25.443$, $P < 0.05$] and remained below the baseline for the remaining 10 h of the recording period [two-way ANOVA, treatment effect: $F(1,11) = 7.905$, $P < 0.05$]. Post hoc analyses showed that the effects on NREMS and REMS were significant in hour 1 (SNK test, $P < 0.05$). A tendency to increase REMS was obvious starting in hour 3, but these changes did not reach the level of significance.

EEG SWA was dose-dependently increased after light onset injection of ghrelin with significant differences between the effects of 0.2 μg and 1 μg ghrelin and between 1 μg and 5 μg ghrelin. Following 0.2 μg ghrelin, EEG SWA did not show changes between the baseline and the experimental day. One μg ghrelin increased EEG SWA [ANOVA, treatment effect $F(1,60) = 9.84$, $P < 0.05$] with significant differences from hour 1 to hour 8 post-injection. Five micrograms of ghrelin injections did not alter EEG SWA.

2.3. *Experiment 3: effects of ghrelin on feeding-restricted (RF) rats*

One microgram of ghrelin induced significant decreases in NREMS in post-injection hours 1 and 2 in RF rats [two-way ANOVA, treatment effect: $F(1,7) = 6.400$, $P < 0.05$]. Post hoc analyses showed that the effect on NREMS was significant in hour 1 (SNK test, $P < 0.05$). In the following hours, NREMS did not differ from baseline values. REMS did not change in the first 2 h, but from hours 3 to 12, a significant decrease was observed [two-way ANOVA, treatment effect: $F(1,6) = 10.814$, $P < 0.05$]. The 1 μg ghrelin-induced NREMS decrease in post-injection hours 1 and 2 was dependent on the feeding protocol. The NREMS decrease in free-feeding rats was significantly higher than that observed in RF rats [ANOVA, group factor: $F(1,24) = 8.259$, $P < 0.05$] (Figs. 3 and 4).

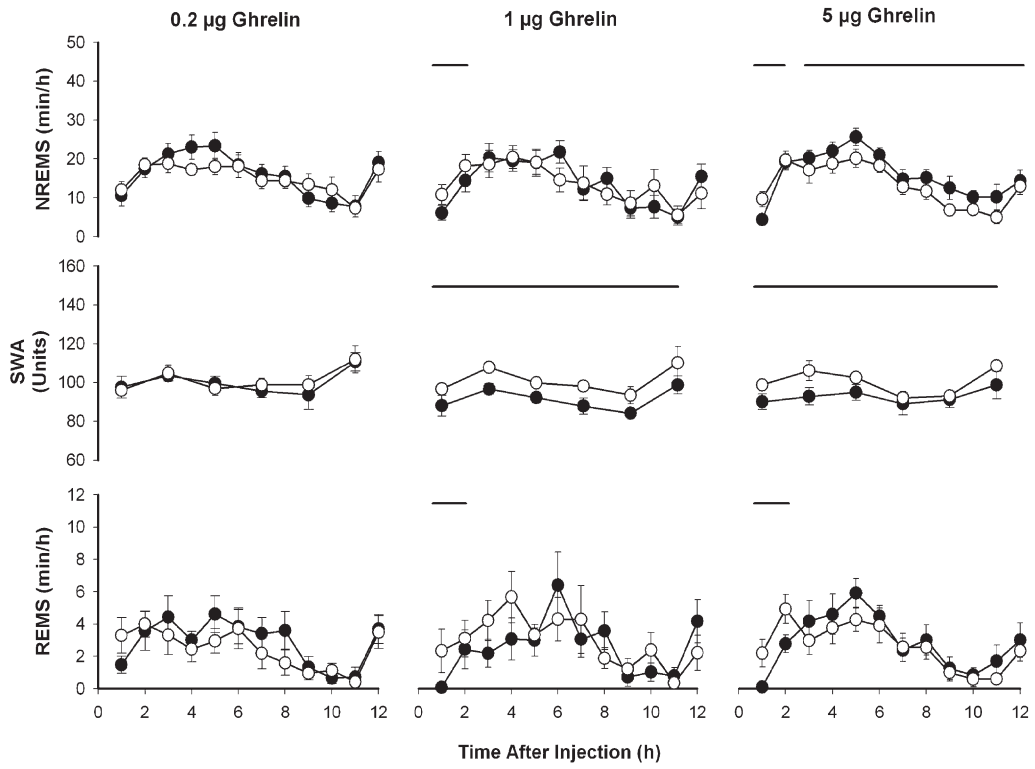

Fig. 2 – The effects of icv administration of ghrelin and PFS on sleep and slow-wave activity (SWA) at dark onset. PFS was injected on baseline day (○), and ghrelin was administered on experimental days (●). The amounts of non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS) were calculated in 1-hour time blocks while amount of EEG SWA were calculated in 2-h time blocks. Error bars: standard error. Horizontal lines above the curves: significant differences between the saline- and ghrelin-treated groups (two-way ANOVA for repeated measures).

Enhancements in EEG SWA after 1 µg ghrelin injection in RF rats were observed [ANOVA, treatment effect: $F(1,54) = 7.809$, $P < 0.05$]. EEG SWA showed treatment-related dependence between free-feeding and RF rats [ANOVA, treatment effect, $F(1,57) = 4.72$, $P < 0.05$].

3. Discussion

Intracerebroventricular injection of ghrelin induced an immediate decrease in NREMS and REMS and also markedly affected EEG delta power in rats. In addition to its sleep modulating effects, ghrelin also stimulated feeding behavior. Ghrelin is primarily implicated in feeding and GH secretion (Kojima et al., 1999; Tschop et al., 2000). Our findings are in agreement with several previous studies reporting that icv injection of ghrelin enhances eating (Tschop et al., 2000; Wren et al., 2000). Rats started to eat quickly after ghrelin injection and continued to eat throughout the first hour after injection. The feeding-inducing property of ghrelin was sufficiently strong that it was able to stimulate food intake at the beginning of the light period, when sleep propensity is the highest (Borbely, 1982) and rats are usually satiated.

Our results are also consistent with previous observations that, in rats, i.v. injections of ghrelin elicit prompt increases in wakefulness at the expense of both NREMS and REMS (Tolle et al., 2002). The effects of GHS-R activation on sleep in other species are less clear. For example, in humans, i.v. bolus injection of GHRP-2 failed to elicit any effect on sleep (Moreno-Reyes et al., 1998), while repeated i.v. boluses of GHRP-6 increased sleep (Frieboes et al., 1999). Intermittent i.v. injections of ghrelin increase slow-wave sleep and decrease REMS in humans (Weikel et al., 2003), while the same research group reported more recently that similar administration of hexarelin, a more potent GHS-R agonist than ghrelin, significantly suppresses stage 4 sleep and EEG delta power (Frieboes et al., 2004). In our experiments, when ghrelin was injected at light onset, the sleep suppressive effects were robust; sleep almost completely disappeared in the first hour of the light period in response to the 1 µg dose. Sleep decreases after dark onset injections were considerably attenuated but still significant in the first 2-h time block, and NREMS was increased during the second half of the night after the injection of 5 µg ghrelin. The reason for the attenuated wakefulness-promoting activities is most likely due to the fact that, under baseline conditions, rats sleep minimal amounts right after dark onset and that can hardly be further suppressed by any experimental manipulations. Similar to our finding, systemic injection of ghrelin during the dark period to rats (Tolle et al., 2002) and mice (Obal et al., 2003), when sleep propensities are low, failed to

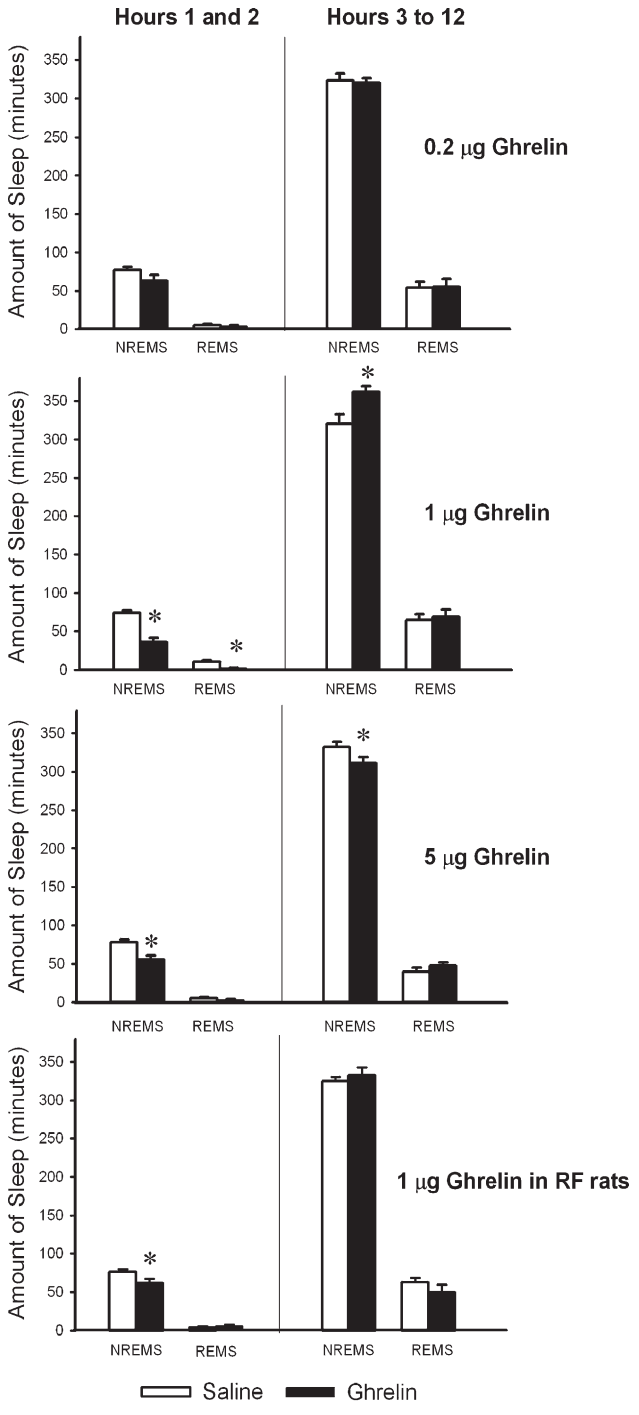

Fig. 3 – The effects of icv administration of ghrelin and PFS on sleep at light onset in free feeding and in restricted feeding (RF) rats. See legends to Fig. 1 for details.

suppress sleep, rather, there was a sleep increase during the night in mice (Obal et al., 2003). The differences in the effect of ghrelin seen between rats, humans, and mice likely resulted from the different routs of administration or may reflect real species specificity in the effect of ghrelin.

The mechanism of the wakefulness-promoting effects of ghrelin is unknown. We directly addressed one possibility in the present study; since rats cannot eat and sleep at the same time, increased eating activity after ghrelin treatment could be responsible for increases in wakefulness in the first 2 h. The wake-promoting effects of ghrelin, however, did not disappear in rats which had no access to food. This strongly suggests that the wake-promoting effects of ghrelin are not simply due to the stimulation of the eating behavior but other activating mechanisms are also involved. GHS receptors have been found in various brain structures implicated in sleep–wake regulation. For example, GHS-R mRNA, detected with in situ hybridization and RNase protection assays, is found in several hypothalamic nuclei, including the anteroventral preoptic nucleus, anterior hypothalamic area, suprachiasmatic nucleus, anterolateral hypothalamic nucleus, ARC, PVN, and tuberomamillary nucleus (Guan et al., 1997; Mitchell et al., 2001). Neurons in the LH also express GHS-Rs (Mitchell et al., 2001) and ghrelin fibers make direct synaptic contact with orexin-producing neurons in this area (Toshinai et al., 2003). A growing body of evidence indicates that ghrelin, in fact, stimulates orexinergic neurons in the LH. For example, icv injection (Lawrence et al., 2002) or local microinjection of ghrelin into the LH (Olszewski et al., 2003) induces increased c-fos protein expression in orexinergic neurons. Also, local application of ghrelin elicits the depolarization and increased firing rates of orexinergic neurons (Yamanaka et al., 2003). Pretreatment with anti-orexin-A IgG and anti-orexin-B IgG attenuates ghrelin-induced feeding suggesting that orexins are involved in the feeding effects of ghrelin (Toshinai et al., 2003). In addition to stimulating feeding, orexins also promote wakefulness and locomotor activity (Sakurai, 2002). It is possible that the wake-promoting effects of ghrelin are due to their stimulatory action on orexinergic cells in the LH. Our preliminary results that microinjection of ghrelin into the LH has potent wake-promoting activities supports this idea (Szentirmai and Krueger, in press(a)). Orexin activates neurons in the tuberomamillary nucleus and the PVN (Huang et al., 2001; Mieda and Yanagisawa, 2002), two nuclei thought to play a role in maintaining wakefulness. GHS-Rs are also present in the TMN and PVN (Guan et al., 1997), and it is possible that, in addition to activating orexinergic input to these sites, ghrelin may directly act in these nuclei. Furthermore, ghrelin activates the HPA axis (Arvat et al., 2001; Wren et al., 2002), and its wake-promoting actions may be due to the stimulation of corticotropin-releasing hormone, adrenocorticotropin hormone and cortisol, hormones all of which inhibit sleep (Ehlers et al., 1986; Opp et al., 1989).

Ghrelin-induced changes in sleep duration have a biphasic pattern. The primary effect on vigilance is a prominent dose-dependent increase in wakefulness which is followed by increases in NREMS during hours 3–12 post-injection in response to 1 μg (given at light onset) and the 5 μg dose (given at dark onset) in freely-feeding rats. We posit that the primary effect of ghrelin is to stimulate wakefulness. Since the half-life of ghrelin is about 30 min (Tolle et al., 2002), it is unlikely that the delayed sleep increases are due to the presence of exogenous ghrelin in biologically relevant quantities. It is possible that these increases in NREMS are secondary to sleep loss and/or increased eating in the first hour(s). Eating is known to elicit postprandial increases in sleep (Danguir, 1987; Zammit et al.,

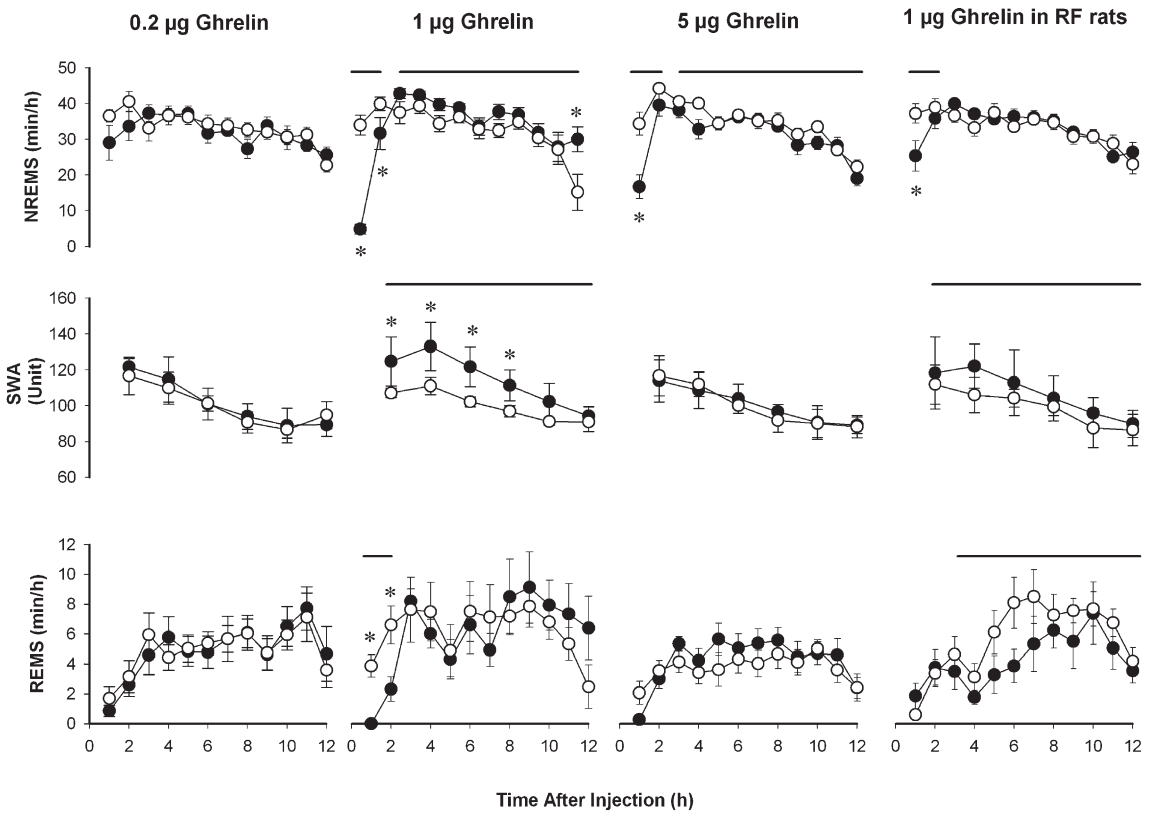

Fig. 4 – The effects of icv administration of ghrelin and PFS on sleep and SWA at light onset. Asterisks denote significant differences between baseline and experimental treatments ($P < 0.05$, Student–Neumann–Keuls test). See legends to Fig. 2 for details.

1992). The finding, that the secondary increases in NREMS are absent in rats that were not allowed to eat after ghrelin treatment strongly suggests that increased feeding could be, at least in part, responsible for those sleep responses.

REMS was also altered by ghrelin. Higher doses of ghrelin given day or night induced a prominent decrease in REMS immediately after injections in agreement with previous observations (Tolle et al., 2002). During the day, injection of 1 µg ghrelin suppressed REMS in the first 2 h in freely fed rats, whereas in RF animals the same treatment decreased REMS during the second part of the light period. A possible explanation for the lack of immediate REMS effects of ghrelin in RF rats is that there was already a tendency towards decreased REMS on the baseline day in this group and it is likely that ghrelin could not elicit further decreases in REMS. The mechanisms responsible for the long-lasting REMS suppressions in the second half of the recording period in RF rats remain to be clarified. One possibility is that the REMS changes may be related to changes in Tcort or body temperature that accompany fasting, since REMS is sensitive to changes in body temperature (Kent et al., 1988). After both the day and night injections of 5 µg ghrelin, a tendency to a slight increase in REMS duration could be observed after a latency of about 2 h. A plausible explanation for this change is that there is unsatisfied hunger after ghrelin injections, which is likely a stressor for the rats; other mild stressors are also known to enhance REMS after a 2- to 3-h delay, and the effects are prolactin-dependent (Bodosi et al., 2000). Alternatively, it is also possible that the REMS-promoting effects of ghrelin are mediated by prolactin. It has been reported that ghrelin stimulates prolactin release (Arvat et al., 2001) and prolactin has a REMS-promoting activity in rats (Roky et al., 1995).

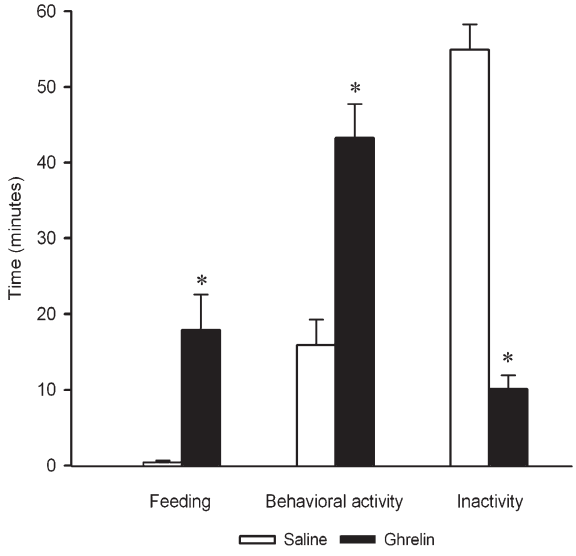

Fig. 5 – The effects of 1 µg ghrelin and saline on behavior in the first hour after the injections. See legends to Fig. 1 for details.

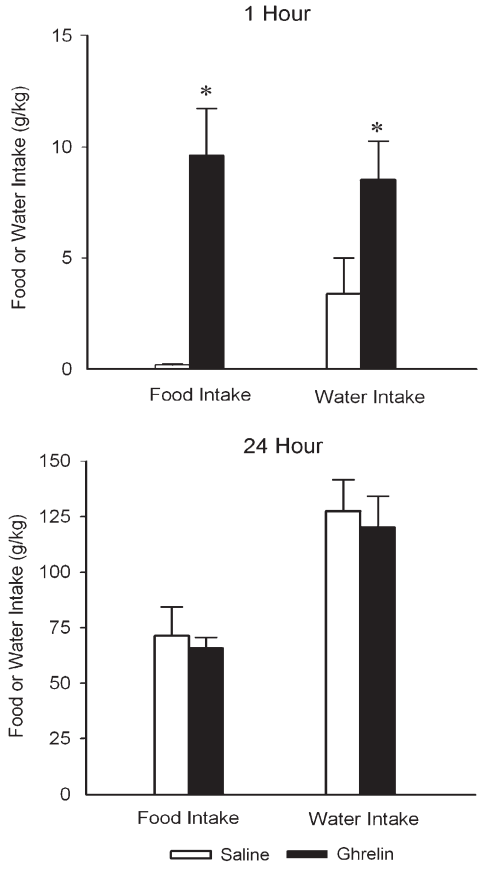

Fig. 6 – The effects of 1 μg ghrelin and PFS on food and water intake after the first and the 24th post-injection hours. See legends to Fig. 1 for details.

Central ghrelin injection elicited opposite changes in NREMS-associated EEG delta power values after light and dark onset injections. Light onset administration of the peptide increased EEG SWA while decreased EEG SWA followed ghrelin given at night. After light onset administration of ghrelin, there was a greater loss of NREMS than after dark onset administration. Since sleep loss is followed by increases in EEG SWA, this may be a partial explanation for the enhancement of EEG SWA observed in the light phase. However, there are data in the literature which suggest that 1–2 h of sleep loss may be too short to elicit such a response (Tobler and Borbely, 1990). Further, after the 5 μg dose, light onset, there was comparable sleep loss, but no increases in EEG SWA. Increased EEG SWA was also observed in RF rats in response to ghrelin, but these changes were comparably lower than that of rats on free-feeding protocol. During the first 2 h after the light onset injection of 1 μg ghrelin in free-feeding rats, the time spent in NREMS decreased, whereas EEG SWA increased. Similar dissociations between NREMS duration and intensity were previously reported. For example, rats fed a cafeteria diet decrease EEG SWA, while NREMS is elevated (Hansen et al., 1998). Similarly, icv infusion of 192 IgG-saporin, a treatment that removes basal forebrain cholinergic neurons increases NREMS at night while causing a decrease in EEG SWA in rats (Kapás et al., 1996). The dissociation between the duration of NREMS and EEG SWA suggests that they are mediated, at least in part, by different pathways; current results are in agreement with this conclusion.

In conclusion, current findings together with previous observations suggest that ghrelin elicits prompt increases in wakefulness and feeding when given before the rest phase of the day in rats. Behavior of the rats in the beginning of the activity period under normal conditions is characterized by typical behavioral patterns that we named “dark onset syndrome”. The behavioral manifestation of the syndrome includes increased eating and motor activity accompanied by high percentage of time spent in wakefulness. Present results are consistent with our hypothesis that ghrelin may have a key role in triggering the behavioral sequence of the dark onset syndrome. There is a marked diurnal rhythm in plasma and hypothalamic ghrelin levels which is linked to feeding cycles. Feeding restriction elevates plasma and hypothalamic ghrelin levels while ghrelin concentrations decline after eating. During the rest phase, when feeding activity is low in rats, plasma and hypothalamic ghrelin levels rise and reach the maximum just before the major eating bout in the first hour of the dark phase (Bodosi et al., 2004). We hypothesize that increasing hypothalamic ghrelin levels may be responsible, at least in part, for triggering behavioral changes characteristic of the beginning of the activity period in rats.

4. Experimental procedures

4.1. *Animals*

Male Sprague–Dawley rats weighing 300–350 g were used. Institutional guidelines for the care and use of research animals were followed and approved by the respective institutional committees.

4.2. *Surgery*

The surgeries were performed using ketamine-xylazine (87 and 13 mg/kg, respectively) anesthesia. Each animal was implanted with stainless steel screw electrodes for electroencephalographic (EEG) recordings over the frontal and parietal cortices and the cerebellum. Stereotaxic equipment was used to insert an intracerebroventricular (icv) cannula into the left lateral cerebral ventricle [0.80 mm posterior from bregma, 1.4 mm lateral from midline, and 4.0 mm ventral from the surface of the skull according to the rat brain atlas by Paxinos and Watson (1997). The guide cannula and the screws were fixed with dental cement (Duracryl) to the skull. The location of the cannula was determined by the gravity method (sudden drop in pressure) during implantation. The drinking response to icv injection of angiotensin was tested 3–4 days after surgery. The rats responding to angiotensin were used in further studies. After recording, trypan blue was injected icv, and the ventricular system was checked for staining. The data refer to only those rats in which the dye stained both the third and the fourth ventricles and the cerebral aqueduct.

4.3. *Sleep–wake recording*

The rats were housed in individual cages. The cages were in a recording room with 12:12-h light–dark cycle (lights on from 8.30 am to 8.30 pm) and with an ambient temperature regulated at 26°C. The rooms were sound attenuated. Water was continuously available. The rats were kept in these conditions for at least 1 week before the operation and for least 10 days of recovery after surgery during which they were connected to the recording cable and habituated to the experimental conditions. 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. Cables from the commutators and electromagnetic transducers were connected to amplifiers. The digitized (128-Hz sampling rate) signals of the EEG and motor activity were collected by computers in an adjacent room. For scoring, the EEG and motor activity signals were restored on the computer screen. Power density values were calculated by fast-Fourier transformation (FFT) for consecutive 8-s epochs in the frequency range of 0.125–20.0 Hz for 0.25-Hz bands and were integrated in 0.5-Hz bins. The states of vigilance were determined for 8-s epochs by the usual criteria as NREMS [high-amplitude EEG slow waves, lack of body movement, predominant EEG power in the delta range (0.125–4.0 Hz)]; REMS (highly regular EEG theta activity with corresponding high FFT theta power, general lack of body movements with occasional twitches); and wakefulness (less regular theta activity, higher delta power than during REMS, frequent body movements). The time spent in each state of vigilance in consecutive 1-h periods and for the 12-h light and dark periods were determined. EEG power values for the 0.125- to 4-Hz delta range during NREMS were integrated and used to characterize sleep intensity, also known as EEG slow-wave activity (SWA). On the baseline day, average EEG SWA values were calculated across the 12-h recording period for each rat to obtain a reference value for each animal. Power densities in 2-h blocks on the baseline day, and the test days were expressed as a percentage of the reference value.

4.4. *Experimental protocol*

Ghrelin (Bachem California Inc.) and pyrogen-free saline (PFS) were intracerebroventricularly injected in a volume of 2 μl. In Experiment 1 with free-feeding rats, the access to food pellets was continuous. Three groups of rats received ghrelin 10–15 min before dark onset in doses of 0.2 μg (n = 11), 1 μg (n = 8), and 5 μg (n = 13). In Experiment 2, another three groups of rats were given the same doses (n = 8, n = 6, n = 12, respectively) before light onset. Each rat was recorded from light onset or dark onset, respectively, for 12 h after injections. In each group, two conditions, a baseline day when 2 μl of PFS was administered and an experimental day when ghrelin was injected, were used. The order of the baseline and experimental days was randomly chosen. Some of the rats (n = 15) were injected with more than one dose of ghrelin; in these cases, the ghrelin days were separated by at least 2 days when no treatment was given to animals. These rats were not selected based on previous responses to ghrelin.

In Experiment 3 with restricted feeding (RF) rats, food pellets were removed at light onset and returned 12 h later at dark onset, each day for at least 10 days before recording. Sleep–wake activity was recorded for 12 h on 2 consecutive days: a baseline day when 2 μl PFS was administered and an experimental day when 1 μg ghrelin was injected. One-half of the rats received PFS on day 1 and ghrelin on day 2, whereas the order of the baseline day and experimental day was reversed for the rest of the rats.

4.5. *Measurement of food and water intake*

Food and water intake were measured in a group of rats (n = 6) injected with 1 μg ghrelin at light onset. This was the same group used in Experiment 2, 1 μg ghrelin, but the food and water intake determinations were done 4 days after the sleep studies. Immediately after injection, animals were returned to cages containing a known amount of chow and water. Food pellets and water bottles were reweighed at 1 and 24 h after the injection. Results are expressed as g food intake/kg body weight ± SE. To detect the behavioral responses to ghrelin, rats were observed through webcams in the adjacent room during the first post-injection hour.

4.6. *Behavioral testing*

To quantify the behavioral responses to ghrelin, the behavior of 6 rats was recorded with the web camera for 60 min after 1 μg ghrelin and PFS injections on two different days and scored at 30-s intervals. The dominant behavior of the previous 30 s of each rat was classified in one of the categories as follows: drinking, feeding, grooming, exploring, and inactivity (irrespective that the rats slept or were obviously awake). For analysis, the following there categories were used, feeding, behavioral activity, which include drinking, grooming, exploring and inactivity.

4.7. *Statistics*

Because of the biphasic effect of ghrelin injections, two-way analysis of variance (ANOVA) for repeated measures was performed for sleep on 1-h time blocks for hours 1 and 2 and for hours 3–12 of the recording period between the baseline day and the experimental days in each group. The treatment effect and the time effect were the two factors of the ANOVA. In addition to the hourly averages, the amount of NREMS and REMS were also calculated for the first 2 h after the injection and for the 3 to 12 post-injection h: the average sleep values were compared between the baseline day and experimental day by paired t test. For statistical analysis of EEG SWA, two-way ANOVA was done on 2-h time blocks for the 12-h recording period. When ANOVA indicated significant variations, the Student–Newman–Keuls (SNK) test was used for post hoc analysis to identify which group and treatment differed from the other groups and treatments. To determine if the effect of ghrelin was dose-related, ANOVA was performed across the effects of the three doses (dependent variable: difference between values on the treatment and baseline day) for NREMS, REMS and EEG SWA on the experimental days. The effects of ghrelin on food and water intake on the baseline and

experimental days were analyzed by paired *t* test. An α-level of $P < 0.05$ was considered to be significant.

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Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats

Éva Szentirmai,[1] Levente Kapás,[2] and James M. Krueger[1]

[1]*Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Neuroscience Program, Washington State University, Pullman, Washington; and* [2]*Department of Biological Sciences, Fordham University, Bronx, New York*

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Szentirmai É, Kapás L, Krueger JM. Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats. *Am J Physiol Regul Integr Comp Physiol* 292: R575–R585, 2007. First published August 17, 2006; doi:10.1152/ajpregu.00448.2006.—Ghrelin, a gut-brain peptide, is best known for its role in the stimulation of feeding and growth hormone release. In the brain, orexin, neuropeptide Y (NPY), and ghrelin are parts of a food intake regulatory circuit. Orexin and NPY are also implicated in maintaining wakefulness. Previous experiments in our laboratory revealed that intracerebroventricular injections of ghrelin induce wakefulness in rats. To further elucidate the possible role of ghrelin in the regulation of arousal, we studied the effects of microinjections of ghrelin into hypothalamic sites, which are implicated in the regulation of feeding and sleep, such as the lateral hypothalamus (LH), medial preoptic area (MPA), and paraventricular nucleus (PVN) on sleep in rats. Sleep responses, motor activity, and food intake after central administration of 0.04, 0.2, or 1 μg (12, 60, or 300 pmol) ghrelin were recorded. Microinjections of ghrelin into the LH had strong wakefulness-promoting effects lasting for 2 h. Wakefulness was also stimulated by ghrelin injection into the MPA and PVN; the effects were confined to the first hour after the injection. Ghrelin's non-rapid-eye-movement sleep-suppressive effect was accompanied by attenuation in the electroencephalographic (EEG) slow-wave activity and changes in the EEG power spectrum. Food consumption was significantly stimulated after microinjections of ghrelin into each hypothalamic site. Together, these results are consistent with the hypothesis that forebrain ghrelinergic mechanisms play a role in the regulation of vigilance, possibly through activating the components of the food intake- and arousal-promoting network formed by orexin and NPY.

lateral hypothalamus; medial preoptic area; paraventricular nucleus; electroencephalogram; slow-wave activity; sleep

GHRELIN IS A 28-AMINO ACID peptide produced by endocrine cells in the gastrointestinal system and by neurons in the central nervous system (21). Circulating ghrelin is derived mainly from the stomach (1). Plasma ghrelin levels inversely correlate with feeding; fasting increases, while food intake reduces, plasma ghrelin concentrations (7, 61). In the brain, ghrelin is produced by arcuate nucleus (ARC) neurons and by a distinct hypothalamic neuronal population in the internuclear region (6). Ghrelin's actions are mediated by the growth hormone secretagogue receptor (GHS-R) (21). GHS-R mRNA is widely distributed in the gastrointestinal tract, sensory vagus fibers, and the brain. Those hypothalamic nuclei that are implicated in the regulation of feeding and/or sleep-wake activity, such as the lateral hypothalamus (LH), paraventricular nucleus (PVN), ARC, dorsomedial hypothalamic nucleus, anteroventral preoptic nucleus, anterior hypothalamic area, suprachiasmatic nucleus, anterolateral hypothalamic nucleus, and the tuberomamillary nucleus (TMN), are relatively abundant in GHS-R (14, 31).

The most widely studied effects of ghrelin are its actions on feeding and its stimulation of growth hormone secretion. In addition, it promotes fat deposition, stimulates gastrointestinal motility, gastric secretion, and the activity of the hypothalamo-pituitary axis (HPA) (22). Ghrelin induces anxiety-like behavior and affects memory (3). Systemic administration of ghrelin strongly stimulates feeding in rats (66), and it enhances food intake and appetite (49, 64) and induces imagination of food (49) in humans. It is hypothesized that circulating ghrelin, acting as a classic hormone, stimulates eating, an action linked to vagal afferents (8) and the ARC (22). Furthermore, ghrelin-producing neurons, together with orexin and neuropeptide Y (NPY) cells, are also part of a food intake-regulatory hypothalamic circuit (22).

Our previous experiments revealed that intracerebroventricular administration of ghrelin suppresses non-rapid-eye-movement sleep (NREMS) and strongly stimulates wakefulness and eating when injected in the rest phase of rats (52). We hypothesized that ghrelin, acting on central GHS-R, may stimulate a behavioral sequence, which includes increased feeding and increased time spent awake. These behavioral manifestations are characteristic of the first hour of the activity period in rats (dark onset syndrome). To characterize the possible hypothalamic sites that may mediate these actions, we studied the sleep and feeding responses to microinjections of three doses of ghrelin in rats. We report herein that ghrelin administration into the LH, medial preoptic area (MPA), and PVN of rats dose dependently increases food intake and the amount of wakefulness and decreases sleep in the first hour after injection.

METHODS

Animals and surgical procedures. Using ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, male Sprague-Dawley rats (275–325 g) were implanted with cortical electroencephalographic (EEG) electrodes and nuchal electromyographic (EMG) electrodes. Stereotaxic equipment was used to insert guide cannulas (26 gauge; Plastics One) bilaterally into the LH ($n = 8$ per dose) and MPA ($n = 7$ per dose) and unilaterally into the PVN ($n = 6–8$ per dose) of the hypothalamus. The coordinates of the tip of the guide cannulas were: 2.1 mm posterior and 2 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for LH; 0.4 mm posterior and 1.85 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for MPA; and 1.80 mm posterior and 0.4 mm lateral to the bregma, and 7.5 mm ventral from the surface of the skull for PVN, according to the

Address for reprint requests and other correspondence: J. M. Krueger, Washington State Univ., College of Veterinary Medicine, Dept. of VCAPP, PO Box 646520, Pullman, WA 99164-6520 (e-mail: Krueger@vetmed.wsu.edu).

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rat brain atlas by Paxinos and Watson (39). The guide cannulas into the MPA and PVN were inserted at a 10° angle from vertical. Substances were administered by a 31-gauge stainless steel injector placed in and projecting 0.5 mm beyond the tip of the cannulas. The position of the cannulas was verified by histology at the end of the experiment. After the surgeries, the animals were placed in individual sleep-recording cages in sound-attenuated, temperature-regulated environmental chambers at 24 ± 1°C ambient temperature on a 12:12-h dark-light cycle (light on at 9:00 AM) for habituation to the experimental conditions for at least 7 days. During this adaptation period, the animals were connected to the recording cables and handled daily to habituate them to the microinjection procedure. Water and food were available ad libitum. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the Institutional Animal Care and Use Committees of Washington State University and Fordham University.

Sleep-wake recordings. The digitized (128-Hz sampling rate) signals of the EEG and EMG were collected by computers. The EEG was filtered >0.1 Hz and <40 Hz. The states of vigilance were visually determined off-line in 10-s epochs by using the conventional criteria we described previously (53). EMG activity served to aid the vigilance-state determination and was not further analyzed. The amount of time spent in each vigilance state was calculated in each hour of the recording period. EEG power density values were calculated for each vigilance state from artifact-free epochs by fast-Fourier transformation for consecutive 10-s epochs in the frequency range of 0.5 to 16.0 Hz in 0.5-Hz bands. Values are means ± SE expressed as percentage of mean total power across the typical frequencies of the behavioral states. In addition, EEG power values for the 0.5- to 4-Hz delta range during NREMS were integrated and used to characterize NREMS intensity, also known as EEG slow-wave activity (SWA). On the baseline day, power density values in the delta range were averaged across the entire 23 h for each rat to obtain a reference value for that rat. SWAs for each hour on the baseline day and the test days were expressed as a percent of that reference value.

Experimental protocol. The treatments were done 10–15 min prior to light onset. The injected volumes were 100 nl per injection site given over 1 min. After the injection, the injectors were left in the guide for an additional minute. On the control day, the animals received 100 nl pyrogen-free isotonic NaCl; on the experimental day, they were injected with ghrelin (0.04 μg, 0.2 μg, or 1 μg/injection site, 12, 60, and 300 pmol, respectively; Bachem California, Torrance, Ca) dissolved in isotonic NaCl (100 nl). The order of the control and experimental days was counterbalanced. Immediately after injection, the animals were returned to their home cages. Food and water were provided ad libitum during the entire recording period. A preweighed amount of chow was placed on a plastic tray at light onset; the pellet leftovers were collected and reweighed 1 h later. Sleep was continuously recorded for 23 h starting from the beginning of light onset.

Verification of cannula placement. To verify the location of the microinjection cannulas, 0.1 μl 5% horseradish peroxidase was injected at the end of the experiment. Rats, anesthetized with isoflurane, were perfused with saline and 4% paraformaldehyde. Brains were removed and kept at 4°C in paraformaldehyde until further examination. The peroxidase-H_2O_2 reaction was visualized by diaminobenzidine in 50-μm thick cresyl violet-stained coronal brain sections. The injection sites were localized with reference to the rat brain atlas by Paxinos and Watson (39). Only animals with correct placement of cannulas were included in the data analysis.

Statistics. Two-way ANOVA for repeated measures was performed on sleep and power spectra data (factors: treatment and time effect or treatment and frequency effect, respectively). For SWA analysis, those hours during which a rat did not have at least 5 min NREMS were excluded. This results in occasionally missing data points. Therefore, instead of repeated-measures ANOVA, two-way ANOVA was performed on SWA. Time spent in sleep and SWA data were analyzed in 1-h time blocks for *hours 1*, *2*, and *3* and separately on 3-h time blocks for *hours 4-23* of the recording period between the baseline day and the experimental days in each group. Average power spectra values during wakefulness and NREMS were analyzed in the first-hour time block after injections. Animals in the first hour after the injections exhibited little if any rapid-eye-movement sleep (REMS), therefore, power spectrum for REMS was analyzed for the first 2-h time block following the injections. When ANOVA indicated significant effects, post hoc comparisons were performed using the Student-Newman-Keuls test to identify which group and treatment differed from the other groups and treatments. Food intake is expressed as gram food intake per kilogram body weight ± SE. The effects of ghrelin on food intake in the first hour after injections were analyzed by paired *t*-test. An α-level of $P < 0.05$ was considered to be significant.

RESULTS

Effects of lateral hypothalamic injection of ghrelin on sleep, EEG, and food intake. The lowest dose of ghrelin (0.04 μg) did not have any significant effect on the amount of wakefulness, NREMS, and REMS and did not affect the SWA of the EEG (Fig. 1). Detailed analysis of the EEG power revealed a significant increase in the 6- to 8-Hz frequency range during wakefulness [ANOVA, treatment × frequency interaction: $F(31,186) = 4.6$, $P < 0.05$] (Fig. 2). There was no significant change in the food intake of the rats in response to 0.04 μg ghrelin (see also Fig. 7).

Administration of 0.2 μg ghrelin significantly increased the time spent in wakefulness and decreased the time in NREMS and REMS [ANOVA *hours 1-3*, treatment effect for wakefulness: $F(1,7) = 16.2$, $P < 0.05$; for NREMS: $F(1,7) = 13.4$, $P < 0.05$; for REMS: $F(1,7) = 27.5$, $P < 0.05$] (Fig. 1). The effects on wakefulness and NREMS were confined to the first 2 h of the recording period, whereas REMS changes were significant in *hours 2* and *3* (Student-Newman-Keuls test). There was a significant effect on EEG SWA in the first 3 h as indicated by ANOVA [treatment × time interaction: $F(2,38) = 3.6$, $P < 0.05$], but post hoc analysis did not show significance in any single hour. Beginning from the fourth hour of the light period, EEG SWA significantly changed [ANOVA *hours 4-23*, treatment × time interaction: $F(6,98) = 2.6$, $P < 0.05$]. EEG power showed a significant increase in the 6.5- to 7.5-Hz frequency range during wakefulness [ANOVA, treatment × frequency interaction: $F(31,217) = 2.8$, $P < 0.05$] and in the 6.5- to 7.5-Hz range during REMS [ANOVA treatment × frequency interaction: $F(31,93) = 2.1$, $P < 0.05$] and a decrease in the 1.5- to 4.5-Hz frequency range during NREMS [ANOVA, treatment × frequency interaction: $F(31,155) = 1.9$, $P < 0.05$] (Fig. 2). Injection of 0.2 μg ghrelin significantly increased the 1-h food intake of the rats from a baseline of 0.65 ± 0.56 to 6.36 ± 1.16 g/kg body wt after ghrelin treatment.

The effects of 1 μg ghrelin injection on sleep were similar to those of the middle dose (Fig. 1). Following ghrelin injection, wakefulness significantly increased and NREMS decreased [ANOVA *hours 1-3*, treatment effect for wakefulness: $F(1,7) = 8.5$, $P < 0.05$; for NREMS: $F(1,7) = 9.9$, $P < 0.05$]. Post hoc analysis showed significant changes in wakefulness and NREMS in the first 2 h after ghrelin treatment. EEG SWA was significantly attenuated in the first hour following ghrelin injection [ANOVA treatment × time interaction: $F(2,36) = 3.9$, $P < 0.05$]. Detailed analysis of the EEG power showed a significant decrease in the 2.5- to 3-Hz range during NREMS

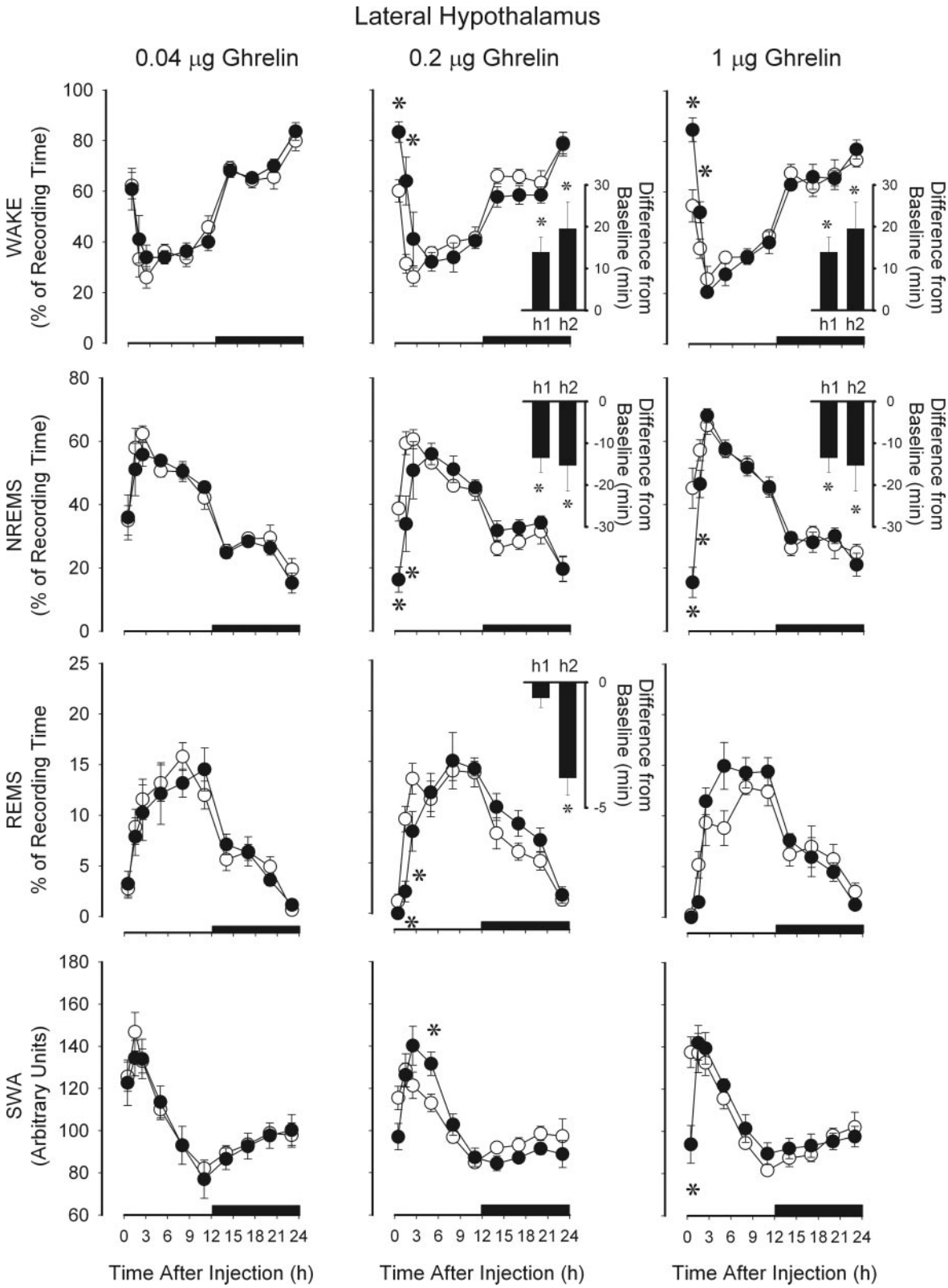

Fig. 1. Wakefulness, non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS), and electroencephalographic (EEG) slow-wave activity (SWA) after ghrelin (●) and isotonic NaCl (○) administration into the lateral hypothalamus. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. *Insets*: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

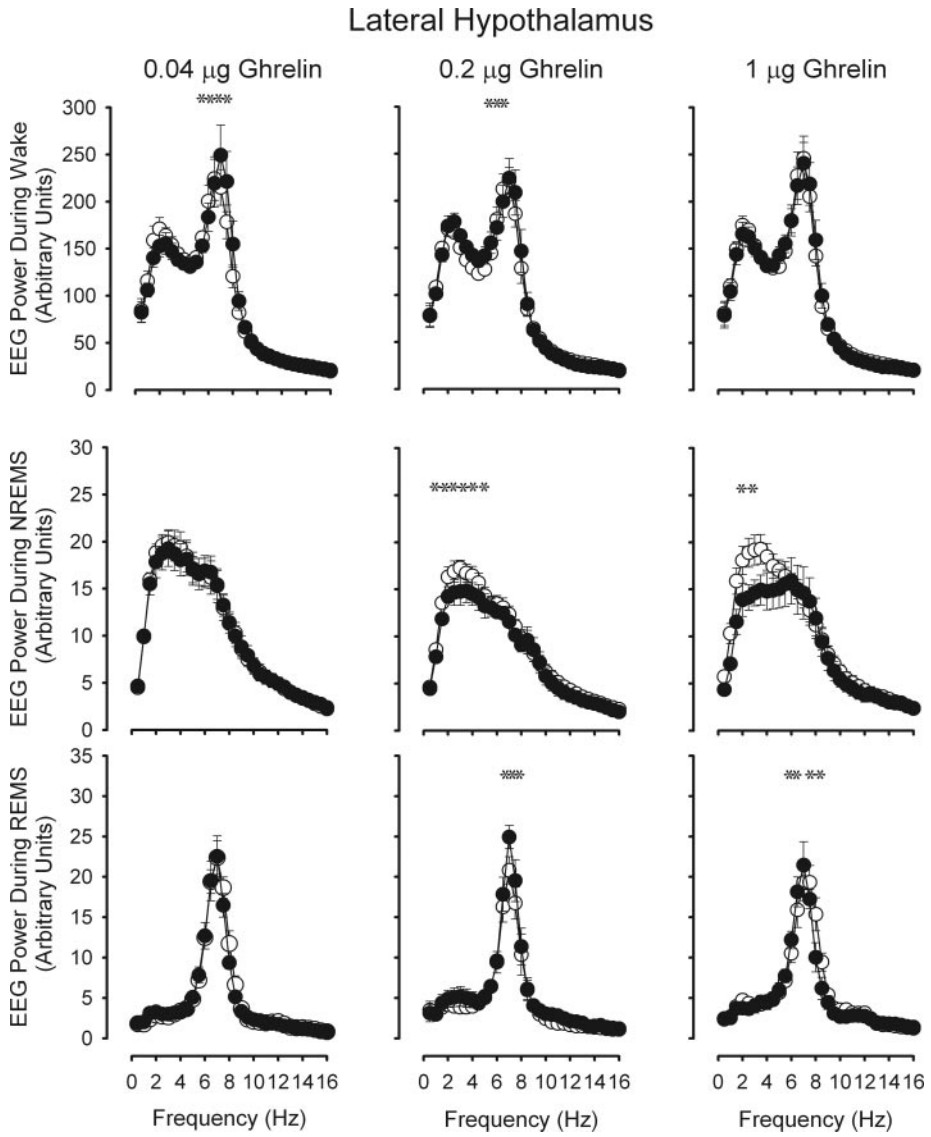

Fig. 2. The EEG power spectra of wakefulness and NREMS averaged over the 1st-hour time block and REMS in the 1st 2-h time block after lateral hypothalamic administration of ghrelin (●) and isotonic NaCl (○). Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

[ANOVA treatment × frequency interaction: $F(31,124) = 1.2$, $P < 0.05$]. During REMS, there was a significant increase in the 6.5- to 7-Hz and a decrease in the 8- to 8.5-Hz frequency ranges [ANOVA treatment × frequency interaction: $F(31,155) = 2.4$, $P < 0.05$] (Fig. 2). One microgram of ghrelin significantly stimulated the 1-h food intake of the rats (0.25 ± 0.14 g/kg body wt after control treatment vs. 7.02 ± 1.75 g/kg body wt after ghrelin treatment) (see also Fig. 7).

Effects of ghrelin injection into the MPA of the hypothalamus on sleep, EEG, and food intake. The lowest dose of ghrelin, 0.04 μg, did not induce any statistically significant change in the time spent in wakefulness, NREMS, or REMS and did not alter the EEG SWA (Fig. 3) or food intake (see also Fig. 7). There was a significant increase in the EEG power in the 7- to 9-Hz frequencies during wakefulness [ANOVA treatment × time interaction: $F(31,186) = 2.0$, $P < 0.05$] and a significant decrease in the 1.5- to 3-Hz frequencies during NREMS [ANOVA treatment × time interaction: $F(31,186) = 3.6$, $P < 0.05$] (Fig. 4).

The middle dose of ghrelin, 0.2 μg, induced a significant increase in time spent awake at the expense of both NREMS and REMS, as indicated by ANOVA [ANOVA h 1–3, treatment effect for wakefulness: $F(1,6) = 26.6$; $P < 0.05$; for NREMS: $F(1,6) = 12.9$; $P < 0.05$; for REMS: $F(1,6) = 15.9$; $P < 0.05$]. Post hoc analysis showed the effects to be confined to the first hour of the recording period. EEG SWA slightly but significantly increased beginning from the fourth hour [ANOVA *hours 4-23*, treatment effect: $F(1,6) = 9.9$, $P < 0.05$] (Fig. 3). EEG power during wakefulness was increased in the 5- to 5.5-Hz frequency range [ANOVA treatment × time interaction: $F(31, 186) = 1.6$, $P < 0.05$], whereas it decreased during NREMS in the 1.5- to 3-Hz frequency range [ANOVA treatment × time interaction: $F(31, 186) = 2.0$, $P < 0.05$] (Fig. 4). Injection of 0.2 μg ghrelin was followed by a significant increase (5.01 ± 0.53 g/kg body wt vs. 0.45 ± 0.23 g/kg body wt after saline injection) in feeding (see also Fig. 7).

The highest dose of ghrelin induced a statistically significant increase in time spent in wakefulness at the expense of NREMS and REMS [ANOVA *hours 1-3*, treatment effect for wakefulness: $F(1,6) = 244.6$; $P < 0.05$; for NREMS: $F(1,6) = 101.6$; $P < 0.05$; for REMS: $F(1,6) = 28.4$; $P <$

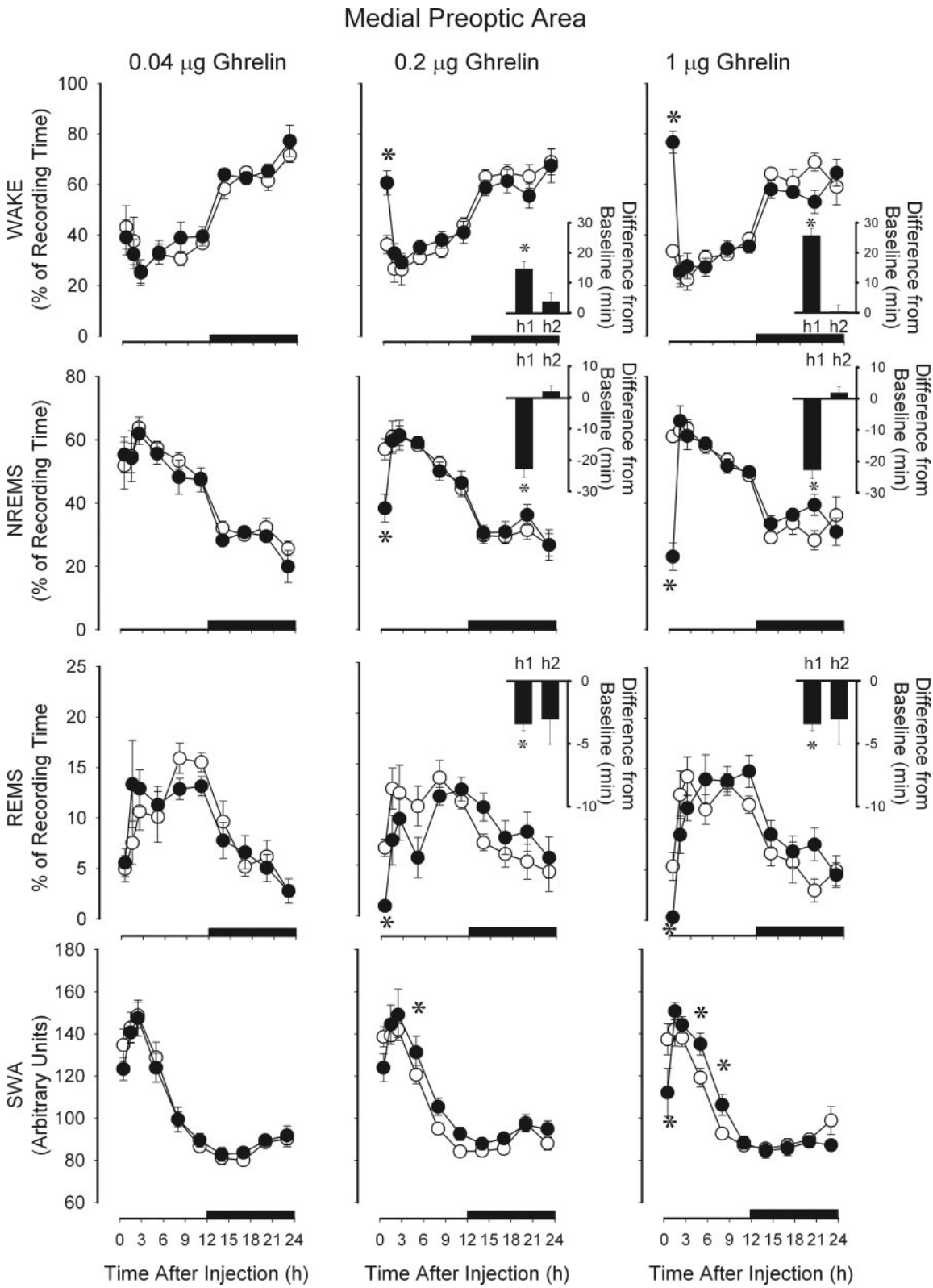

Fig. 3. Wakefulness, NREMS, REMS, and EEG SWA after ghrelin (●) and isotonic NaCl (○) administration into the medial preoptic area. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. *Insets*: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

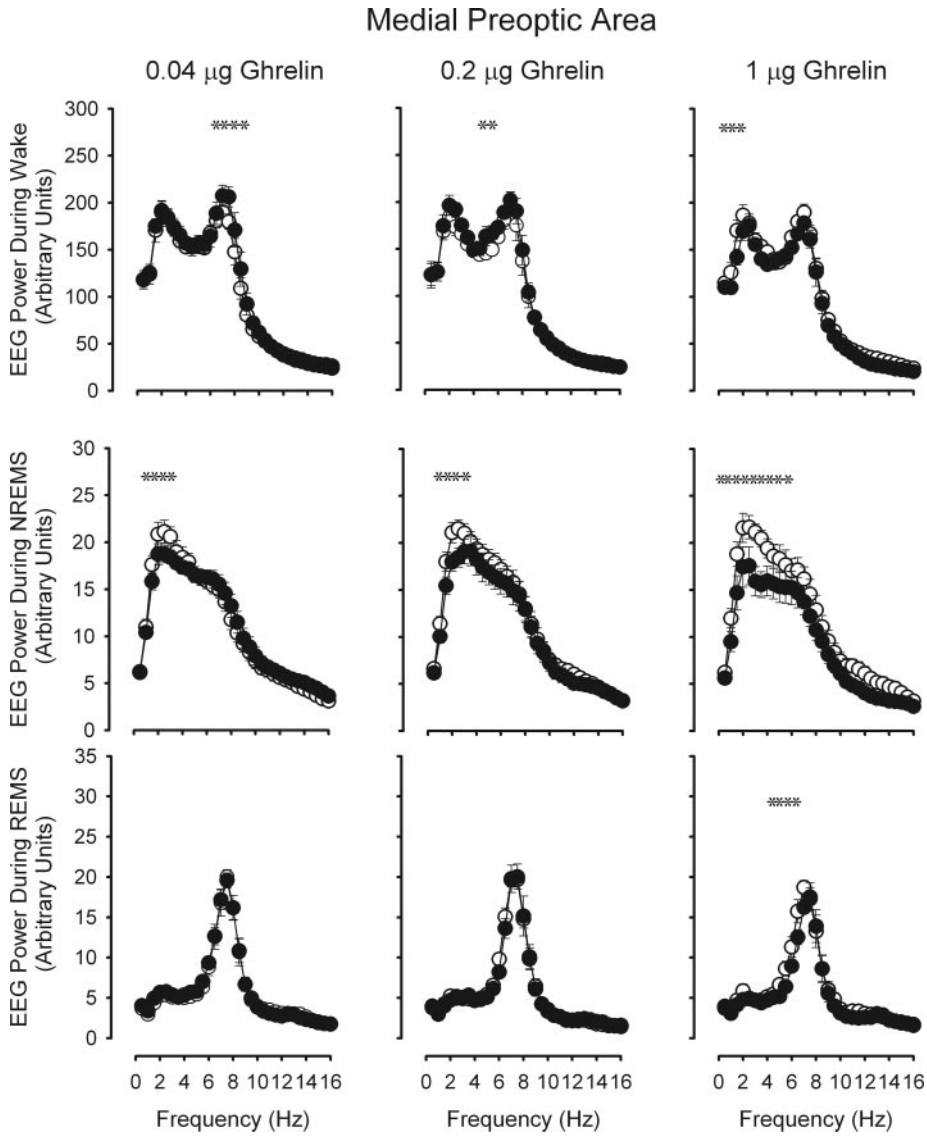

Fig. 4. EEG power spectra of wakefulness and NREMS in the 1st-hour time block and REMS in the 1st 2-h time block after administration of ghrelin (●) and isotonic NaCl (○) into the medial preoptic area. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

0.05]. The effects were confined to the first hour of the recording period. The NREMS changes in the first hour were accompanied by a significant decrease in EEG SWA [ANOVA *hours 1-3*, treatment × time interaction: $F(2,34) = 4.7$, $P < 0.05$]. The initial decrease in EEG SWA was followed by an increase beginning from the fourth hour [ANOVA *hours 4-23*, treatment × time interaction: $F(6,84) = 3.4$, $P < 0.05$] (Fig. 3). The EEG power during wakefulness and sleep was also affected (Fig. 4). One microgram of ghrelin induced a decrease in EEG power in the 1- to 3.5-Hz frequency range during wakefulness [ANOVA treatment effect: $F(1,6) = 18.1$, $P < 0.05$], in the 0.5- to 7-Hz frequencies during NREMS [ANOVA treatment effect: $F(1,6) = 15.2$, $P < 0.05$] and in the 5.5- to 7-Hz frequencies during REMS [ANOVA treatment effect: $F(1,6) = 8.09$, $P < 0.05$]. Food intake was significantly stimulated by 1 μg ghrelin injection (0.74 ± 0.34 g/kg body wt on the control day and 7.44 ± 1.26 g/kg body wt on the treatment day) (see also Fig. 7).

Effects of ghrelin injection into the PVN of the hypothalamus on sleep, EEG, and food intake. The lowest dose of ghrelin failed to induce any significant change in NREMS, REMS, SWA (Fig. 5), or food intake (see also Fig. 7) when injected into the PVN.

Injection of 0.2 μg ghrelin did not change the time spent awake, in NREMS or REMS and there was no significant effect on EEG SWA (Fig. 5). Detailed analysis of EEG power revealed a significant suppression in the 2.5- to 4.5-Hz frequency range during NREMS [ANOVA, treatment × frequency interaction: $F(31,186) = 1.8$, $P < 0.05$] (Fig. 6). Food intake was significantly increased from a baseline of 0.82 ± 0.35 g/kg body wt to 4.52 ± 1.14 g/kg body wt in response to 0.2 μg ghrelin injection (Fig. 7).

One microgram of ghrelin induced a statistically significant increase in time spent awake in the first hour after injection [ANOVA *hours 1-3*, treatment × time interaction: $F(2,14) = 3.8$; $P < 0.05$], which was accompanied by a significant decrease in NREMS [ANOVA *hours 1-3*, treatment × time interaction: $F(2,14) = 4.5$, $P < 0.05$]. EEG SWA did not change in response to 1 μg ghrelin injection (Fig. 5). There was a significant increase in EEG power in the 5- to 5.5-Hz and 7.5-Hz frequencies during wakefulness

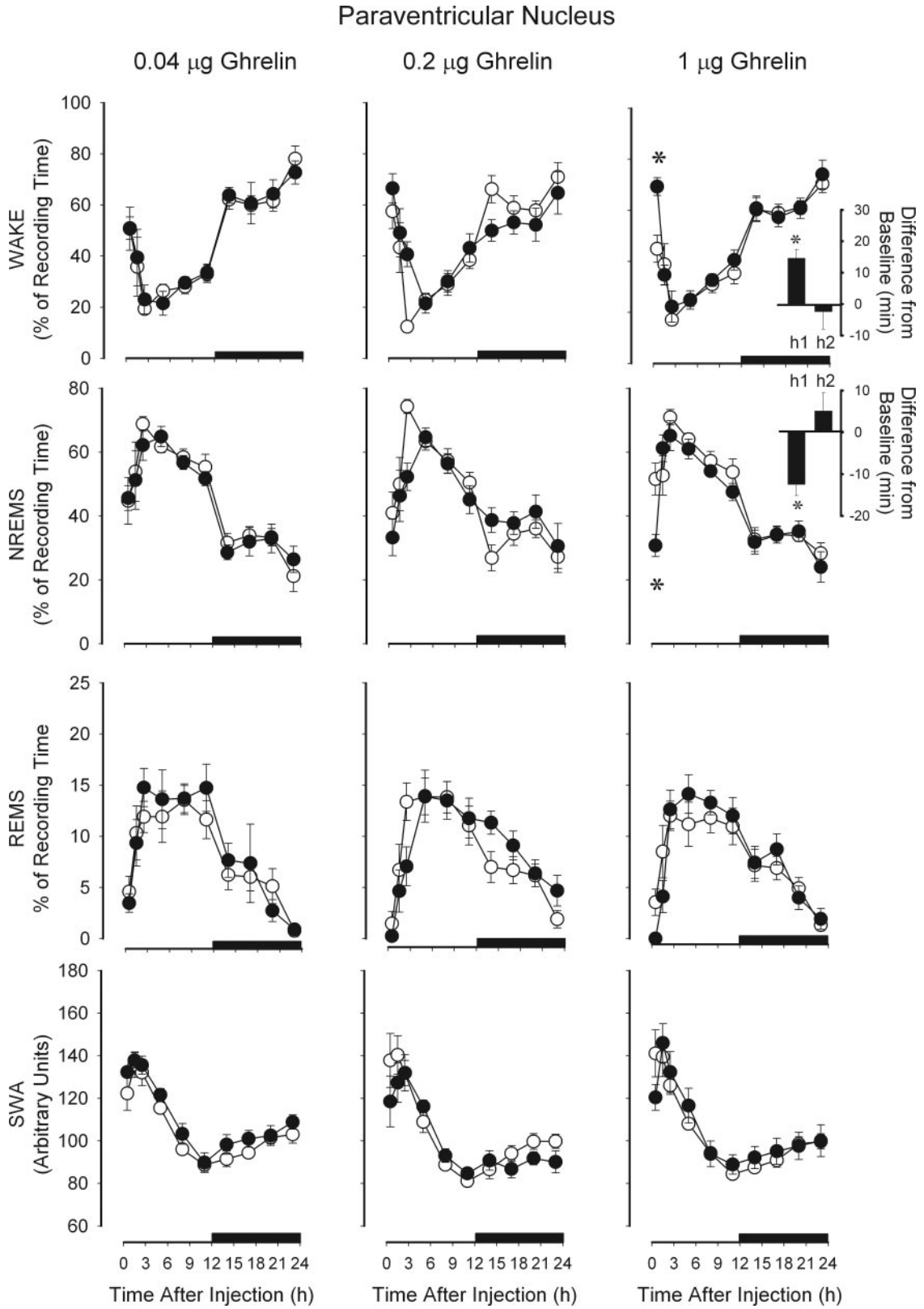

Fig. 5. Wakefulness, NREMS, REMS, and EEG SWA after ghrelin (●) and isotonic NaCl (○) administration into the paraventricular nucleus. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. *Insets*: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

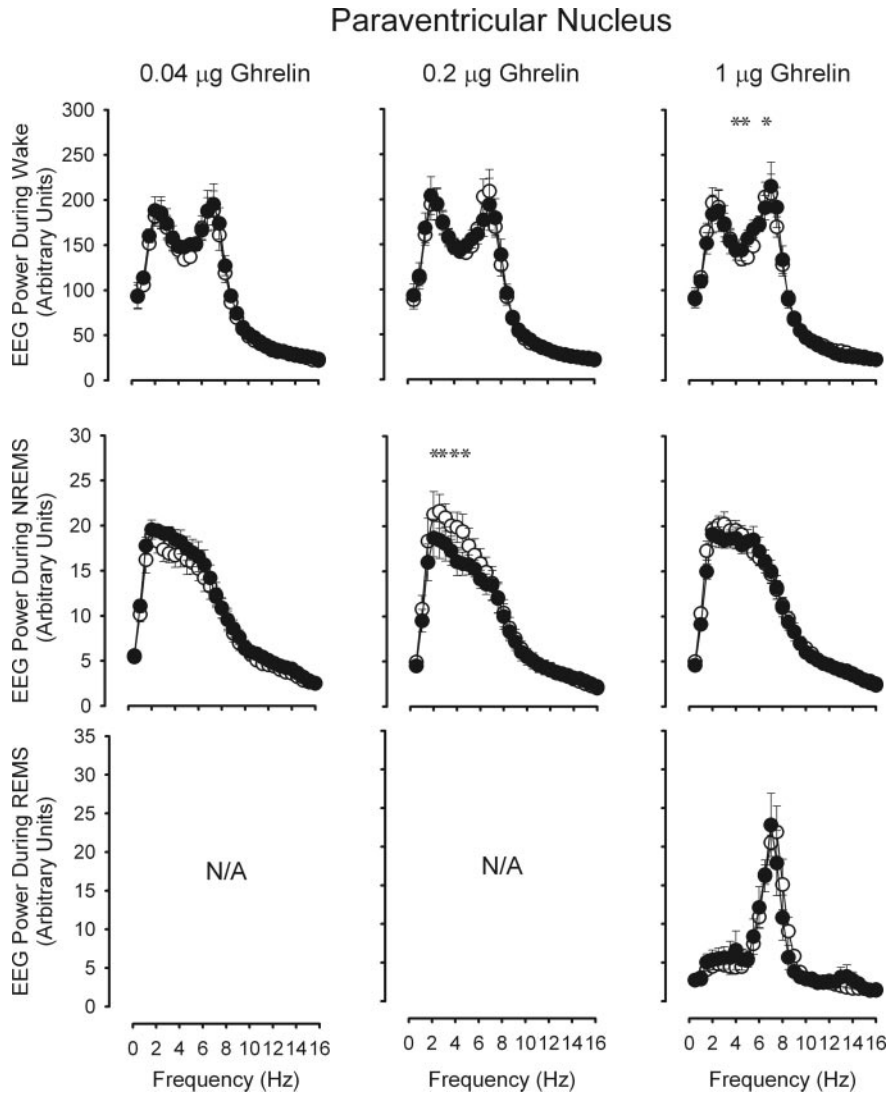

Fig. 6. The EEG power spectra of wakefulness and NREMS in the 1st-hour time block and REMS in the 1st 2-h time block after administration of ghrelin (●) and isotonic NaCl (○) into the paraventricular nucleus. After the injection of 0.04 and 0.2 μg ghrelin, only 3 rats had REMS. Due to the small number of animals, statistical analysis was not performed on these data, and the results are not shown. N/A, not available. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

[ANOVA treatment × time interaction: $F(31,186) = 1.9$, $P < 0.05$] (Fig. 6). Injection of 1 μg ghrelin into the PVN of the rats induced a significant (~4-fold increase) in food intake (Fig. 7).

DISCUSSION

Our findings indicate that microinjections of ghrelin into the LH, MPA, and PVN increase wakefulness, suppress NREMS, and REMS and affect EEG power in rats. Furthermore, our results are consistent with previous observations reporting multiple hypothalamic sites that are sensitive to the orexigenic action of ghrelin (65). Together, these findings are consistent with the hypothesis that brain ghrelinergic mechanisms play a role in the regulation of vigilance and feeding.

Intracerebroventricular (52) and systemic (56) injections of ghrelin during the light period suppress sleep in rats. Also, intracerebroventricular injection of ghrelin (19) and des-acyl ghrelin (58) increases spontaneous locomotor activity of rats. The effects of GHSs on sleep in other species are less consistent and dependent on the timing of the administration. In humans, repeated intravenous bolus injections of ghrelin increase slow-wave sleep and decrease REMS (63). Pulsatile administration of GHRP-6, a GHS-R agonist, increases sleep (12), whereas hexarelin, a more potent agonist, suppresses slow-wave sleep and EEG SWA (11). Single intravenous bolus injection of a third GHS-R agonist, GHRP-2, failed to induce any change on sleep in humans (32). Systemic injection of ghrelin at dark onset stimulates NREMS in control mice but not in a mutant strain lacking the GHRH-receptor (35). In the present experiment, microinjection of ghrelin into various sites of the hypothalamus elicited a prompt and robust increase in wakefulness and decrease in sleep.

The ghrelin-induced increase in wakefulness was most marked when the peptide was injected into the LH. The effects of intracerebroventricular injection of 1 μg ghrelin in our previous study were similar to those seen after the intra-LH injection of 0.2 μg in the present experiments; increasing the intracerebroventricular dose to 5 μg did not result in a further enhancement of the wake-promoting activity (52). A similar ceiling-like phenomenon is seen after LH microinjection. The lack of a significant effect on REMS after the 1-μg dose may be due to the already short REMS after the control injections; it is likely that ghrelin could not elicit further decreases.

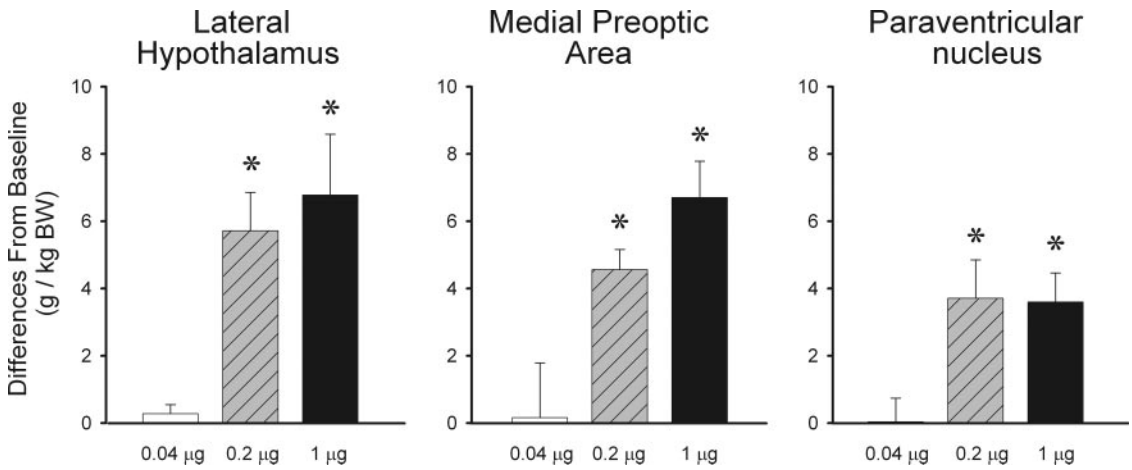

Fig. 7. Food intake after ghrelin administration into the lateral hypothalamus, medial preoptic area, and paraventricular nucleus in the 1st hour after injection. BW, body weight. Error bars: SE. *Significant differences between baseline day and experimental day ($P < 0.05$, paired *t*-test).

According to the classic view, the posterior/LH is a "wake-center" since Von Economo (62) observed excessive sleepiness in patients with the damage of this region. Electrolytic (51) or excitotoxic (47) lesions of the LH, however, yielded inconsistent results. The LH regained the interest of sleep researchers when it became apparent that orexin is produced almost exclusively by LH neurons (34). Orexinergic mechanisms play a central role in the maintenance of wakefulness. Orexin-containing neurons innervate forebrain and brain stem structures that are implicated in arousal (40, 60). Orexins stimulate wakefulness when injected intracerebroventricularly (15) or into the lateral POA, PVN, TMN, or locus coeruleus (2, 18, 29, 48). Orexinergic neurons discharge during active wakefulness, and they are silent in slow-wave sleep (25, 30). Narcolepsy is linked to the lack of orexin and/or orexin receptors (5, 26). There is a close relationship between ghrelin and orexin in the LH. GHS-Rs are present in the LH (31). Ghrelin-containing axons project to the LH and form direct synaptic contact with orexin-producing neurons (57). Intracerebroventricular or local application of ghrelin into the LH of rats activates orexin cells (24, 37, 57, 67). Orexinergic mechanisms may contribute to the feeding effects of ghrelin since pretreatment with orexin antibodies attenuates ghrelin-induced eating and the effect of ghrelin was significantly reduced in orexin-deficient mice (57). We hypothesize that the wake-promoting effects of ghrelin in the LH may also involve the activation of orexinergic mechanisms.

Ghrelin injection into the MPA also increased the amount of wakefulness. The importance of the MPA in the hypothalamic sleep-regulating system is well-documented. Lesion of the preoptic area suppresses sleep (28), electrical (17) or thermal stimulation (46) of the MPA increases sleep, and sleep deprivation induces c-*fos* expression in the MPA (44). Intra-MPA microinjection of adenosine agonist (55), TNF-α (23), and growth hormone-releasing hormone (68) enhances sleep, whereas prostaglandin E2 (27) and octreotide (16) induce arousal. Similar to ghrelin, microinjection of orexin-A into the MPA increases time spent awake (10). GHS-R has been detected in the MPA. Feeding is increased after local ghrelin application to this area (65). The latter finding was confirmed in our present study. It is possible that ghrelin's wake- and feeding-promoting effects are mediated through MPA nitric oxide (NO). NO-producing mechanisms are implicated in the regulation of sleep (20) and feeding (33). Microinjection of a NO-donor into the MPA increased arousal (45). The GH secretion- and feeding-stimulatory actions of ghrelin are NO-dependent (13, 43).

The PVN plays an important role in arousal, autonomic, and behavioral responses to stressors (41). It has reciprocal connections with arousal centers, such as locus coeruleus and raphe nuclei (4, 59). Lesion of the PVN decreases REMS sleep and abolishes the circadian sleep-wake cycles (42). Microinjections of orexin (48) or histamine (50) into the PVN elicit arousal responses. The PVN is also one of the major targets for ghrelin to induce feeding; injections of ghrelin induce c-*fos* expression in the PVN (36) and stimulate eating (65). Similar to prior findings (65), we did not observe a clear dose-response relationship on feeding after PVN injections of ghrelin. Ghrelin injection into the PVN also enhanced wakefulness and suppressed NREMS, but this region appears to be the least sensitive among the three sites for sleep effects. Ghrelin did not reduce REMS, which may be due to the already short REMS on the baseline day at the beginning of the light period. It is possible that ghrelin's actions in the PVN are mediated, in part, through NPYergic mechanisms. Central administration of NPY induces wakefulness in rats (53). NPY-producing neurons in the PVN express the GHS-R (14) and receive ghrelin-positive neuronal projections from the ARC (6). In the PVN, ghrelin binds to the GHS-R localized on the presynaptic terminals of NPY neurons, stimulating NPY release (6). Alternatively, it is possible that ghrelin's wake-promoting ability in the PVN is due to the activation of the HPA axis. Ghrelin facilitates corticotropin-releasing hormone (CRH) in the PVN through stimulating GABA-release from NPY neurons (6). CRH inhibits sleep (9, 38). Recent findings suggest that there is indeed a functional interaction in regulating digestive functions within the PVN between ghrelin and NPY and ghrelin and CRH. Intra-PVN injection of ghrelin stimulates colonic motor function, an effect that is inhibited by local pretreatment with a NPY1- or CRH-receptor antagonist (54). Activation of the PVN may also contribute to the wake-promoting effects of the intra-LH-injected ghrelin since the LH-infusion of ghrelin activates neurons in the PVN as shown by enhanced c-Fos immunoreactivity (36).

Ghrelin microinjections also markedly affected EEG power. NREMS-associated EEG delta power, particularly the 1.5- to 4-Hz frequencies, was significantly attenuated in the first hour after 1 μg ghrelin injections into the LH and MPA. Rats had very little NREMS, which is a possible explanation for the attenuated EEG power. The immediate decrease was followed by an increase in EEG SWA after injections into the MPA. Sleep loss, in general, is followed by increases in EEG SWA. It is possible that the delayed increase in EEG SWA is due to the sleep loss in the first hour after ghrelin treatment. The impact of ghrelin on the EEG power extends to wakefulness and REMS. During wakefulness, EEG power in the 6- to 8-Hz frequencies was increased, suggesting that ghrelin induces not only quantitative but also qualitative changes in wakefulness. Similar EEG power increases in the 6.5- to 7.5-Hz frequencies during REMS after LH microinjections also occurred.

The wake-promoting and food intake-stimulating effects of ghrelin are in the same dose range after both intracerebroventricular (52) and, as the present experiments indicate, hypothalamic injection. Orexigenic hormones may reduce wakefulness indirectly due to their primary effects on feeding. One possibility is that, since sleep and feeding are mutually exclusive behaviors, an increase in feeding might result in shortened sleep time. The other possibility is that hunger, which is an assumed effect of feeding-stimulatory hormones in rats, may cause discomfort that could also interfere with sleep. Our current finding, however, that intra-PVN injection of 0.2 μg of ghrelin stimulated feeding as strongly as did the higher, wake-promoting dose, but did not affect sleep, indicates that the assumed hunger and increased eating activity do not necessarily interfere with sleep. Our previous finding that rats with no access to food also respond with increased wakefulness to central injection of ghrelin (52) suggests that the wake-promoting activity of ghrelin is not due to increased feeding behavior. We hypothesize that increased wakefulness and increased feeding are two parallel outputs of a hypothalamic ghrelin-sensitive circuitry that also involves NPY-ergic and orexinergic neurons and possibly NO and CRH. The activation of this mechanism triggers the behavioral sequence characteristic of the first hours of the activity period in rats (dark onset syndrome). In summary, the present study provides further evidence about the role of ghrelin in sleep-wake regulation. The current findings confirm the notion that ghrelin, as a part of a network, integrates homeostatic processes, such as metabolism and sleep.

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Permanent address of É. Szentirmai: Department of Physiology—A, Szent-Györgyi Medical and Pharmaceutical Center, University of Szeged, Szeged, Hungary H-6720.

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Central administration of neuropeptide Y induces wakefulness in rats

E. Szentirmai[1,2] and J. M. Krueger[2]

[1]*Department of Physiology, A. Szent-Györgyi Medical and Pharmaceutical Center, University of Szeged, Szeged, Hungary; and* [2]*Neuroscience Program, Washington State University, Pullman, Washington*

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Szentirmai, E. and J. M. Krueger. Central administration of neuropeptide Y induces wakefulness in rats. *Am J Physiol Regul Integr Comp Physiol* 291: R473–R480, 2006. First published February 23, 2006; doi:10.1152/ajpregu.00919.2005.—Neuropeptide Y (NPY) is a well-characterized neuromodulator in the central nervous system, primarily implicated in the regulation of feeding. NPY, orexins, and ghrelin form a hypothalamic food intake regulatory circuit. Orexin and ghrelin are also implicated in sleep-wake regulation. In the present experiments, we studied the sleep-modulating effects of central administration of NPY in rats. Rats received intracerebroventricular injection of physiological saline or three different doses of NPY (0.4, 2, and 10 μg in a volume of 4 μl) at light onset. Another group of rats received bilateral microinjection of saline or 2 μg NPY in the lateral hypothalamus in a volume of 0.2 μl. Sleep-wake activity and motor activity were recorded for 23 h. Food intake after the control and treatment injections was also measured on separate days. Intracerebroventricular and lateral hypothalamic administration of NPY suppressed non-rapid-eye-movement sleep and rapid-eye-movement sleep in rats during the first hour after the injection and also induced changes in electroencephalogram delta power spectra. NPY stimulated food intake in the first hour after both routes of administration. Data are consistent with the hypothesis that NPY has a role in the integration of feeding, metabolism, and sleep regulation.

food intake; lateral hypothalamus; electroencephalogram; fast-Fourier transformation; slow-wave activity

NEUROPEPTIDE Y (NPY) is widely distributed in high concentrations in the central nervous system and acts as a neurohormone and neuromodulator. The main source of NPY in the brain is the hypothalamus, particularly the arcuate nucleus (ARC), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), suprachiasmatic nucleus (SCN), and the brain stem (2). NPY is implicated in the regulation of several physiological processes, such as food intake (24, 38), hormone secretions (13, 23, 42), circadian rhythms (1), thermoregulation (19), and blood pressure (7).

NPY is part of the widely studied hypothalamic food intake regulatory circuit that also involves orexin, ghrelin, agouti-related peptide, and melanin-concentrating hormone. All of these neuropeptides stimulate food intake when injected in the cerebral ventricle or in various hypothalamic nuclei (9, 20, 34–36, 46). The NPY receptor family includes at least six subtypes from which the Y1 and Y5 are implicated in the regulation of food intake. Both receptors are present in the PVN, ARC, medial preoptic area, SCN, supraoptic nucleus, and in the lateral hypothalamus (LH; see Ref. 44). The NPY Y1 receptor may be involved in mediating behavioral effects other than feeding, since mice lacking NPY Y1 receptor show reduced locomotor activity (33).

NPY-immunoreactive neurons, originating from the ARC, innervate orexinergic cells in the LH (16). Orexin-immunoreactive axon terminals from the LH end on NPYergic neurons in the ARC (16, 28). Ghrelin is known to act through NPYergic pathways in the ARC (22, 37) to stimulate feeding. Orexin is known to play an important role in maintaining wakefulness (36). Orexin stimulates wakefulness when injected in the cerebral ventricles (14) or in the PVN, DMH, and LH (8). The lack of orexin and/or orexin receptors is linked to narcolepsy (26). Ghrelin also inhibits sleep in rats when injected in the cerebral ventricle (41) or various hypothalamic nuclei (unpublished observations).

Little is known concerning the potential role of NPY in sleep regulation. In one study, visual inspection of the electroencephalograms (EEG) suggested that NPY induces a reduction in desynchronized EEG activity and an increase in synchronized and mixed activity in rats (49). Ehlers at al. (11) found that high doses of NPY decrease EEG power of all frequencies in rats but does not influence sleep onset or the amount of non-rapid-eye-movement sleep (NREMS). In humans, repeated intravenous injection of NPY was reported to promote sleep and reduce sleep latency when given to young normal male subjects (3). The same research group in a more recent study that was done in older male and female patients with depression using age-matched controls found no change in sleep time, only shortened sleep latency after repeated intravenous administration of NPY (15). The aim of our experiments was to study sleep and EEG responses to centrally injected NPY in rats.

METHODS

Animals. Male Sprague-Dawley rats, weighing 275–300 g at the time of surgeries, were used. Rats were housed individually in Plexiglas cages in temperature-controlled (23 ± 1°C) environmental chambers at a 12:12-h light-dark cycle (light on at 9:00 A.M.). Water and food were available ad libitum. Institutional guidelines for the care and use of research animals were followed and approved by the Institutional Animal Care and Use Committees.

Surgery. Rats were anesthetized by intraperitoneal injection of a ketamine and xylazine mixture (87 and 13 mg/kg, respectively). Stainless steel screw electrodes for EEG recordings were placed over the frontal (1.5 mm anterior and 1.5 mm lateral to the bregma) and parietal (4 mm posterior and 2 mm lateral to the bregma) cortices, and electromyographic (EMG) electrodes were implanted in the dorsal neck muscle. Stereotaxic equipment was used to insert an intracerebroventricular cannula (22-gauge; Plastics One) in the right lateral cerebral ventricle [coordinates of the tip of the guide cannula: 0.80

Address for reprint requests and other correspondence: J. M. Krueger, Washington State Univ., College of Veterinary Medicine, Dept. of VCAPP, P.O. Box 646520, Pullman, WA 99164-6520 (e-mail: Krueger@vetmed.wsu.edu).

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mm posterior and 1.5 mm lateral to the bregma, and 4.0 mm ventral from the surface of the skull, according to the rat brain atlas by Paxinos and Watson (32)] or microinjection cannulas (26 G) bilaterally in the LH (coordinates of the tip of the guide cannula: 2.1 mm posterior, 2 mm lateral, and 7.8 mm ventral). The size of the injector cannulas were 30 gauge for the intracerebroventricular cannula and 33 gauge for the microinjection cannulas: both extended 0.5 mm beyond the tip of the guide. The guide cannulas and the screws were fixed with dental cement to the skull.

Verification of cannula placement. The location of the intracerebroventricular cannula was determined by the gravity method (sudden drop in pressure) during implantation, and the drinking response to intracerebroventricular injection of angiotensin (Bachem, Torrance, CA) was tested 3–4 days after surgery and also after the end of the experiments. To verify the location of the microinjection cannulas in the LH, 0.2 μl of 5% horseradish peroxidase was injected in the cannulas at the end of the experiment. Rats, anesthetized with isoflurane were perfused with saline and 4% paraformaldehyde. Brains were removed and kept at 4°C in paraformaldehyde until further examinations. The peroxidase-H_2O_2 reaction was visualized by diamonobenzidine in 100-μm-thick neutral red-stained coronal brain sections. The spread of the injections was <1 mm, as indicated by the enzyme reaction. The injection sites were localized with reference to the rat brain atlas (32 and Fig. 1).

Sleep-wake recording. After surgery, a 7- to 10-day recovery period followed, and then the rats were connected to the recording cable and habituated to the experimental conditions for an additional 5 days. The recording cables were attached to commutators. Cables from the commutators were connected to amplifiers. The digitized (128-Hz sampling rate) signals of the EEG and EMG were collected by computers. The EEG was filtered <0.1 Hz and >40 Hz. EMG activity served the purpose of aiding in determining the vigilance state of the animals. The states of vigilance were determined off-line in 10-s epochs by using the conventional criteria as NREMS [high-amplitude EEG waves, lack of body movement, predominant EEG power in the delta range (0.5–4.0 Hz)]; rapid-eye-movement sleep [REMS; highly regular low-amplitude EEG, dominance of theta activity with corresponding high fast-Fourier transformation (FFT) theta (4.5–8 Hz) power, general lack of body movements with occasional twitches]; and wakefulness (less regular low-amplitude EEG, the lack of the visible theta dominance, and frequent body movements). The amount of time spent in each vigilance state was calculated in 1-h time blocks. Power density values were calculated for each vigilance state by FFT for consecutive 10-s epochs in the frequency range of 0.5–16.0 Hz for 0.5-Hz bands. In addition, EEG power values for the 0.5- to 4-Hz delta range during NREMS were integrated and used to characterize NREMS intensity, also known as EEG slow-wave activity (SWA). Those epochs that contained EEG artifacts were excluded from the FFT analyses.

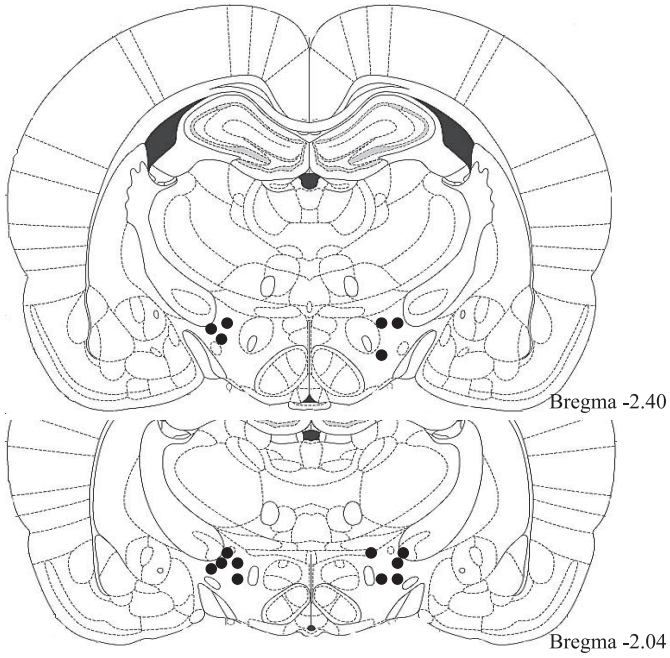

Fig. 1. Representation of the microinjection sites in the lateral hypothalamus [Paxinos and Watson (32)]. Each rat received bilateral microinjections. Values denote distances from bregma.

Experimental procedures. In *experiment 1,* rats were injected intracerebroventricularly with NPY (Bachem California) or pyrogen-free isotonic NaCl (PFS) 10–15 min before light onset. The three doses of NPY were of 0.4 μg ($n = 8$), 2 μg ($n = 9$), and 10 μg ($n = 8$) injected in a volume of 4 μl. In each group, two conditions, a baseline day when 4 μl of PFS was administered and an experimental day when NPY was injected, were used. The order of the baseline and experimental days was chosen randomly. Some of the rats were injected with more than one dose of NPY; at least 1 wk separated the injections. These rats were not selected based on previous responses to NPY. In *experiment 2,* another group of rats ($n = 8$) with bilateral intrahypothalamic cannulas received 2 μg NPY/injection on each side in a volume of 0.2 μl on the experimental day and equal volumes of PFS on the control day. Microinjections took place over a 1-min period, and the microinjection cannulas were left in place for one additional minute. The rats were adapted to the experimental procedures for at least 7 days before the experiments; by the time of the recording, the injection procedures did not cause any visible stress or discomfort to the rats. Each rat was recorded beginning at 9:00 A.M. for 23 h, starting immediately after injections.

Measurement of food intake. Food intake was measured in each group of rats 4 days after the sleep studies. The experimental procedures were similar to those above. Immediately after injection, animals were returned to their home cages where a known amount of chow had been placed. Food pellets were reweighed 1 h after injection. Results are expressed as gram food intake per kilogram body weight ± SE.

Statistics. Two-way ANOVA for repeated measures was performed on sleep and power spectra data (factors: treatment and time effect or treatment and frequency effect, respectively). Those hours, during which a rat did not have at least 5 min NREMS, were excluded from the SWA analysis, resulting in missing data points. Therefore, instead of repeated-measures ANOVA, two-way ANOVA was performed on SWA. Time spent in sleep and SWA data were analyzed in 1-h time blocks for *hours 1*, *2*, and *3* and on 3-h time blocks for *hours 4–23* of the recording period between the baseline day and the experimental days in each group. Average power spectra values during each vigilance state were analyzed in the first 3 h after injections. When ANOVA indicated significant effects, the Student-Newman-Keuls test (SNK test) was used for post hoc analysis to identify which group and treatment differed from the other groups and treatments. The episode numbers, the average episode duration of NREMS and REMS, and the effects of NPY on food intake in the first hour after the injection were analyzed by paired *t*-test. When at least half of the rats did not have a REMS episode in that hour, statistical analysis on average REMS episode duration was not performed. An α-level of $P < 0.05$ was considered to be significant.

RESULTS

Effects of NPY intracerebroventricular injection on sleep. Intracerebroventricular injection of NPY elicited decreases in NREMS and REMS in the first hour after injection (Fig. 2). The lowest dose (0.4 μg NPY) had a statistically significant effect on NREMS, as indicated by ANOVA (Table 1), that was

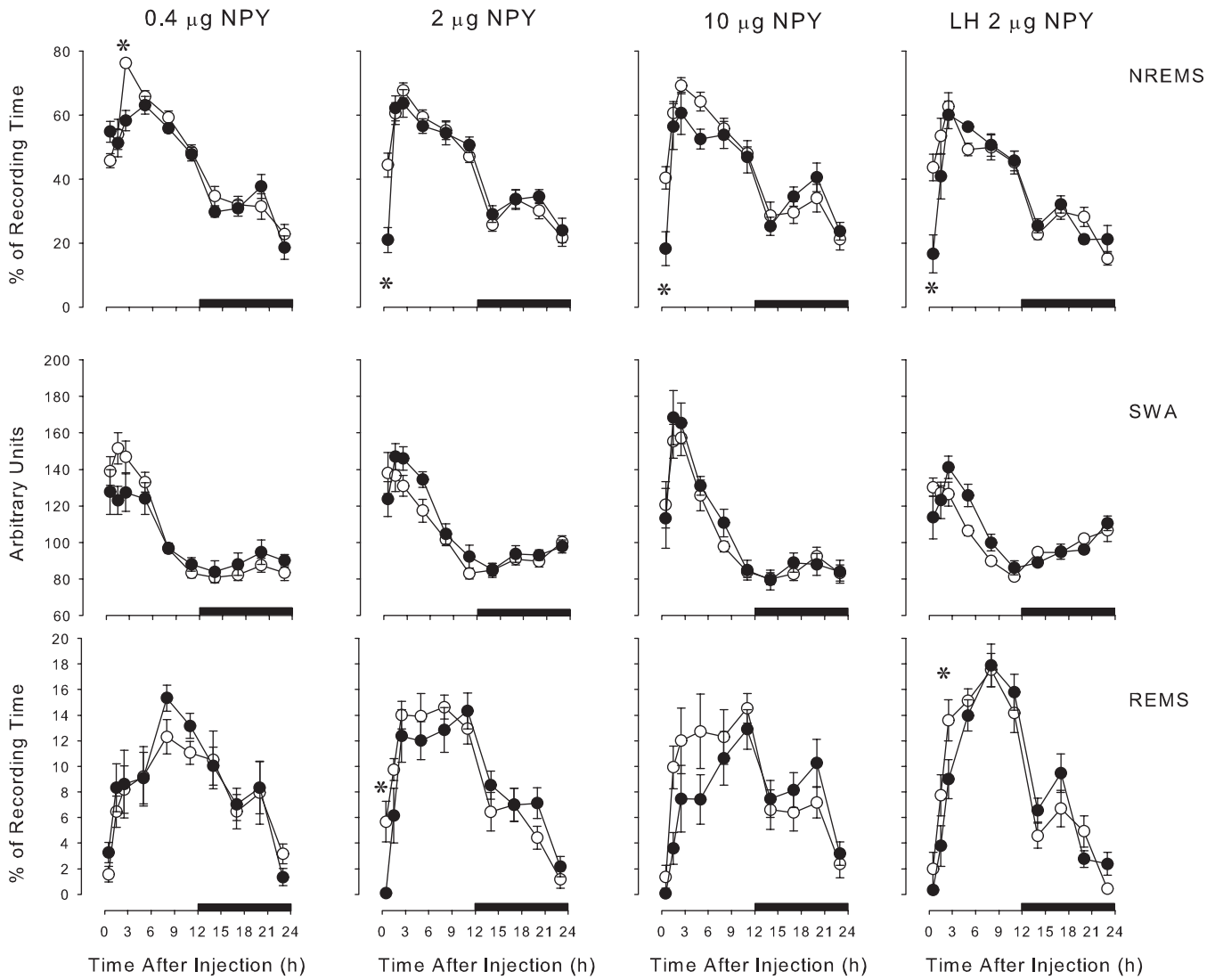

Fig. 2. Effects of intracerebroventricular (icv) and lateral hypothalamic administration of neuropeptide Y (NPY; ●) and pyrogen-free physiological saline (PFS; ○) on sleep and slow-wave activity (SWA). The amounts of non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS), and electroencephalogram (EEG) SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. Error bars indicate SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).

confined to the third hour after the injection (SNK test, Fig. 2); the biological significance of this isolated difference in NREMS between the baseline and experimental day is questionable. There was no significant effect on the total episode number and episode duration of NREMS and REMS and on EEG SWA after the 0.4-μg dose of NPY (Table 2 and Fig. 2). The detailed analysis of EEG power spectrum in the first 3 h revealed significant decreases in the NREMS power spectrum in the 0.5- to 4.5-Hz frequency band; wake and REMS EEG were not affected (Fig. 3). There was no significant change in the food intake of the rats in response to 0.4 μg NPY (Fig. 4).

Administration of 2 μg NPY had significant effects on both NREMS and REMS, as indicated by ANOVA (Table 1). The effects were confined to the first hour; NREMS decreased from a baseline of 26.6 ± 2.2 to 12.6 ± 2.3 min after NPY treatment. REMS virtually disappeared in *hour 1* on the test day. The reduced time spent in sleep may have resulted from a significant decrease in the average duration of NREMS episodes and a significant decrease in the number of REMS episodes (Table 2). There was a tendency toward decreased NREMS episode number, but statistical analyses did not show significant differences. The EEG SWA did not change significantly. Detailed analysis of the EEG showed a significant increase in EEG power spectrum in the 4- to 7-Hz frequency band during wake and REMS (Fig. 3). NPY (2 μg) significantly increased the food intake of the rats (Fig. 4).

The 10-μg NPY injection was also followed by a significant decrease in both NREMS and REMS amount, as indicated by ANOVA (Table 1). Post hoc analyses showed significant suppression in NREMS in *hour 1*. The NREMS decrease may be because of the significant decrease in the number of NREMS episodes; the changes in average NREMS episode duration were not significant. In *hour 1*, on the baseline day, rats already had a minimal amount of REMS, and on the NPY day, they had no REMS at all. Injection of 10 μg NPY did not change the EEG SWA. The EEG power spectra did not show

Table 1. *Effects of icv and lateral hypothalamic administration of NPY on NREMS, REMS amount, EEG SWA, and EEG power spectra of vigilance states*

	NPY, μg									
	0.4		2		10		LH (2 μg NPY)			
	Treatment	Interaction	Treatment	Interaction	Treatment	Interaction	Treatment	Interaction		
NREMS amount	$F(1,7) = 2.287$ NS	$F(9,63) = 3.402$*	$F(1,8) = 1.864$ NS	$F(9,72) = 4.735$*	$F(1,8) = 16.219$*	$F(9,63) = 3.564$*	$F(1,7) = 3.654$NS	$F(9,63) = 4.104$*		
REM amount	$F(1,7) = 2.088$ NS	$F(9,63) = 0.539$ NS	$F(1,8) = 9.885$*	$F(9,72) = 2.483$	$F(1,7) = 13.051$*	$F(9,63) = 2.26$*	$F(1,7) = 0.515$	$F(9,63) = 2.883$*		
SWA	$F(1,140) = 1.939$ NS	$F(9,140) = 1.835$ NS	$F(1,156) = 3.271$ NS	$F(9,156) = 1.263$ NS	$F(1,132) = 0.498$ NS	$F(9,132) = 0.476$ NS	$F(1,136) = 0.96$NS	$F(9,136) = 2.378$*		
Wake power spectra	$F(1,7) = 0.188$ NS	$F(31,217) = 0.523$ NS	$F(1,8) = 4.885$ NS	$F(31,248) = 7.304$*	$F(1,7) = 0.479$ NS	$F(31,217) = 1.247$ NS	$F(1,7) = 1.993$ NS	$F(31,217) = 3.638$*		
NREMS power spectra	$F(1,7) = 3.917$ NS	$F(31,217) = 6.426$*	$F(1,8) = 0.143$ NS	$F(31,248) = 0.619$ NS	$F(1,7) = 0.416$ NS	$F(31,217) = 0.212$ NS	$F(1,7) = 0.083$ NS	$F(31,217) = 0.714$ NS		
REMS power spectra	$F(1,7) = 1.442$ NS	$F(31,217) = 1.038$ NS	$F(1,8) = 6.555$*	$F(31,248) = 6.576$*	$F(1,7) = 0.724$ NS	$F(31,217) = 1.431$ NS	$F(1,7) = 0.247$ NS	$F(31,217) = 0.734$ NS		

icv, Intracerebroventricular; NPY, neuropeptide Y; LH, lateral hypothalamus; NREMS, non-rapid-eye-movement sleep; REMS, rapid-eye-movement sleep; SWA, slow-wave activity; EEG, electroencephalogram. *$P < 0.05$, significant difference between baseline and treatment condition. NS, nonsignificant difference between baseline and treatment condition.

any significant difference in any vigilance state (Fig. 3). The highest dose of NPY significantly increased the food intake of the rats (Fig. 4).

Effects of NPY lateral hypothalamic injection on sleep. The effects of NPY lateral hypothalamic injections on sleep and feeding were similar to those observed after intracerebroventricular treatment (Fig. 1). Time spent in NREMS and REMS was decreased significantly in *hours 1* and *3*, respectively (Table 1). NREMS episode number significantly decreased in *hour 1*, and there was a tendency toward a decrease in average NREMS episode duration as well (Table 2). EEG SWA increased in response to the injection starting from *hour 3*; however, post hoc analyses did not show significance in any particular hour. The detailed analyses of EEG power spectra showed a slight but statistically significant increase of the EEG power spectrum in the 6- to 7.5-Hz frequency band during the wake period (Fig. 2). Food intake was enhanced significantly by lateral hypothalamic injection of NPY (Fig. 4).

DISCUSSION

Intracerebroventricular and lateral hypothalamic administration of NPY suppressed NREMS and REMS in rats when injected at light onset. In addition, it also stimulated food intake in the first hour after both routes of administration. NPY is primarily implicated in feeding regulation. Our findings confirm previous studies that central injection of NPY increases food intake in rats (24, 39).

Previous reports concerning NPY's sleep-modulating effect did not yield consistent results. In rats, intracerebroventricular injection of NPY 3 h after light onset failed to change the amount of time spent in slow-wave sleep (11); the differences in the results between that study and current one may be attributed to the different time of injection. In humans, repeated intravenous bolus injections of NPY during the dark period promoted NREMS and had no effect on sleep EEG spectra in normal young male subjects (3). The same research group carried out a more recent study in older male and female patients with depression (15). NPY infusion caused the shortening of NREMS and REMS latencies but did not affect the time spent in stage 2 sleep, slow-wave sleep, REMS, or total sleep time. There was no significant difference in the responsiveness to NPY between the depressed and control groups. In our experiments, when NPY was injected at light onset, the sleep-suppressive effects were robust; both NREMS and REMS decreased significantly in the first hour of the light period, and REMS practically disappeared. The decrease in NREMS amount in the first hour after the injection is clearly reflected in the decreased total number of NREMS episodes; nevertheless, there was a tendency toward decreased average duration of NREMS episodes as well. After NPY injection, REMS completely disappeared in the first hour of the light period; however, the amount of REMS on the baseline day was also relatively low.

The mechanism through which NPY promotes wakefulness is unknown. NPY-immunoreactive cell bodies are present in the ARC, PVN, SCN, DMH, and LH, nuclei implicated in feeding and sleep-wake regulation. NPY Y1 and Y5 receptors, which are mainly involved in the food intake stimulatory activity of NPY, are also present in these hypothalamic nuclei

Table 2. *Total NREMS and REMS episode number and the average NREMS and REMS episode duration after icv and lateral hypothalamic administration of NPY in the first hour after the injections*

	NREMS Episode No.	Average NREMS Episode Duration, min	REMS Episode No.	Average REMS Episode Duration, min
Baseline	6.8±0.8	4.4±0.5	0.6±0.3	1.6±0.2
NPY (0.4 μg)	8.4±1.1	4.4±0.6	1.3±0.4	1.9±0.4
Baseline	7.2±0.7	4.0±0.5	1.5±0.4	2.3±0.3
NPY (2 μg)	5.2±1.1	2.5±0.4*	0.1±0.1*	NA
Baseline	8.1±0.6	3.1±0.3	0.4±0.3	NA
NPY (10 μg)	4.4±0.9*	2.6±0.8	0.0±0.0	NA
Baseline	6.9±0.9	4.5±0.8	0.5±0.3	NA
LH (2 μg NPY)	3.8±1.3*	2.4±0.7	0.2±0.2	NA

Values are means ± SE. *$P < 0.05$, significant difference between baseline and treatment condition. NA, not available.

(44). NPY-containing axon terminals innervate orexinergic neurons in the LH. Intracerebroventricular injection of NPY increases c-*fos* immunoreactivity in the ARC, PVN (25), and lateral hypothalamic orexinergic neurons (6). Besides stimulating feeding, orexins promote wakefulness and locomotor activity (36); therefore, it is also possible that NPY's stimulatory action on orexinergic cells in the LH mediates the wake-promoting effect of NPY. This notion is supported by our observation that NREMS decreased in response to lateral hypothalamic injection of NPY. A reciprocal relationship exists between NPY and orexinergic neurons. Intracerebroventricular administration of orexins stimulates NPY expression in

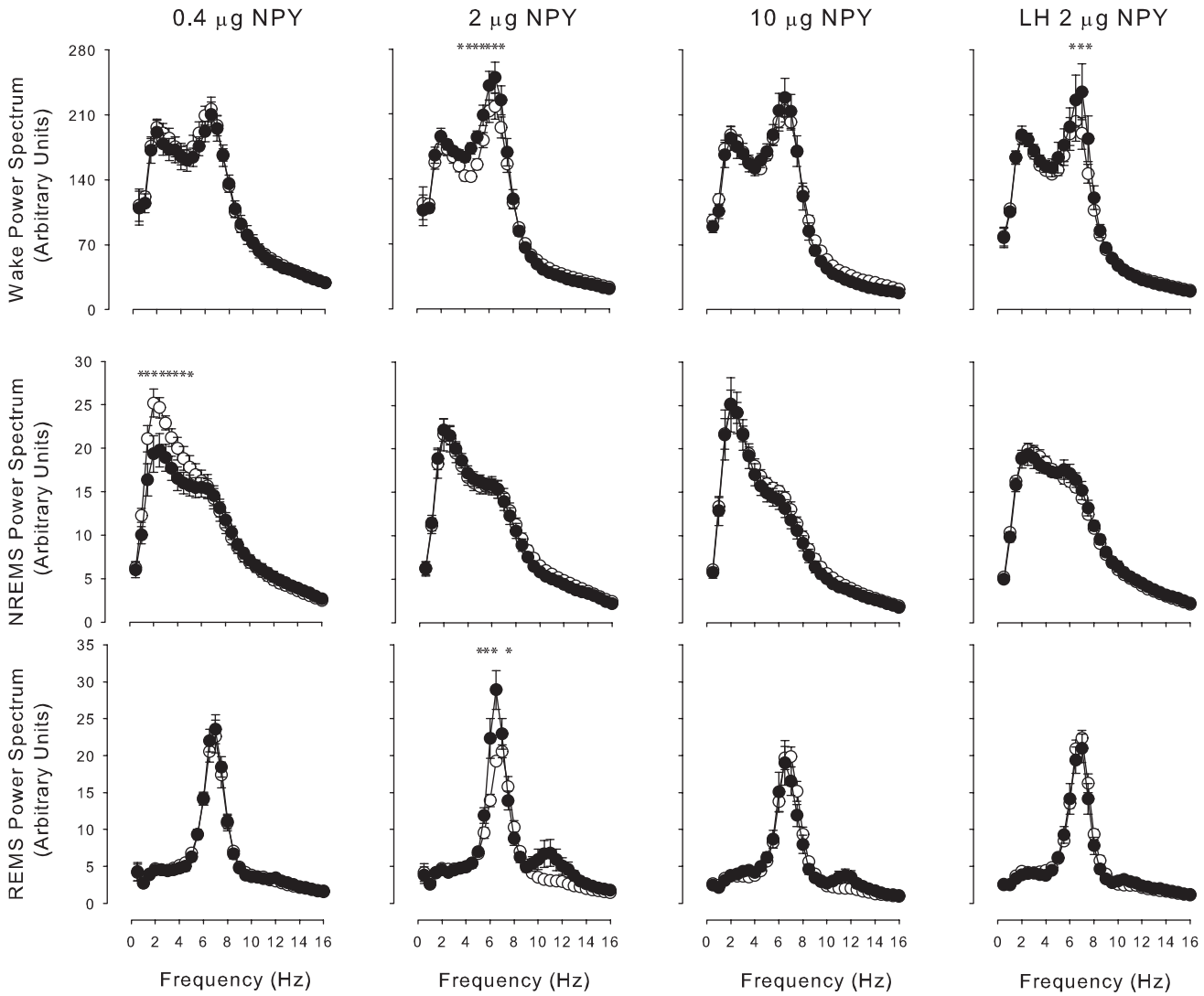

Fig. 3. EEG power spectra of wake, NREMS, and REMS in the first 3-h time block after icv and lateral hypothalamic administration of NPY and PFS. See legend to Fig. 2 for details.

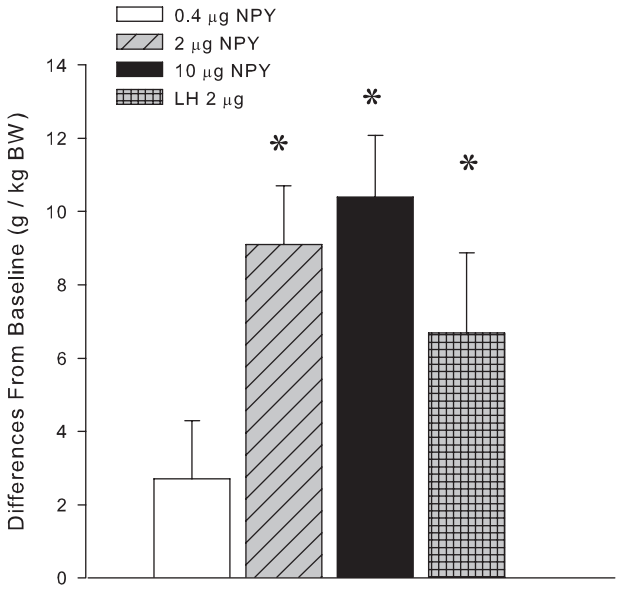

Fig. 4. Effects of icv and lateral hypothalamic (LH) administration of NPY and PFS on food intake in the 1st h after injection. Error bars indicate SE. *Significant differences between baseline day and experimental day ($P < 0.05$, paired *t*-test).

the ARC (27). Orexinergic neuron terminals originating from the LH form synapses on NPY-immunoreactive cells in the ARC and also have close contact with NPYergic cells in the PVN (16). Orexin receptor immunoreactivity is present on NPY neurons in the ARC (4). The feeding stimulatory effect of orexin may be mediated, at least partly, by NPY, since orexin-induced feeding is inhibited by pretreatment with NPY receptor antagonists (17, 47). Conversely, orexin antiserum significantly attenuates the feeding response to NPY (30). In addition to orexinergic neurons, NPY activates other mechanisms in the hypothalamus known to be involved in promoting arousal. For example, NPY stimulates corticotropin-releasing hormone (CRH) release and increases CRH gene expression in the PVN (40). CRH is known to inhibit sleep (10, 31). Therefore, CRH is another candidate for mediating the wakefulness-stimulating effect of NPY.

The sleep-suppressing and food intake-promoting activities of NPY in rats are very similar to those observed after central injection of ghrelin. Intracerebroventricular administration of ghrelin decreases NREMS and REMS dose dependently and increases food intake of rats in *hour 1* after the injection (41). Ghrelin and NPY are part of the hypothalamic food intake regulatory circuit. Both peptides strongly promote feeding behavior in rats (5), and we found that both suppress sleep. Sleep and feeding are mutually exclusive behaviors; therefore, any increase in feeding might result in less sleep time. In our previous study, the wakefulness-enhancing activity of ghrelin was not a direct consequence of its food intake-promoting activity because ghrelin also induced wakefulness in rats that had no access to food. In the present study, the sleep effect of NPY in food-deprived rats was not examined; nevertheless, it is possible that NPY, similarly to ghrelin, suppresses sleep not exclusively because it enhances a competitive behavior such as feeding. There is a growing body of evidence suggesting that NPY may mediate the food intake-promoting activity of ghrelin. Ghrelin receptors are located on NPY neurons in the ARC (43). Intracerebroventricular injection of ghrelin induces c-*fos* (29) and NPY mRNA expression in the ARC (37) and stimulates electrophysiological activity in NPY neurons (22). Ghrelin stimulates the synthesis and release of NPY in the ARC (45). Ghrelin-induced feeding is decreased by pretreatment with NPY antibodies or receptor antagonists (29).

In addition to its sleep-modulating effect, NPY also induced changes in EEG power spectra. The 0.4-μg dose significantly decreased the EEG power spectrum in the 0.5- to 4.5-Hz frequency range during NREMS, whereas the 2-μg dose induced an increase in the power spectra during wake and REMS in the 4- to 7-Hz frequency band in the first 3 h after the injections. In contrast, previous studies found that intracerebroventricular injection of NPY induced the slowing of high-frequency theta activity simultaneously with the speeding up of low-frequency theta waves in cortex, hippocampus, and amygdala in rats (11). Two studies from the same research group found increased synchronized and mixed EEG activity after central administration of NPY in rats (12, 49). The differences between the results of the studies above and ours may be because of the different experimental conditions. In our experiment, we recorded the EEG from the beginning of the light onset, starting immediately after the injection. In the above experiments, NPY was injected 2 h after light onset, and the EEG recording started 20 min after the injection and lasted for ~2 h during which the animals were removed from their home cages. The timing of NPY injection could be a crucial factor, since NPY is known to modulate the activity of the SCN (48).

In conclusion, the current findings suggest that NPY elicits a prompt increase in wakefulness and feeding when given before the rest phase of the day. The food intake-enhancing and the wakefulness-promoting effects of NPY are in the same dose range, since the lowest dose that did not induce significant food intake also failed to cause a biologically relevant difference in sleep. Increased eating activity accompanied by a high percentage of time spent awake are characteristics of the first and second hours of the active period of rats under normal conditions ("dark-onset syndrome"). Orexins, NPY, and ghrelin are parts of the hypothalamic food intake regulatory circuit. It is possible that the activation of the same circuit results in wakefulness, which is independent, at least in part, from the food intake-inducing actions. Additional evidence supporting the role of NPY in sleep regulation include that chronic REMS deprivation increases NPY expression in the rat hypothalamus (21) and NPY-like immunoreactivity shows a diurnal rhythm in the SCN and ARC, with a significant peak before onset of the dark period (18). We hypothesize that increasing hypothalamic NPY levels may be responsible, at least in part, for triggering behavioral changes characteristic of the dark-onset syndrome.

GRANTS

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