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

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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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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.

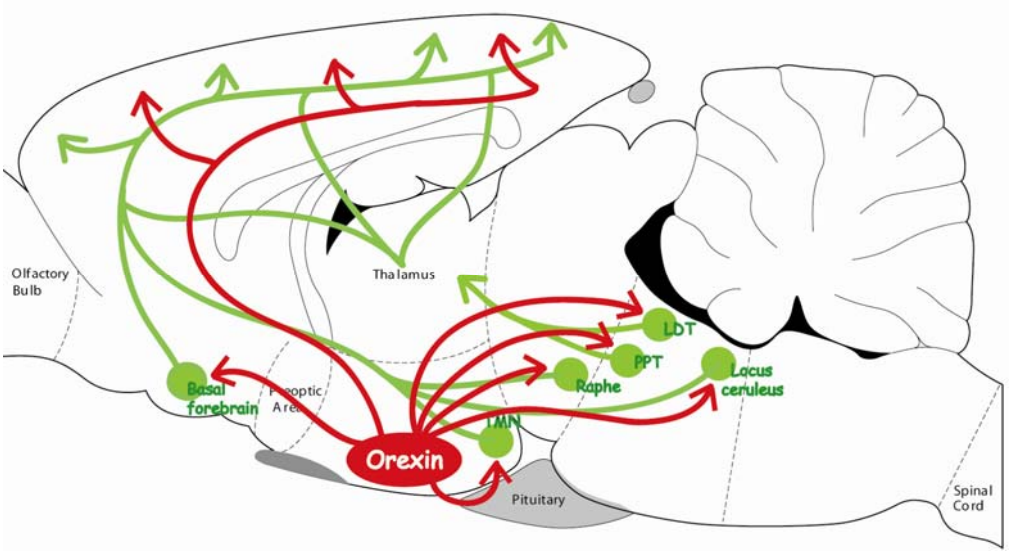

Figure 1. Wakefulness-promoting centers in the brain. Schematic sagittal drawing of the rat brain showing the major centers and pathways involved in promoting wakefulness. LDT: laterodorsal tegmental nucleus, PPT: pedunculopontine tegmental nucleus, TMN: tuberomamillary nucleus.

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.

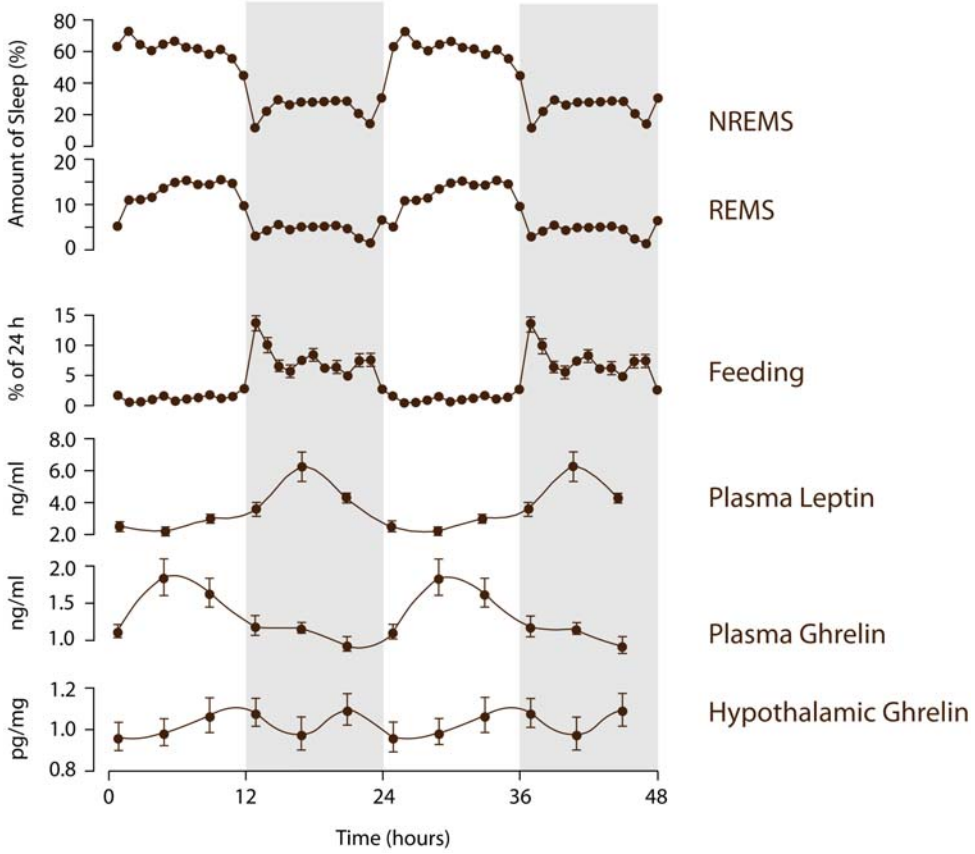

Figure 2. Diurnal rhythms of non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS), feeding activity, plasma leptin and ghrelin levels and hypothalamic ghrelin contents in free-feeding rats. Sleep and feeding activity values are the average of 3 consecutive days from 51 rats and expressed 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.

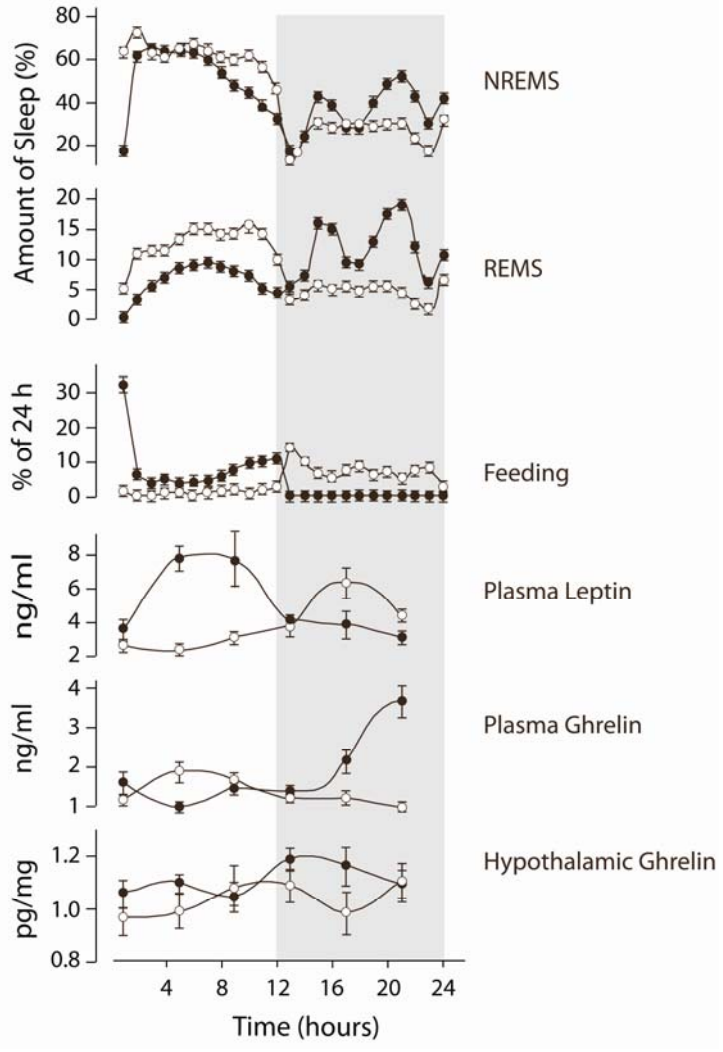

Figure 3. The effects of restricted feeding on the amount of NREMS and REMS, feeding activity, plasma leptin and ghrelin levels and hypothalamic ghrelin content. Data are obtained from 55 rats. Open symbols: baseline day, solid symbols: restricted feeding day. See legend for Figure 2 for details. Modified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

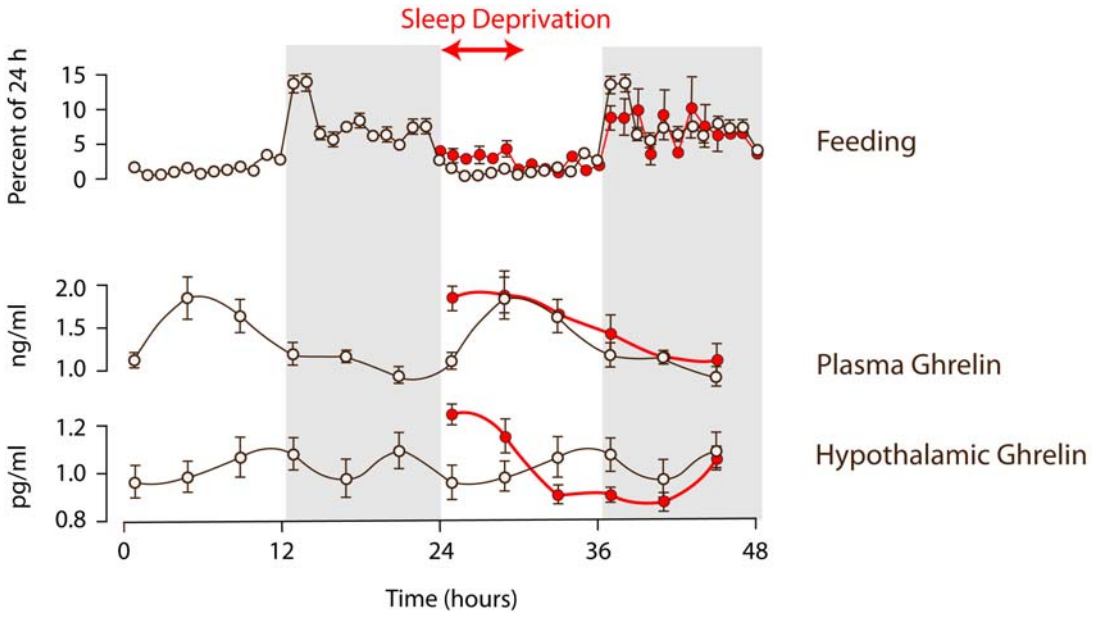

Figure 4. The effects of 5 hours of sleep deprivation on feeding activity, plasma ghrelin levels and hypothalamic ghrelin contents. Open symbols: baseline day, solid red symbols: sleep deprivation day. Data are obtained from 47 rats. Red arrow represents the 5 hours of sleep deprivation period started 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, $F(1,12) = 9.8$, $p < 0.05$].

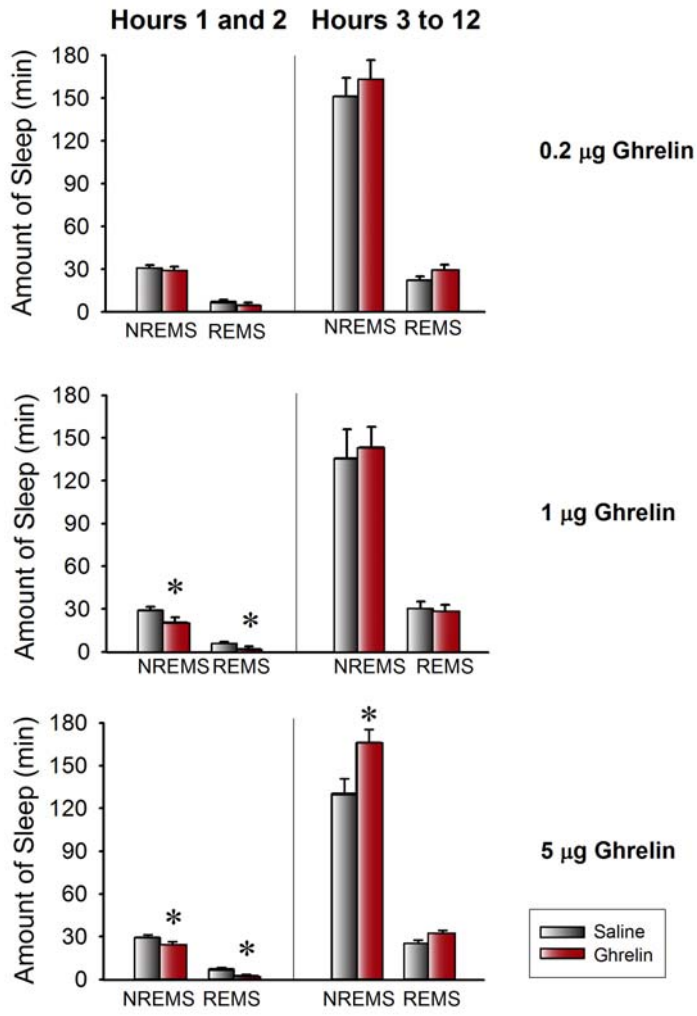

Figure 5. The effects of intracerebroventricular injection of 3 doses of ghrelin on NREMS and REMS. Sleep is expressed in minutes, as time spent in NREMS and REMS during the 2- or 10-hour period. Asterisk denote significant differences between control and test day ($p < 0.05$, paired *t* test). Modified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

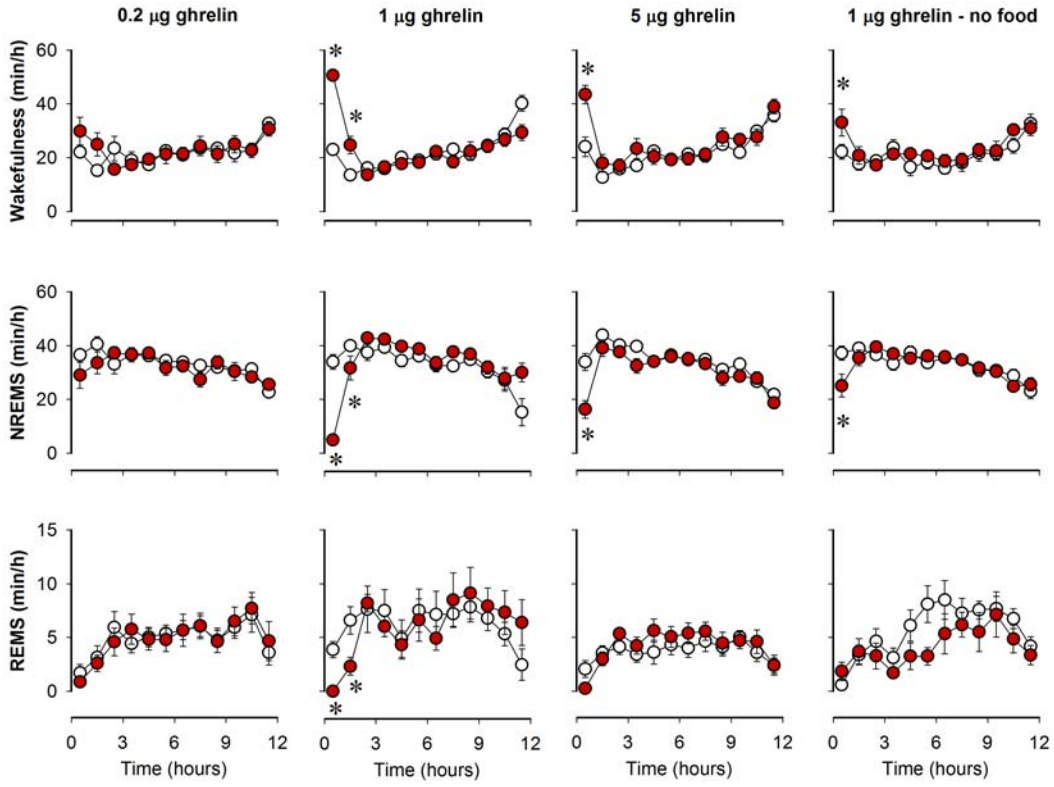

Figure 6. The effects of icv injection of 3 doses of ghrelin on wakefulness, NREMS and REMS in free-feeding and 1μg ghrelin in feeding restricted rats. Open symbols: baseline day, solid red symbols: ghrelin day. Time 0: time of injections. Asterisks denote significant differences between control and 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).

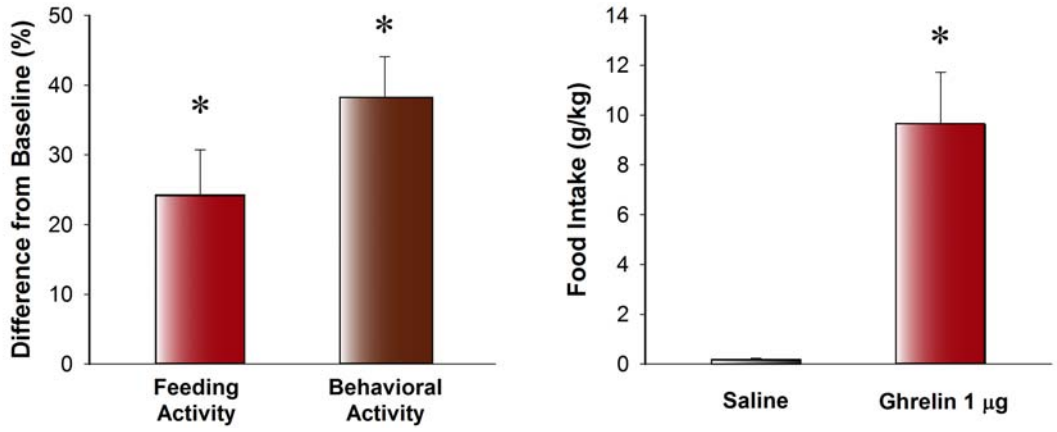

Figure 7. The effects of icv administration of 1 µg ghrelin on feeding and behavioral activity (left panel) and on 1-h food intake (right panel) in rats. Food intake is expressed as g consumed food/kg body weight. Asterisks denote significant differences between control and experimental day ($p < 0.05$, 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

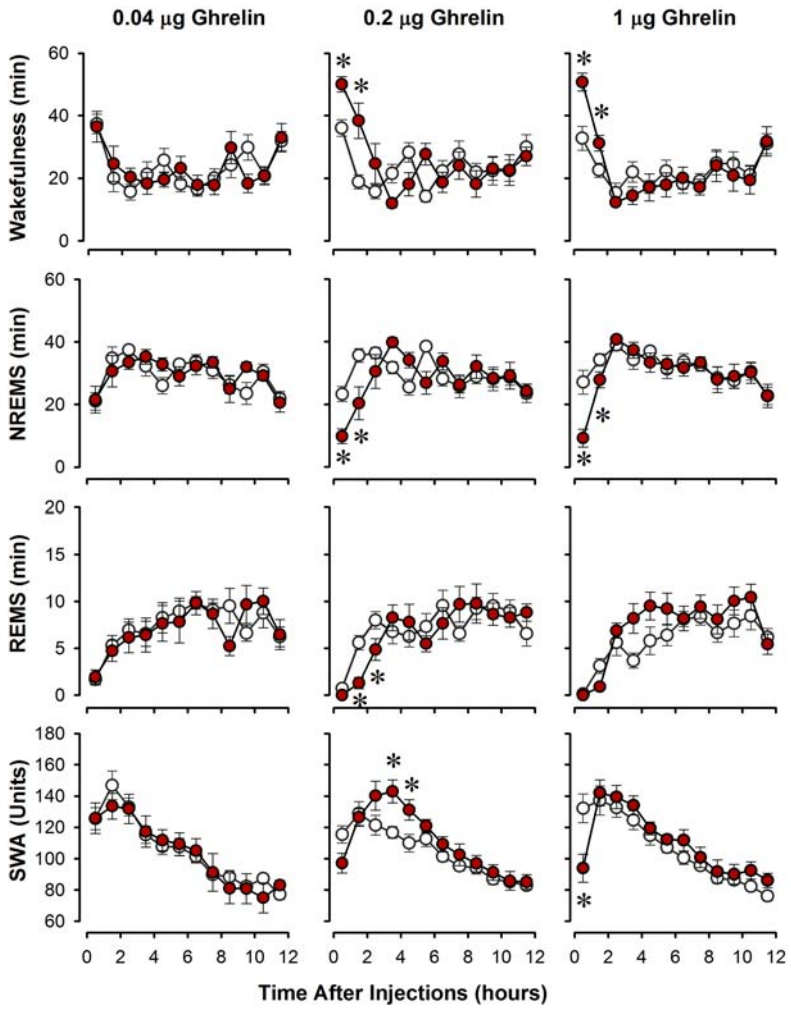

Figure 8. The effects of lateral hypothalamic (LH) administration of three doses of ghrelin on wakefulness, NREMS, REMS and electroencephalographic (EEG) slow-wave activity (SWA). Open symbols: baseline day, solid red symbols: ghrelin day. Time 0: time of injections. Data are expressed in minutes, as 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).

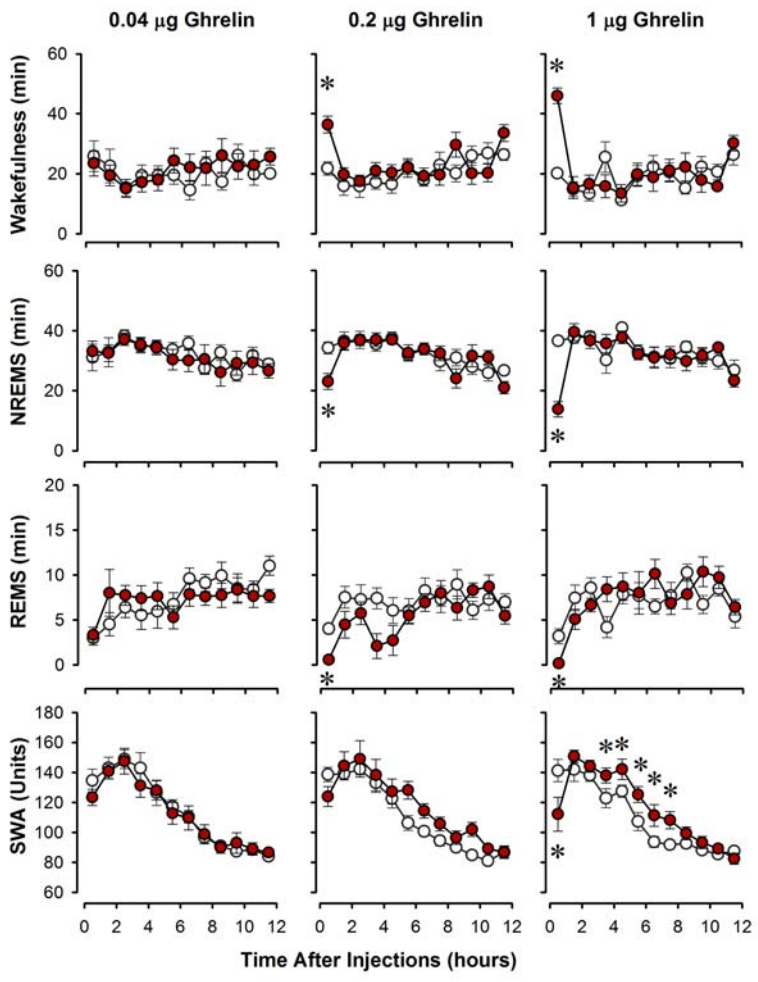

Figure 9. The effects of 3 doses of ghrelin microinjection into the medial preoptic area (MPA) on wakefulness, NREMS, REMS and EEG SWA. See legend to Figure 8 for details. Modified from Szentirmai et al., 2007, Figure 1.

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

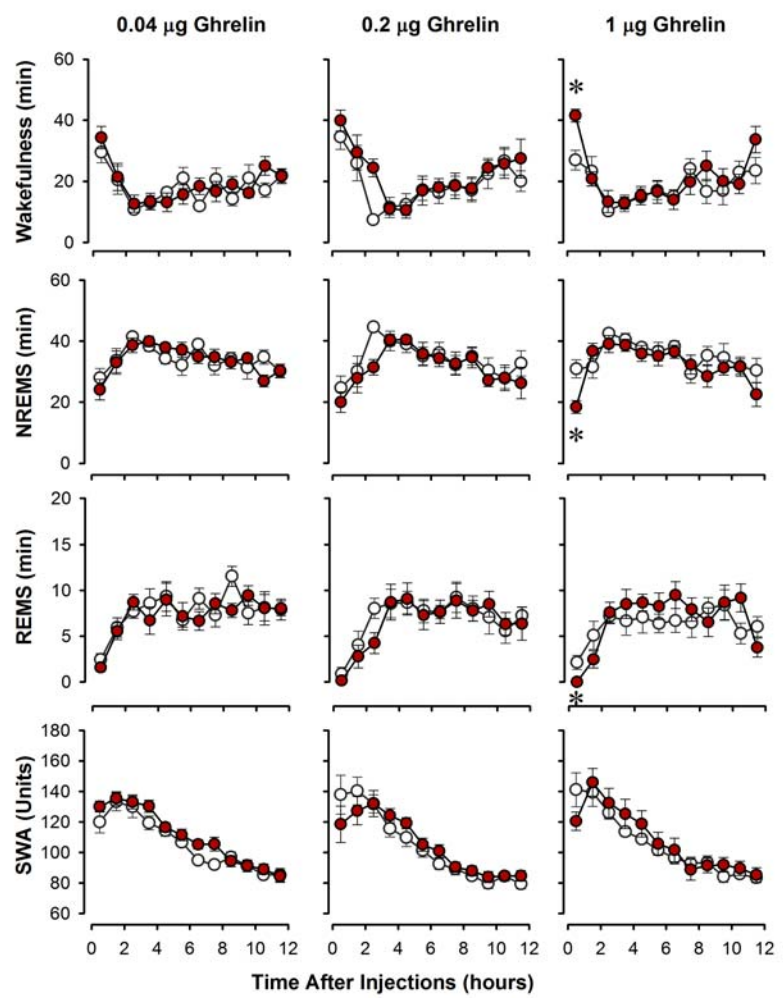

Figure 10. The effects of 3 doses of ghrelin microinjection into the paraventricular nucleus (PVN) on wakefulness, NREMS, REMS and EEG SWA. See legend to Figure 8 for details. Modified from Szentirmai et al., 2007, Figure 5.

(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

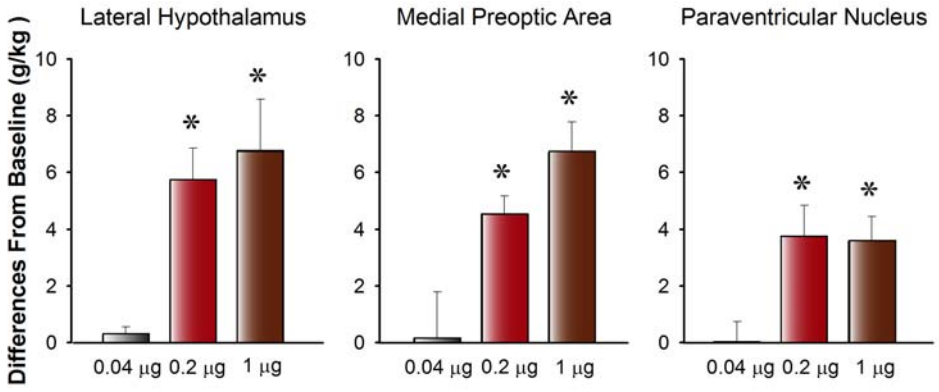

Figure 11. The effects of 3 doses of ghrelin microinjected into the LH, MPA or PVN on food intake. Data are expressed as gram food intake per kg body weight ± SE and plotted as differences from baseline. *: Significant differences from baseline ($p < 0.05$, paired *t*-test). Modified from Szentirmai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

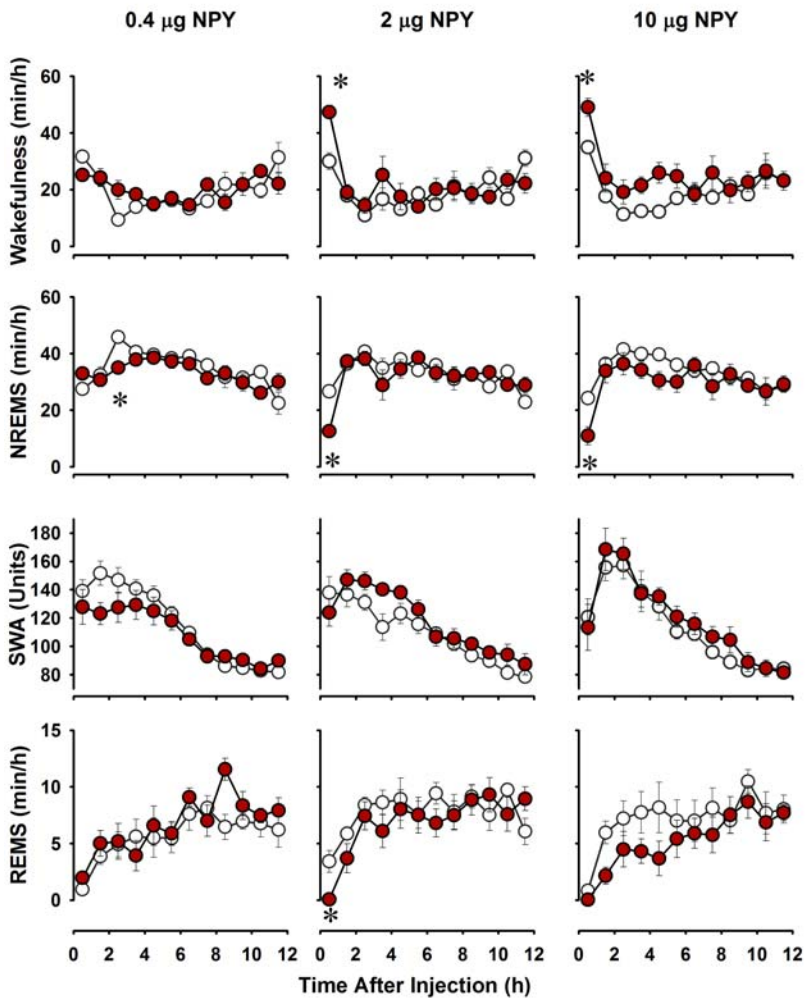

Figure 12. The effects of 3 doses of icv injection of neuropeptide Y (NPY) on wakefulness, NREMS, REMS and EEG SWA. Open symbols: baseline day, solid red symbols: NPY day. Time 0: time of injections. Data are expressed in minutes, as time spent in wakefulness, NREMS and REMS during the 1-hour period. 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).

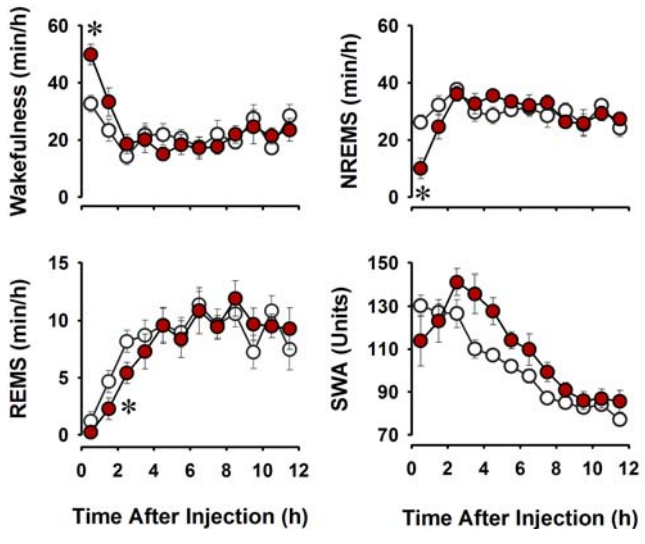

Figure 13. The effects of 2 µg NPY microinjection into the LH on wakefulness, NREMS, REMS and EEG SWA in rats. See legends to Figure 12 for details. Modified from Szentirmai and Krueger, 2006, Figure 2.

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.

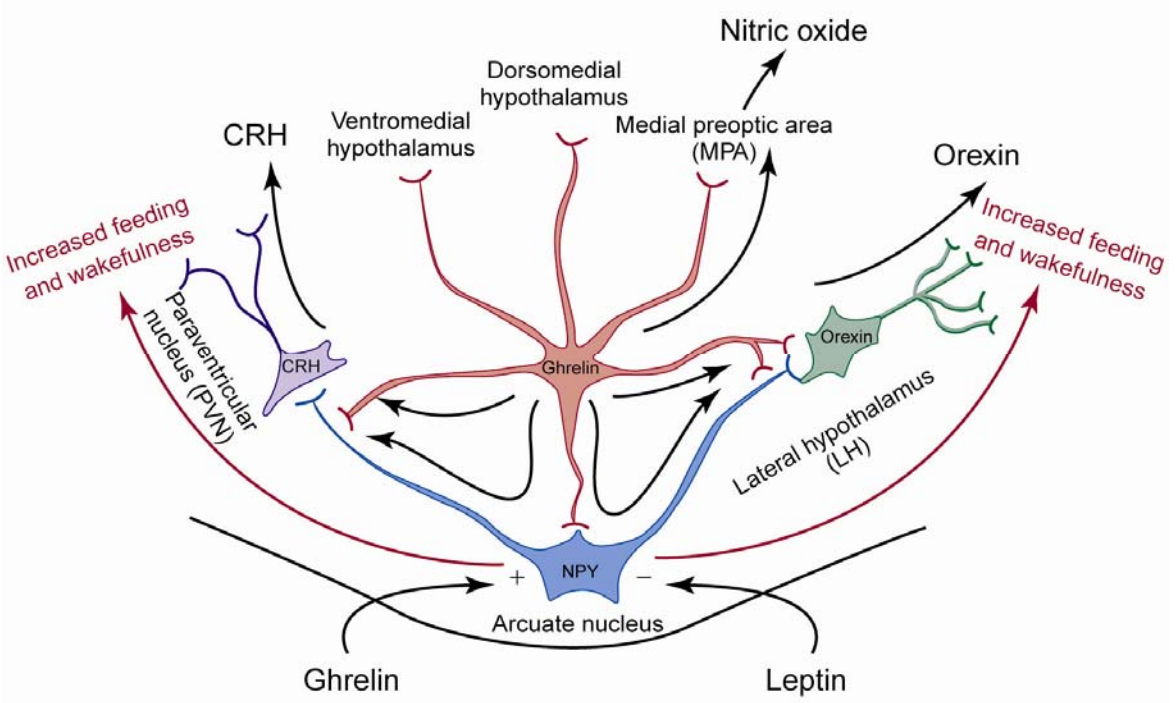

Figure 14. Schematic drawing of the hypothalamic ghrelin-NPY–orexin circuit with possible pathways mediating the wakefulness-promoting effects of ghrelin and NPY. Hypothalamic ghrelin-, orexin-, and NPYergic neurons form a well-characterized circuit that is implicated in the regulation of food intake 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.

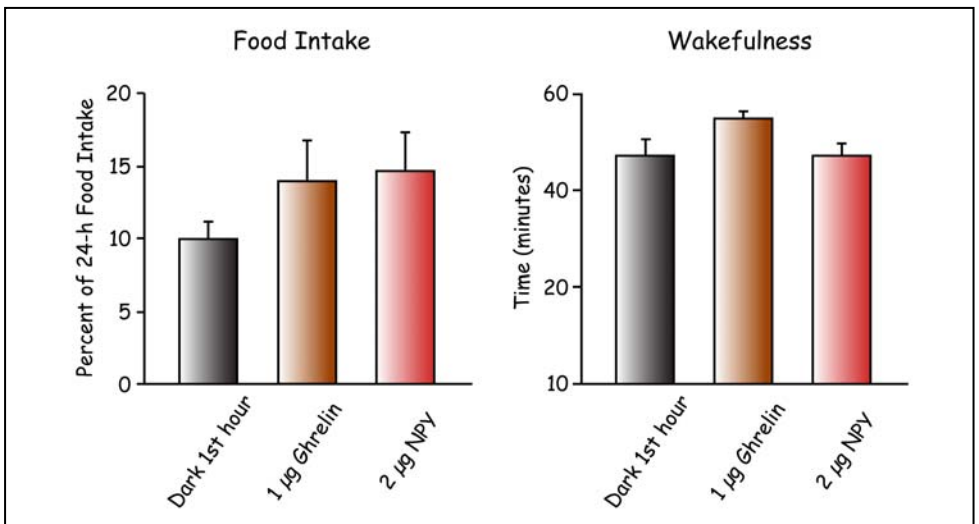

Figure 15. Food intake and the amount of wakefulness during the first hour of the dark phase under normal conditions and in the first hour of light after icv administration of ghrelin and NPY in rats. Central injections of ghrelin and NPY stimulates eating and wakefulness in the light period. These changes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

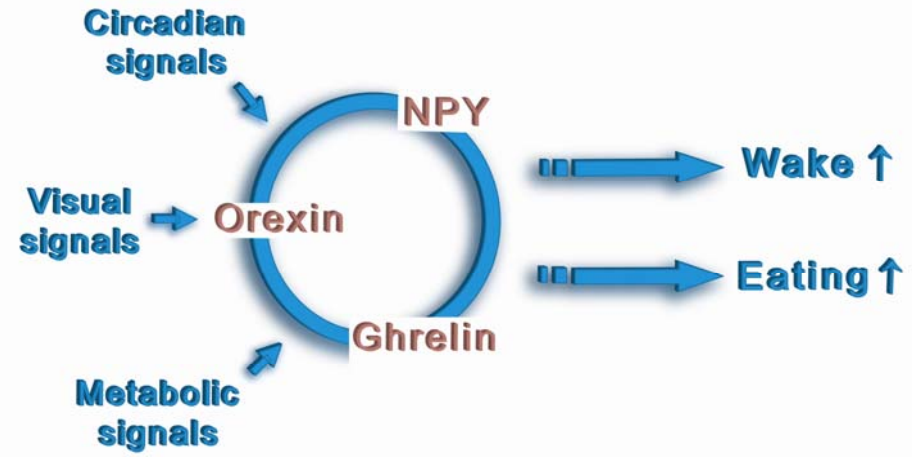

Figure 16. Schematic drawing of the inputs to and outputs of the hypothalamic network formed by ghrelin, orexin and NPY neurons.

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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