Summary of Doctoral Thesis

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

Éva Szentirmai, M.D.

Department of Physiology, Faculty of Medicine
University of Szeged

Szeged
2010

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

Summary of Doctoral Thesis

Éva Szentirmai, M.D.

Department of Physiology, Faculty of Medicine
University of Szeged

Szeged
2010

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-wake activity-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. In mammals and birds, three types of vigilance states can be distinguished by electroencephalogram (EEG), i.e., wake, rapid-eye movement sleep (REMS) and non-rapid-eye movement sleep (NREMS). These states are fundamentally different in terms of regulation and associated physiological, neurological, and psychological features.

The neuronal pathways that promote arousal

In the first half of the 20th century, the landmark observations of von Economo, Moruzzi and Magoun provided the first evidence for a neurological basis for wakefulness and arousal. 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. The brain contains multiple wakefulness-promoting centers that reside in the brainstem, thalamus, hypothalamus and basal forebrain. 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.

A 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. Orexinergic neurons diffusely project and innervate the cerebral cortex and also excite other arousal centers, such as the basal forebrain, locus ceruleus, tuberomamillary nucleus and raphe nucleus. Lack of the orexin peptide or functional orexin receptors result in narcolepsy in humans, dogs and mice. Orexin-producing neurons are also part of a food intake regulatory circuit in the hypothalamus. Orexin, as the name implies, also promotes eating.

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

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 nucleus of the thalamus. Bidirectional synaptic connections exist among ghrelin-, NPY and orexin-producing neurons (Figure 1). In the ARC, ghrelin stimulates the production and release of NPY. In the LH, ghrelin activates orexinergic neurons directly and also indirectly via NPY. Ghrelin stimulates CRH neurons by promoting the release of NPY from axon terminals in the PVN.

The activation of the circuit leads to increased food intake whereas 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. Based on the localization and organization of the ghrelin – NPY – orexin circuit we hypothesized that this circuit is shared by feeding and arousal. 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.

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 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) on sleep were tested. Injections were done 10-15 minutes before dark onset.

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. Food intake after the light onset administration of 1 μg ghrelin was also measured in the same group of animals 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. The order of the baseline day and experimental day was balanced.

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 medial preoptic area (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. Food and water were provided *ad libitum* during the entire recording period. Food intake was measured 1 hour after injections. Sleep was recorded for 23 h 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. 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.

RESULTS

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

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. 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. Plasma leptin and ghrelin displayed distinct diurnal rhythms with their peak values occurring at opposite times of the day. The leptin maximum followed the dark onset-elicited eating peak and occurred 5 hours after dark onset. The ghrelin peak preceded the eating peak and occurred 5 hours 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.

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

Restriction of feeding to the light period significantly altered the sleep-wake activity of rats. NREMS decreased by 11% during the light period and increased by 10% during the dark. The normal diurnal rhythm with more NREMS during the day was maintained. Restricted feeding fundamentally altered the diurnal rhythm of REMS. REMS decreased throughout the light period and increased in the dark period, resulting in more REMS at night than during the day. The daily, 24-hour total amount of NREMS and REMS did not change. During the feeding restriction, 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 ± 2.2% of the total daily activity, and this value was significantly higher 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. In response to restricted feeding, diurnal rhythms of ghrelin and leptin 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 plasma leptin and ghrelin and hypothalamic ghrelin.

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

Five hours of sleep deprivation was followed by significant increases in the duration of both NREMS and REMS. 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 significantly stimulated during sleep deprivation. During the 5 hours of sleep deprivation, 11.1 ± 2.9% of the 24-hour feeding activity occurred. Sleep deprivation did not alter plasma concentration of leptin. In contrast, plasma ghrelin increased significantly in the first hour of sleep deprivation. Hypothalamic ghrelin was highly responsive to sleep deprivation, displaying statistically significant biphasic

variations. Ghrelin contents of the hypothalamus increased significantly during sleep deprivation and dropped below baseline after sleep deprivation.

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

One μg ghrelin induced significant decreases in NREMS and REMS in hours 1 and 2. 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. Similar tendencies in REMS were observed but the changes only reached the level of significance in hours 1 and 2.

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.

Ghrelin induced significant dose-dependent changes in wakefulness, NREMS and REMS in hours 1 and 2 after injection. 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. 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. In the following hours, NREMS did not differ from baseline values. REMS did not change in the first 2 hours but from hours 3 to 12 a significant decrease was observed. The 1 μg ghrelin-induced NREMS decrease in hours 1 and 2 was significantly higher in free-feeding rats than in feeding restricted animals. 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 observed in 10 minutes after the injection and eating continued throughout the first hour of the light period resulting in higher food intake than after the control injection.

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

Administration of 0.2 μg ghrelin significantly increased the time spent in wakefulness and decreased the time in NREMS and REMS. 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. In hours 4 and 5 after injection, EEG slow-wave activity (SWA) was

significantly elevated. Injection of 0.2 µg ghrelin significantly increased the 1-hour food intake of the rats from a baseline of 0.65 ± 0.56 g/kg BW to 6.36 ± 1.16 g/kg BW after ghrelin treatment. Similarly to the middle dose, 1 µg ghrelin injection increased wakefulness and decreased NREMS in the first two hours after ghrelin treatment. EEG SWA was significantly attenuated in the first hour following ghrelin injection. 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).

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

The middle dose of ghrelin, 0.2 µg, induced a significant increase in wakefulness at the expense of both NREMS and REMS in the first hour of the recording period. EEG SWA was significantly increased beginning from the fourth hour. Injection of 0.2 µg ghrelin was followed by a significant increase in food intake (5.01 ± 0.53 g/kg BW vs. 0.45 ± 0.23 g/kg BW after saline injection). The highest dose, 1 µg ghrelin induced a statistically significant increase in wakefulness and decrease in NREMS and REMS. 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. The initial decrease in EEG SWA was followed by an increase beginning from the fourth hour. 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).

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

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. Food intake 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, which was accompanied by a significant decrease in NREMS. EEG SWA did not change in response to 1 µg ghrelin injection. Injection of 1 µg ghrelin into the PVN of the rats induced a significant, about 4-fold increase, in food intake.

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

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 in response to NPY treatment. REMS virtually disappeared in hour 1 after NPY injection. The reduced time spent in sleep mainly resulted from a significant decrease in the average duration of NREMS episodes and a significant decrease in the number of REMS episodes. The EEG SWA was not altered by 2 μg NPY. Food intake in the first hour after NPY injection increased significantly compared to baseline. 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. *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. 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 highest dose of NPY significantly increased the food intake in the first hour after injection.

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

Wakefulness was significantly elevated in the first hour after the injections. The amounts of NREMS and REMS were significantly decreased. NREMS episode number significantly decreased in hour 1 and there was a tendency toward decrease in average NREMS episode duration, as well. 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.

DISCUSSION

The major findings of the experiments presented here are the following. 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. Food restriction to the light period reverses REMS, plasma ghrelin and leptin rhythms and sleep deprivation increases plasma and hypothalamic ghrelin levels. 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. 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. 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 administration and microinjections of ghrelin into the LH, MPA and PVN increase food intake. 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. Previous studies on the effects of systemically administered ghrelin did not yield to consistent findings. In humans, increased and decreased sleep and no effect on sleep have been reported while intraperitoneal administration of ghrelin at dark onset increased NREMS in mice. 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. 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. 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. We posit that the primary effect of ghrelin is to stimulate wakefulness. It is possible that 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 while eating is known to elicit postprandial increases in sleep. 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. The mechanism of the wakefulness-promoting effects of ghrelin in rats is unknown. Ghrelin's food intake-promoting effect is mediated by a central action involving primarily the NPY-signaling pathway in the ARC of the hypothalamus. 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. 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. 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. 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.

Ghrelin receptors have been found in various hypothalamic structures implicated in sleep-wake regulation such as anteroventral preoptic nucleus, anterior hypothalamic area, SCN, anterolateral hypothalamic nucleus, ARC, PVN, and TMN. To test possible hypothalamic sites that may mediate ghrelin's wake-promoting action we characterized sleep and food intake after intrahypothalamic ghrelin microinjections. Ghrelin injection into the LH, MPA or PVN increased the amount of wakefulness in the first hour after injection. 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.

There are several possible mechanisms that may mediate the wake-promoting effect of ghrelin; these mechanisms are summarized on Figure 1. One possibility is that ghrelin activates orexinergic and NPY-ergic arousal mechanisms. Ghrelin-containing neurons innervate orexin-producing cells in the LH and icv or local application of ghrelin into the LH of rats activates orexin neurons. The stimulatory effect of ghrelin on feeding is known to be mediated through NPY-ergic mechanisms in the ARC. In the PVN, the presynaptic terminals

of NPY-producing neurons express GHS-R and ghrelin-positive neuronal projections from the ARC stimulate NPY release. Another possibility is that ghrelin acts through the activation of the hypothalamic--pituitary--adrenal axis; corticotrop-releasing hormone (CRH) release is stimulated by ghrelin in the PVN and CRH is known to inhibit sleep.

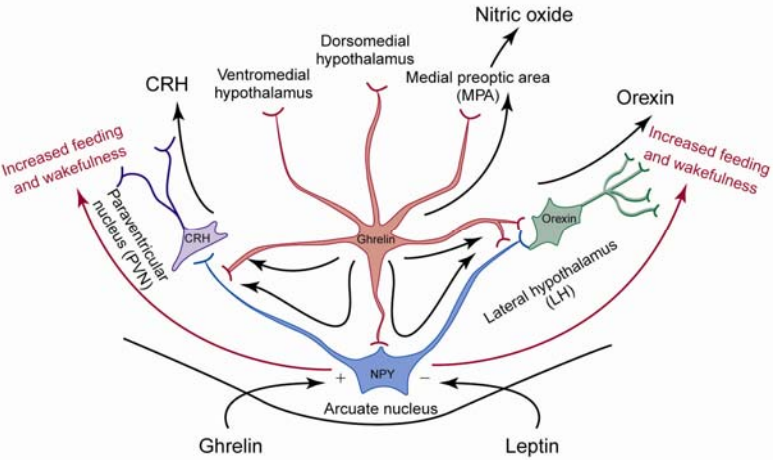

Figure 1. Schematic drawing of the hypothalamic ghrelin-NPY–orexin circuit with possible pathways mediating the wakefulness-promoting effects of ghrelin and NPY. Hypothalamic ghrelin-, orexin-, and NPYergic neurons form a well-characterized circuit that is implicated in the regulation of food intake. We posit that the activation of the same circuit promotes wakefulness. Ghrelin elicits wakefulness when injected into the cerebral ventricle, LH, PVN, or MPA. Ghrelin activates 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. In the MPA, ghrelin stimulates NO formation. Hypothalamic orexin, CRH and NO are all known to stimulate wakefulness and are possible outputs of the ghrelin-orexin-NPY circuit.

Previous studies concerning NPY's sleep-modulating effect did not yield consistent results. 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. 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. Orexinergic axon terminals, originating from the LH, form synapses on NPY-immunoreactive cells in the ARC. Orexin receptors are present on NPY neurons in the ARC. 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. Conversely, orexin antiserum significantly attenuates the feeding response to NPY. In addition to orexinergic neurons, NPY activates CRH release and increases CRH gene expression in the PVN and CRH is known to inhibit sleep.

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 2). The first hours of the dark, behav-iorally 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.

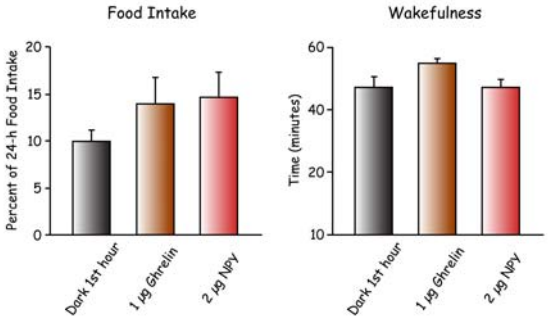

Figure 2. Food intake and wakefulness during the first hour of the dark phase under normal conditions and in the first hour of light after icv administration of ghrelin and NPY in rats. Central injections of ghrelin and NPY stimulates eating and wakefulness in the light period. These changes are characteristic 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. 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. 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. 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.

ACKNOWLEDGEMENT

I would like to thank all of those who helped me in completing this work.

I wish to express my gratitude to Professors György Benedek and Gábor Jancsó for making it possible for me to join the Department of Physiology as a medical student to conduct research and to participate in the neuroscience PhD program.

I am thankful to my supervisor, Dr. Ferenc Obál Jr. for granting me the opportunity to work in his laboratory and introducing me to sleep research. My interest in research was ignited by his enthusiasm and immense knowledge in science.

I am especially grateful to Dr. James M. Krueger, for taking over the mentorship and guidance of my research at Washington State University. His perpetual energy and enthusiasm in research had motivated me throughout the 4 years and 4 months I spent working in his laboratory. Without his continuing encouragement, outstanding support and trust in me I would not be where I am in my career.

I am grateful to Dr. Levente Kapás for his collaboration, inspiration and his guidance in statistical analysis. The stimulating discussions about science, his insightful comments and valuable advice about my work and thesis have been of a great value for me.

I would also like to thank my co-authors, Drs. Balázs Bodosi, János Gardi for their work and help preparing the manuscripts. Special thanks to Dr. Ildikó Hajdú for teaching me the basic techniques used in the experiments in addition to being a co-author of several papers.

I am thankful to Péter Liszli, Szilveszter Tóth, Kálmán Hermann and Richard Brown for their excellent technical assistance. Special thanks to Anikó Jász for her secretarial assistance.

I would like to thank my colleagues, Drs. Zoltán Lelkes, Lynn Churchill, Ping Taishi, Chris Davis and Stewart Bohnet, for their help and for creating a friendly and inspiring environment to work in.

Finally, I would like to thank my family for supporting me throughout my studies, for their continuous encouragement, understanding and patience during the years.

The research was supported by grants from the Hungarian National Scientific Research Fund (OTKA-T-043156) and Ministry of Health Grant ETT103 04/2003 to Dr. Ferenc Obál Jr., and by a grant to Dr. James M. Krueger from the National Institutes of Health (NS27250).

Peer-reviewed publications directly related to the thesis

1. 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.
   IF: 3.405
2. **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.
   IF: 2.341
3 **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.
   IF: 3.661
4. **Szentirmai É**, Krueger JM. Central administration of neuropeptide Y induces wakefulness in rats. *Am J Physiol* 291(2): R473-R480, 2006.
   IF: 3.685

Reviews and book chapters directly related to the thesis

1. Kapás L, **Szentirmai É.** Sleep regulatory factors. In: Monti, J., Sinton, C. and Pandi-Perumal, S. R. (Eds.), The Neurochemistry of Sleep and Wakefulness. Cambridge University Press, UK, 2008, pp. 315-336.
2. **Szentirmai É**., Kapás L. and Krueger J.M. Interactive regulation of sleep and feeding. In: Kryger, M. H. (ed.), Atlas of Clinical Sleep Medicine, Philadelphia: Saunders Elsevier, 2010, pp. 53-56.
3. **Szentirmai, É**., L. Kapás and J. M. Krueger. Hormones and sleep. In: Breed, M. D. and Moore, J. (Eds.), Encyclopedia of Animal Behavior, Oxford: Academic Press, 2010, *in press*.

Peer-reviewed publications not directly related to the thesis

1. Gardi J, **Szentirmai É**, Hajdu I, Obál F Jr., Krueger JM. The somatostatin analog, octreotide, causes accumulation of growth hormone-releasing hormone and depletion of angiotensin in the rat hypothalamus. *Neurosci Lett* 315: 37-40, 2001.
2. Hajdu I, **Szentirmai É**, Obál F Jr., Krueger JM. Different brain structures mediate drinking and sleep suppression elicited by the somatostatin analog, octreotide, in rats. *Brain Res* 994: 115-123, 2003.
3. **Szentirmai É**, Krueger J.M. Obestatin alters sleep in rats. *Neurosci Lett* 404 (1-2): 222-226, 2006.
4. Alföldi P, Kapás L, **Szentirmai É**, Taishi P, Gardi J, Peterfi Z, Kacsóh B, Krueger JM. The somatotropic axis in sleep and thermoregulation: a tribute to Ferenc Obál, Jr. (1948-2004). *J Therm Biol* 31(1-2): 30-39, 2006.
5. **Szentirmai É**, Kapás L, Sun Y, Smith RG, Krueger JM. Spontaneous sleep and homeostatic sleep regulation in ghrelin knockout mice. *Am J Physiol* 293(1): R510-R517, 2007.
6. Majde JA, Bohnet S, Ellis GA, Churchill L, Leyva-Grado V, Wu M, **Szentirmai É**, Rehman A, Krueger JM. Detection of mouse-adapted human influenza virus in the olfactory bulbs of mice within hours after intranasal infection. *J Neuro Virol* 13: 399-409, 2007.
7. **Szentirmai É**, Yasuda T, Taishi P, Wang M, Churchill L, Bohnet S, Magrath P, Kacsóh B, Jimenez L, Krueger JM. Growth hormone-releasing hormone: cerebral cortical sleep-related EEG actions and expression. *Am J Physiol*. 293: R922-R930, 2007.

8. Kapás L, Bohnet GS, Traynor RT, Majde JA**, Szentirmai É**, Magrath P, Taishi P, Krueger JM. Spontaneous and influenza virus-induced sleep are altered in TNF-α double-receptor deficient mice. *J Appl Physiol*. 105(4):1187-98, 2008.
9. **Szentirmai É**., Kapás L., Sun Y., Smith R.G., Krueger JM. The preproghrelin gene is required for normal integration of thermoregulation and sleep in mice. *Proc. Natl. Acad. Sci. USA*, 106(33):14069-14074, 2009.
10. **Szentirmai É**., Kapás L., Sun Y., Smith R.G., Krueger JM. Restricted feeding-induced sleep, activity and body temperature changes in normal and preproghrelin deficient mice. *Am J Physiol*. 298(2):R467-77, 2010.

Reviews and book chapters not directly related to the thesis

1. Krueger JM, **Szentirmai É,** Kapás L. The Biochemistry of Sleep Function. In: Amlaner, C. and Fuller, P. M (eds.), Basics of Sleep Guide, 2nd edition, Sleep Research Society, 2009, pp. 69-74.