Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats

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

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Methods

Animals and surgical procedures. Using ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, male Sprague-Dawley rats (275–325 g) were implanted with cortical electroencephalographic (EEG) electrodes and nuchal electromyographic (EMG) electrodes. Stereotaxic equipment was used to insert guide cannulas (26 gauge; Plastics One) bilaterally into the LH (n = 8 per dose) and MPA (n = 7 per dose) and unilaterally into the PVN (n = 6–8 per dose) of the hypothalamus. The coordinates of the tip of the guide cannulas were: 2.1 mm posterior and 2 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for LH; 0.4 mm posterior and 1.85 mm lateral to the bregma, and 7.8 mm ventral from the surface of the skull for MPA; and 1.80 mm posterior and 0.4 mm lateral to the bregma, and 7.5 mm ventral from the surface of the skull for PVN, according to the
rnat brain atlas by Paxinos and Watson (39). The guide cannulas into the MPA and PVN were inserted at a 10° angle from vertical. Substances were administered by a 31-gauge stainless steel injector placed in and projecting 0.5 mm beyond the tip of the cannulas. The position of the cannulas was verified by histology at the end of the experiment. After the surgeries, the animals were placed in individual sleep-recording cages in sound-attenuated, temperature-regulated environmental chambers at 24 ± 1°C ambient temperature on a 12:12-h dark-light cycle (light on at 9:00 AM) for habituation to the experimental conditions for at least 7 days. During this adaptation period, the animals were connected to the recording cables and handled daily to habituate them to the microinjection procedure. Water and food were available ad libitum. Institutional guidelines for the care and use of research animals were followed and protocols were approved by the Institutional Animal Care and Use Committees of Washington State University and Fordham University.

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

Experimental protocol. The treatments were done 10–15 min prior to light onset. The injected volumes were 100 nl per injection site given over 1 min. After the injection, the injectors were left in the guide for an additional minute. On the control day, the animals received 100 nl pyrogen-free isotonic NaCl; on the experimental day, 100 nl ghrelin (see also Fig. 7). The lowest dose of ghrelin (0.04 μg) did not have any significant effect on the amount of wakefulness, NREMS, and REMS and did not affect the SWA of the EEG (Fig. 1). Detailed analysis of the EEG power revealed a significant increase in the 6- to 8-Hz frequency range during wakefulness [ANOVA, treatment × frequency interaction: F(31,186) = 4.6, P < 0.05] (Fig. 2). There was no significant change in the food intake of the rats in response to 0.04 μg ghrelin (see also Fig. 7).

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

The effects of 1 μg ghrelin injection on sleep were similar to those of the middle dose (Fig. 1). Following ghrelin injection, wakefulness significantly increased and NREMS decreased [ANOVA hours 1-3, treatment effect for wakefulness: F(1,7) = 8.5, P < 0.05; for NREMS: F(1,7) = 9.9, P < 0.05]. Post hoc analysis showed significant changes in wakefulness and NREMS in the first 2 h after ghrelin treatment. EEG SWA was significantly attenuated in the first hour following ghrelin injection [ANOVA treatment × time interaction: F(2,26) = 3.9, P < 0.05]. Detailed analysis of the EEG power showed a significant decrease in the 2.5- to 3-Hz range during NREMS

**RESULTS**

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

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Fig. 1. Wakefulness, non-rapid-eye-movement sleep (NREMS), rapid-eye-movement sleep (REMS), and electroencephalographic (EEG) slow-wave activity (SWA) after ghrelin (*) and isotonic NaCl (○) administration into the lateral hypothalamus. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. Insets: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day (P < 0.05, Student-Neumann-Keuls test).
During REMS, there was a significant increase in the 6.5- to 7-Hz and a decrease in the 8- to 8.5-Hz frequency ranges [ANOVA treatment \times frequency interaction: \( F(31,124) = 1.2, P < 0.05 \)]. One microgram of ghrelin significantly stimulated the 1-h food intake of the rats (0.25 ± 0.14 g/kg body wt after control treatment vs. 7.02 ± 1.75 g/kg body wt after ghrelin treatment) (see also Fig. 7).

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

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

The highest dose of ghrelin induced a statistically significant increase in time spent in wakefulness at the expense of NREMS and REMS [ANOVA hours 1–3, treatment effect for wakefulness: \( F(1,6) = 244.6, P < 0.05 \); for NREMS: \( F(1,6) = 101.6, P < 0.05 \); for REMS: \( F(1,6) = 28.4, P < 0.05 \)].
Fig. 3. Wakefulness, NREMS, REMS, and EEG SWA after ghrelin (●) and isotonic NaCl (○) administration into the medial preoptic area. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. Insets: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day (P < 0.05, Student-Neumann-Keuls test).
The effects were confined to the first hour of the recording period. The NREMS changes in the first hour were accompanied by a significant decrease in EEG SWA [ANOVA hours 1-3, treatment × time interaction: F(2,34) = 4.7, P < 0.05]. The initial decrease in EEG SWA was followed by an increase beginning from the fourth hour [ANOVA hours 4-23, treatment × time interaction: F(6,84) = 3.4, P < 0.05] (Fig. 3). The EEG power during wakefulness and sleep was also affected (Fig. 4). One microgram of ghrelin induced a decrease in EEG power in the 1- to 3.5-Hz frequency range during wakefulness [ANOVA treatment effect: F(1,6) = 18.1, P < 0.05], in the 0.5- to 7-Hz frequencies during NREMS [ANOVA treatment effect: F(1,6) = 15.2, P < 0.05] and in the 5.5- to 7-Hz frequencies during REMS [ANOVA treatment effect: F(1,6) = 8.09, P < 0.05]. Food intake was significantly stimulated by 1 μg ghrelin injection (0.74 ± 0.34 g/kg body wt on the control day and 7.44 ± 1.26 g/kg body wt on the treatment day) (see also Fig. 7).

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

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

One microgram of ghrelin induced a statistically significant increase in time spent awake in the first hour after injection [ANOVA hours 1-3, treatment × time interaction: F(2,14) = 3.8; P < 0.05], which was accompanied by a significant decrease in NREMS [ANOVA hours 1-3, treatment × time interaction: F(2,14) = 4.5, P < 0.05]. EEG SWA did not change in response to 1 μg ghrelin injection (Fig. 5). There was a significant increase in EEG power in the 5- to 5.5-Hz and 7.5-Hz frequencies during wakefulness
Fig. 5. Wakefulness, NREMS, REMS, and EEG SWA after ghrelin (•) and isotonic NaCl (○) administration into the paraventricular nucleus. The amounts of wakefulness, NREMS, REMS, and EEG SWA were calculated in 1-h time blocks for the first 3 h after the injection and in 3-h time blocks for the remaining part of the recording period. Insets: changes in wakefulness, NREMS, and REMS from baseline (in minutes) in the 1st and 2nd hour after ghrelin injections. Horizontal dark bars: dark phase of the day. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student–Neumann–Keuls test).
[ANOVA treatment × time interaction: $F(31,186) = 1.9$, $P < 0.05$] (Fig. 6). Injection of 1 µg ghrelin into the PVN of the rats induced a significant (~4-fold increase) in food intake (Fig. 7).

**DISCUSSION**

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

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

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

**Fig. 6.** The EEG power spectra of wakefulness and NREMS in the 1st-hour time block and REMS in the 1st 2-h time block after administration of ghrelin (●) and isotonic NaCl (○) into the paraventricular nucleus. After the injection of 0.04 and 0.2 µg ghrelin, only 3 rats had REMS. Due to the small number of animals, statistical analysis was not performed on these data, and the results are not shown. N/A, not available. Error bars: SE. *Significant differences between baseline and experimental day ($P < 0.05$, Student-Neumann-Keuls test).
According to the classic view, the posterior/LH is a “wake-center” since Von Economo (62) observed excessive sleepiness in patients with the damage of this region. Electrolytic (51) or excitotoxic (47) lesions of the LH, however, yielded inconsistent results. The LH regained the interest of sleep researchers when it became apparent that orexin is produced almost exclusively by LH neurons (34). Orexinergic mechanisms play a central role in the maintenance of wakefulness. Orexin-containing neurons innervate forebrain and brain stem structures that are implicated in arousal (40, 60). Orexins stimulate wakefulness when injected intracerebroventricularly (15) or into the lateral POA, PVN, TMN, or locus coeruleus (2, 18, 29, 48). Orexinergic neurons discharge during active wakefulness, and they are silent in slow-wave sleep (25, 30). Narcolepsy is linked to the lack of orexin and/or orexin receptors (5, 26). There is a close relationship between ghrelin and orexin in the LH. GHS-Rs are present in the LH (31). Ghrelin-containing axons project to the LH and form direct synaptic contact with orexin-producing neurons (57). Intracerebroventricular or local application of ghrelin into the LH of rats activates orexin cells (24, 37, 57, 67). Orexinergic mechanisms may contribute to the feeding effects of ghrelin since pretreatment with orexin antibodies attenuates ghrelin-induced eating and the effect of ghrelin was significantly reduced in orexin-deficient mice (57). We hypothesize that the wake-promoting effects of ghrelin in the LH may also involve the activation of orexinergic mechanisms.

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

The PVN plays an important role in arousal, autonomic, and behavioral responses to stressors (41). It has reciprocal connections with arousal centers, such as locus coeruleus and raphe nuclei (4, 59). Lesion of the PVN decreases REMS sleep and abolishes the circadian sleep-wake cycles (42). Microinjections of orexin (48) or histamine (50) into the PVN elicit arousal responses. The PVN is also one of the major targets for ghrelin to induce feeding; injections of ghrelin induce c-fos expression in the PVN (36) and stimulate eating (65). Similar to prior findings (65), we did not observe a clear dose-response relationship on feeding after PVN injections of ghrelin. Ghrelin injection into the PVN also enhanced wakefulness and suppressed NREMS, but this region appears to be the least sensitive among the three sites for sleep effects. Ghrelin did not reduce REMS, which may be due to the already short REMS on the baseline day at the beginning of the light period. It is possible that ghrelin’s actions in the PVN are mediated, in part, through NPYergic mechanisms. Central administration of NPY induces wakefulness in rats (53). NPY-producing neurons in the PVN express the GHS-R (14) and receive ghrelin-positive neuronal projections from the ARC (6). In the PVN, ghrelin binds to the GHS-R localized on the presynaptic terminals of NPY neurons, stimulating NPY release (6). Alternatively, it is possible that ghrelin’s wake-promoting ability in the PVN is due to the activation of the HPA axis. Ghrelin facilitates corticotropin-releasing hormone (CRH) in the PVN through stimulating GABA-release from NPY neurons (6). CRH inhibits sleep (9, 38). Recent findings suggest that there is indeed a functional interaction in regulating digestive functions within the PVN between ghrelin and NPY and ghrelin and CRH. Intra-PVN injection of ghrelin stimulates colonic motor function, an effect that is inhibited by local pretreatment with a NPY1- or CRH-receptor antagonist (54). Activation of the PVN may also contribute to the wake-promoting effects of the intra-LH-injected ghrelin since the LH-infusion of ghrelin activates neurons in the PVN as shown by enhanced c-Fos immunoreactivity (36).
Ghrelin microinjections also markedly affected EEG power. NREMS-associated EEG delta power, particularly the 1.5- to 4-Hz frequencies, was significantly attenuated in the first hour after 1 μg ghrelin injections into the LH and MPA. Rats had very little NREMS, which is a possible explanation for the attenuated EEG power. The immediate decrease was followed by an increase in EEG SWA after injections into the MPA. Sleep loss, in general, is followed by increases in EEG SWA. It is possible that the delayed increase in EEG SWA is due to the sleep loss in the first hour after ghrelin treatment. The impact of ghrelin on the EEG power extends to wakefulness and REMS. During wakefulness, EEG power in the 6- to 8-Hz frequencies was increased, suggesting that ghrelin induces not only quantitative but also qualitative changes in wakefulness. Similar EEG power increases in the 6.5- to 7.5-Hz frequencies during REMS after LH microinjections also occurred.

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

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GRANT

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