Inhibition of ghrelin-induced feeding in rats by pretreatment with a novel dual orexin receptor antagonist

Mariko So 1,2 • Hirofumi Hashimoto 2 • Reiko Saito 2,3 • Yukiyu Yamamoto 3 • Yasuito Motojima 2 • Hiromichi Ueno 2 • Satomi Sonoda 2 • Mitsuhiro Yoshimura 2 • Takashi Maruyama 2 • Koichi Kusuhara 3 • Yoichi Ueta 2

Received: 22 November 2016 / Accepted: 22 December 2016 / Published online: 4 January 2017
© The Author(s) 2017, corrected publication 2019

Abstract Orexin-A and -B, and ghrelin are potent orexigenic peptides. The effects of ACT-462206, a novel dual orexin receptor antagonist (DORA), on ghrelin-induced feeding were examined in adult male Wistar rats. Hyperphagia induced by the intracerebroventricular (icv) administration of ghrelin was significantly suppressed for at least 2 h by pretreatment with icv administration of DORA. A marked increase was observed in the number of neurons showing Fos immunoreactivity in the paraventricular nucleus, arcuate nucleus and lateral hypothalamic area (LHA), 90 min after icv administration of ghrelin. Pretreatment with DORA significantly decreased the number of Fos-immunoreactive (IR) neurons; however, Fos immunoreactivity remained significantly increased. Double-immunostaining for Fos and orexin-A showed that many orexin-A-IR neurons in the LHA coexisted with Fos immunoreactivity after icv administration of ghrelin, but their number was reduced significantly by DORA pretreatment. These results suggest that centrally administered ghrelin may activate the orexinergic and non-orexinergic pathways responsible for the regulation of feeding.

Keywords Food intake • Fos • Ghrelin • Immunohistochemistry • Orexin receptor antagonist

Introduction

Orexin-A and -B discovered as orexigenic neuropeptides, were derived from a common precursor, prepro-orexin [1, 2]. They bind to G-protein coupled receptors called orexin receptor type 1 and type 2 (identical to hypocretin receptor-1 and -2), which display 64% homology [1, 2]. Although orexin-A binds to orexin receptor type 1 and type 2 with similar affinities, orexin-B has a 10-fold greater affinity for orexin receptor type 2 than for type 1 [1, 2]. Orexins increase intracellular Ca2+ concentration via activation of the Gq/phospholipase C/protein kinase C pathway [1]. Pharmacological blockade of orexin receptor type 1 inhibited feeding and reduced body weight gain in rodents [3]. Genetic mutation in orexin receptor type 2 was found in narcoleptic dogs [4].

Orexin-producing neurons are located mainly in the lateral hypothalamic area (LHA), which is known as the feeding center, and they project their axon terminals to various brain regions, including the hypothalamic nuclei and the brainstem [5–7]. Their receptors are distributed widely in the central nervous system (CNS) [8] and are well-characterized pharmacologically [9]. The deficiency of orexin or orexin receptor causes narcolepsy in mice, dog and humans [4, 10–12]. The linkage revealed between the orexin/orexin receptor system and the regulation of sleep/wakefulness has helped in the development of drugs for sleep disorders. In particular, dual orexin receptor
antagonists (DORAs) such as almorexant and suvorexant have been chosen as therapeutic drugs for patients with insomnia [13–15].

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), was isolated from the stomach [16], and recognized as a potent orexigenic peptide [17–19]. Although ghrelin is predominantly produced and secreted by the stomach, ghrelin-producing neurons and their receptors have been identified in the CNS, including the hypothalamus [17]. Central and peripheral administration of ghrelin increases food intake dramatically in rats and mice [17–20]. Previous studies have demonstrated that central and peripheral administration of ghrelin activates various brain regions, including the feeding-related areas such as the paraventricular nucleus (PVN) and the arcuate nucleus (Arc) in rats [6, 21]. This was observed by Fos immunohistochemistry, which is widely used as an indicator for neuronal activity in the CNS.

Centrally administered ghrelin-induced feeding was significantly attenuated by pretreatment with antibodies against orexin-A and -B [22]. Ghrelin-induced food intake in orexin knockout mice was significantly reduced compared to that in the wild type [22]. These observations suggest that ghrelin-induced feeding may be mediated, at least in part, via the central orexinergic pathway.

Recently, DORAs have been developed for patients with insomnia. To our knowledge, there is a lack of information on the central effects of DORAs on ghrelin-induced feeding. In the present study, we first examined the effects of intracerebroventricular (icv) administration of a novel DORA, ACT462206, which is one of the derivatives from the proline sulfonamide [23], on centrally administered ghrelin-induced feeding in conscious rats. Second, Fos immunohistochemistry was performed to examine the effects of pretreated ACT462206 on centrally administered ghrelin-activated neurons in the hypothalamus, in particular in the PVN, the Arc, and the LHA in rats. Finally, double-immunostaining for Fos and orexin-A were performed to examine the effects of pretreated ACT462206 on ghrelin-activated orexin-A-producing neurons in the LHA in rats.

Materials and methods

Animals

Adult male Wistar rats at 6–8 weeks of age, weighing 250–420 g, were housed individually in plastic cages, in an air-conditioned room (24 ± 1 °C) under a 12-h light (0700-1900)/12-h dark (1900-0700) cycle.

All procedures in the present study were performed in accordance with the guidelines on the use and care of laboratory animals as put forward by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

Surgical procedures

For icv administration of solutions, all rats were implanted with stainless steel cannulas aimed at the lateral ventricle as previously described [21]. In brief, the rats were anesthetized [sodium pentobarbital, 50 mg/kg body weight, intraperitoneal (ip) injection] and placed in a stereotaxic frame. A stainless steel guide cannula (550-μm outer diameter, 10-mm length) was implanted stereotaxically [24], and the tip of the cannula was 1.0 mm above the left cerebral ventricle. Two stainless steel anchoring screws were fixed to the skull, and the cannula was secured in place by using acrylic dental cement.

After the surgery, the rats were returned to their cages and allowed to recover for at least 5 days. They were handled every day and housed in cages before the start of the experiments.

Icv administration of solutions

For the icv injection of ACT462206, ghrelin, and saline, a stainless steel injector (300-μm, outer diameter) was introduced through the cannula at a depth of 1.0 mm beyond the end of the guide cannula. The total volume of the solutions of ACT462206 (or saline) and ghrelin (or saline) injected into the lateral ventricle was 5 μL. A novel DORA, ACT462206, [(2S)-N-(3,5-dimethylphenyl)-1-[4-methoxyphenyl)sulfonyl]-2-pyrrolidinecarboxamide] was purchased from TOCRIS Bioscience (Bristol, UK). Rat ghrelin was purchased from the Peptide Institute (Minoh, Japan). ACT462206 and ghrelin were dissolved in sterile 0.9% saline.

Experimental procedures

Measurement of food and water intake

To examine the effects of ACT462206 on ghrelin-induced feeding, we administered ghrelin (1 nmol) or saline 30 min after icv administration of ACT462206 (0.1 and 15 nmol) or saline. After icv administration of ACT462206 or saline, the rats were put into metabolic cages at 11:30 am. The dosage of ghrelin was chosen according to our previous study [21]. We measured the cumulative food intake and the cumulative water intake at 0.5, 1, 1.5, 2, 3, 6, and 24 h.
after icv injection of ghrelin or saline. The number of rats in each group was 4–7.

**Fos immunohistochemistry and double immunohistochemical staining for Fos and orexin-A**

Thirty minutes after the icv administration of DORA (15 nmol) or saline, we administered ghrelin (2 nmol) or saline. Ninety minutes after the icv injection of ghrelin or saline, the rats were deeply anesthetized by ip injection of sodium pentobarbital (50 mg/kg). They were then perfused transcardially with 100 mL of 0.1 M phosphate buffer (PB; pH 7.4) containing heparin (1000 units/L) and 150 mL of a fixative containing 4% paraformaldehyde (PFA). The brains were removed and postfixed with 4% PFA for 48 h at 4 °C. The tissues were then cryoprotected in 20% sucrose in 0.1 M PB for 24 h at 4 °C. For immunostaining, serial section (30 μm thick) were cut using a microtome (REM-700; Yamato Kohki Industrial Co., Ltd., Saitama, Japan). The sections were rinsed twice with 0.1 M phosphate buffered saline (PBS), followed by washing in 0.1 M Tris buffer (pH 7.6) containing 0.3% Triton X-100. Floating sections were incubated with 1% hydrogen peroxide for 60 min, followed by a rabbit polyclonal anti-Fos protein antiserum (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500 in 0.1 M PBS containing 0.3% Triton X-100 at 4 °C for 4 days. After washing in 0.3% Triton X-100/PBS for 20 min, the sections were further incubated for 120 min with a biotinylated secondary antibody solution (1:250) and finally with an avidin–biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) for 120 min. The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.02% diaminobenzidine (DAB) and 0.05% hydrogen peroxide for 3 min. For orexin-A immunostaining, the sections were sequentially incubated with rabbit antiserum for orexin-A antibody (lot no. 297980A1, Alpha Diagnostic Intl., Inc., San Antonio, TX, USA) diluted at 1:10,000 in 0.1 M PBS containing 0.3% Triton X-100 at 4 °C for 5 days. The avidin–biotin peroxidase complex was visualized with nickel-sulfate-enhanced DAB. Fos-immunoreactive (IR) neurons were observed as dark brown nuclei, whereas orexin-A-IR neurons were observed as a violet cytoplasmic and axonal precipitate (Fig. 4A). The sections were mounted on gelatin-coated glass slides, and then air-dried, dehydrated in 100% ethanol, cleared with xylene, and covered with a cover slip. The number of Fos-IR and orexin-A-IR neurons of the bilateral hypothalamic area (the PVN, the Arc, and the LHA) was counted in selected six sections according to coordinates given in the rat brain atlas [24] and averaged in all rats. These were counted by two researchers and each group was double-blinded.

**Statistical analysis**

Data are represented as the mean deviation from the control (percentage) ± SEM of the cumulative food intake and number of Fos-IR and orexin-A-IR neurons. Each group within an experiment was compared with the saline-treated group. The data were analyzed using a one-way fractional ANOVA followed by Bonferroni and Tukey–Kramer corrections for multiple comparisons. All statistical analyses were performed using JMP 11 (SAS, Tokyo) and SPSS 21 (SPSS, Osaka). The statistical significance was set at P less than 0.05.

**Results**

**Effects of DORA, ACT462206 on ghrelin-induced feeding**

Icv administration of DORA at small doses (0.1 nmol) did not suppress ghrelin (1 nmol)-induced feeding (data not shown). Icv administration of DORA (15 nmol) potently suppressed the centrally administered ghrelin-induced feeding at 0.5–2 h after icv administration of ghrelin (1 nmol; Fig. 1Aa). There was no difference among all groups at 24 h after icv administration of the solutions (Fig. 1Ab). Cumulative water intake was comparable among all groups after icv administration of DORA (15 nmol) at all time points (Fig. 1Ba, b).

**Fos-IR neurons in the PVN, the Arc, and the LHA 90 min after icv administration of solutions**

The distribution of Fos-IR neurons in the PVN (Fig. 2Aa–d), the Arc (Fig. 2Ba–d), and the LHA (Fig. 2Ca–d) was observed at 90 min after icv administration of the solutions. Icv administration of ghrelin induced Fos immunoreactivity in the entire range of the PVN; in particular, potent increases were observed in the parvocellular division of the PVN (Fig. 2Ab), the ventral part of the Arc (Fig. 2Bb), and the perifornical LHA (Fig. 2Cb). In rats pretreated with DORA before icv administration of ghrelin, the number of Fos-IR neurons decreased dramatically in the PVN (Fig. 2Ad), the Arc (Fig. 2Bd), and the LHA (Fig. 2Cd).

The numbers of Fos-IR neurons in the PVN, the Arc, and the LHA were counted (Fig. 3). Icv administration of ghrelin (2 nmol) caused a significant increase in the number of Fos-IR neurons in the PVN, Arc, and LHA. However, pretreatment of DORA significantly decreased the number of Fos-IR neurons induced by icv administration of ghrelin in the PVN, Arc, and LHA. However, Fos immunoreactivity remained significantly increased in the
PVN, the Arc, and the LHA at 90 min after icv administration of ghrelin with DORA when compared to that at 90 min after icv administration of saline without DORA or icv administration of saline with DORA (Fig. 3).

Effects of DORA, ACT462206 on ghrelin-induced Fos-IR in orexin A-IR neurons

The distribution of orexin-A-IR neurons expressing Fos immunoreactivity at 90 min after icv administration of ghrelin with DORA was compared to that at 90 min after icv administration of saline without DORA or icv administration of saline with DORA (Fig. 3).

Effects of DORA, ACT462206 on ghrelin-induced Fos-IR in orexin A-IR neurons

The distribution of orexin-A-IR neurons expressing Fos immunoreactivity at 90 min after icv administration of ghrelin with DORA was compared to that at 90 min after icv administration of saline without DORA or icv administration of saline with DORA (Fig. 3).

Discussion

In the present study, we demonstrated that icv administration of DORA, ACT462206, a novel orexin receptor antagonist, significantly suppressed centrally administered ghrelin-induced feeding in rats. In addition, Fos immunohistochemistry revealed that centrally administered ghrelin induced Fos expression in the PVN, the Arc, and the LHA, which are areas related to feeding behavior. Fos expression was significantly inhibited by pretreatment with DORA; however, it remained significantly increased in those nuclei when compared to that after icv administration of ghrelin without DORA. It is...
worth noting that Fos expression of orexin-A-IR neurons induced by icv administration of ghrelin was significantly inhibited by pretreatment with DORA, but the Fos expression in orexin-A-IR neurons remained significantly increased.

The suppressive effect of DORA on centrally administered ghrelin-induced feeding suggests that centrally administered ghrelin-induced feeding may be mediated via the central orexin pathway. This result is in agreement with the results of a previous study, which showed that centrally administered ghrelin-induced feeding was significantly attenuated in anti-orexin-A and -B IgG pretreated rats and orexin knockout mice [22].

In the present study, the suppressive effects of pretreatment with DORA (15 nmol) on feeding were observed at 1.5 and 2 h, but not at 3, 6, and 24 h after icv administration of ghrelin. Boss et al. [23] reported that IC$_{50}$ of orexin receptor type 1 and type 2 is 60 and 11 nM, respectively, and t$_{1/2}$ is 1.6–3.1 h after intravenous injection of ACT462206 (1 mg/kg body weight) in rats. These pharmacological characteristics seem to be reasonable to understand the obtained results in the present study.

The possibility that icv administration of DORA decreased the arousal levels, and then inhibited feeding after icv administration of ghrelin in rats has not been excluded. Although we did not investigate the locomotor activity after pretreatment of DORA, the rats pretreated with DORA drank water as same amount as saline pretreated group (Fig. 1Ba, b). Thus, we presume that the pretreatment of DORA did not change locomotor activity, and it may be said that decreased ghrelin-induced food intake with pretreatment of DORA was not due to decreased locomotor activity. Boss et al. also reported that ACT462206 could pass through the blood brain barrier into the CNS [23]. Thus, further studies are required to evaluate whether a peripheral administration,
such as intravenous and oral administration, of ACT462206 can inhibit peripherally/centrally administered ghrelin-induced feeding in rats.

GHSR is widely distributed in the CNS, including the hypothalamus [25, 26]. The results of our study as well as previous studies demonstrated that both central and peripheral administration of ghrelin induced Fos expression in various regions, including the hypothalamic nuclei [6, 21]. The hypothalamic nuclei are important sites that regulate feeding and energy metabolism [6, 21, 27–30]. The neurons located in the PVN, the Arc, and the LHA express GHSR [6, 21]. Centrally administered ghrelin may induce Fos expression in those neurons via GHSR directly because the number of neurons expressing Fos immunoreactivity remained significantly increased by pretreatment with DORA (Fig. 3). However, the decrease in number of neurons expressing Fos immunoreactivity in the PVN, the Arc, and the LHA by pretreatment with DORA may be activated by the central orexin pathway via orexin receptors.

Previous studies reported that ghrelin and orexin may have sex differences such as feeding, behavior, and

![Image](image_url)
expression levels [31–34]. This evidence suggested that ghrelin-induced feeding via the orexin pathway may have a sex difference. Further studies are required to examine the different effects of ghrelin on orexin-mediated feeding in male and female rats.

In the PVN, corticotropin-releasing hormone (CRH) neurons express orexin receptors [35]. Orexin-containing neurons in the LHA also express CRH type 1 and type 2 receptors [35]. In the present study, pretreatment with DORA attenuated the number of Fos-expressing neurons in the PVN. Although we did not analyze the number of neurons with Fos expression in the parvocellular division of the PVN that contain CRH neurons, Fos-IR neurons induced after icv administration of ghrelin seem to be reduced in the parvocellular division relative to the magnocellular division in the PVN by pretreatment with DORA (Fig. 2Aa, d, Ba, d, Ca, d). The result suggests that orexin receptors may be expressed in CRH neurons in the PVN and is consistent with the previous study [35].

It is hypothesized that centrally administered ghrelin may activate orexin-containing neurons in the LHA via GHSR, and these neurons project their axons to the CRH neurons. CRH neurons in the PVN also project their axons to orexin neurons in the LHA, and activate orexin neurons in the LHA via CRH type 1 and type 2 receptors [35]. This interdependence between orexin neurons in the LHA and CRH neurons in the PVN may be important for stress responses [35].

In the Arc, the neurons synthesize neuropeptides such as neuropeptide Y (NPY)/agouti-related protein (AgRP), which are orexigenic neuropeptides, and proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART), which are anorexie neuropeptides, and regulate the feeding behavior [25–30]. NPY/AgRP neurons in the Arc express GHSR and orexin receptors [25, 26]. Thus, both orexin and ghrelin activate NPY/AgRP neurons in the Arc, and stimulate feeding. Another possible explanation is that orexin or ghrelin directly activated GABAergic neurons then inhibit POMC/CART neurons in the Arc, which results in hyperphagia. The results shown in Fig. 3 are reasonable because pretreatment of DORA significantly attenuated Fos expression in the Arc.

In the LHA, approximately 70% of orexin-A-IR neurons expressed Fos immunoreactivity after icv administration of ghrelin (Fig. 4D). However, pretreatment of DORA significantly reduced Fos immunoreactivity in orexin-A-IR neurons into approximately 15% in the LHA. Neuronal circuits involving the orexin—orexin receptor signaling may exist in the LHA. As orexin-A and -B are derived from a common precursor, prepro-orexin [1, 2], we performed immunohistochemistry only for orexin-A in the present study. However, the detailed mechanism of orexin-A or -B responsible for feeding regulation induced by centrally administered ghrelin remains unclear. We propose investigation of the distinct mechanism of these peptides which are related to feeding regulation, using orexin-A-specific or orexin-B-specific antagonist.

In conclusion, DORA can inhibit centrally administered ghrelin-induced feeding via the central orexin pathway in rats. The physiological relevance of the other pathways activated by centrally administered ghrelin, independent of the orexin pathway, should be clarified by further studies.

Acknowledgements We would like to thank Ms. Yuki Nonaka for her technical assistance.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Funding This study was funded by Grant-in-Aid for Scientific Research (C) number 26461535 and number 16k08537 from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Research Activity Start-up number 15H0680 from the Japan Society for the Promotion of Science (JSPS).

Ethical approval All procedures performed in this study were in accordance with the guidelines on the use and care of laboratory animals as put forward by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, duplication, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

References

1. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M (1999) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 92:573–585

2. de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, Fukuhara C, Battenberg EL, Gautvik VT, Bartlett FS 2nd, Frankel WN, van den Pol AN, Bloom FE, Gautvik KM, Sutcliffe JG (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci USA 95:322–327

3. Smart D, Haynes AC, Williams G, Arch JR (2002) Orexins and the treatment of obesity, Eur J Pharmacol 440:199–212

4. Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98:365–376

5. Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K (1999) Distribution of orexin neurons in the adult rat brain. Brain Res 827:243–260

6. Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M (1999) J Physiol Sci (2018) 68:129–136
Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96:748–753

7. Peyron C, Tighe DK, van den Pol AN, de Lecca L, Hellier HC, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18:9996–10015

8. Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LH, Guan XM (1998) Distribution of orexin receptor mRNA in the rat brain. FEBS Lett 438:71–75

9. Thompson MD, Xhaard H, Sakurai T, Rainero I, Kukkonen JP (2014) OX1 and OX2 orexin/hypocretin receptor pharmacogenetics. Front Neurosci. doi:10.3389/fnins.2014.00057

10. Hara J, Beuckmann CT, Nambu T, Willie JT, Chenellini RM, Sinton CM, Sugiyama F, Yagami K, Goto K, Yanagisawa M, Sakurai T (2001) Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron 30:345–354

11. Peyron C, Faraco J, Rogers W, Nishino S, Ripley B, Overeem S, Charnay Y, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y (2013) Orexin (hypocretin) receptor agonists and antagonists for treatment of sleep disorders. Rationale and current status. CNS Drugs 91:975–985. doi:10.1007/s40263-012-0036-8

12. Nishino S, Ripley B, Overeem S, Lammers GJ, Bouras C, Kucherlapati R, Nishino S, Mignot E (2000) Distribution of orexin receptor mRNA in the rat brain. J Neurosci 18:9996–10015

13. Michelson D, Snyder E, Paradis E, Chengan-Liu M, Snavely DB, Hutzellman J, Walsh JK, Krystal AD, Benca RM, Cohn M, Lines C, Roth T, Herring WJ (2014) Safety and efficacy of sозворен during 1-year treatment of insomnia with subsequent abrupt treatment discontinuation: a phase 3 randomised, double-blind, placebo-controlled trial. Lancet Neurol 13:461–471. doi:10.1016/S1474-4422(14)70053-5

14. Hoever P, Dorffner G, Beneš H, Penzel T, Danker-Hopfe H, Barbanoj MJ, Pillar G, Saletu B, Polo O, Kunz D, Zeilhofer J, Berg S, Partinen M, Bassetti CL, Högl B, Ebrahim IO, Holsboer-Trachsler E, Bengtsson H, Peyer Y, Hemmeter UM, Chiossi E, Hajak G, Dingemanse J (2012) Orexin receptor antagonism, a new sleep-enabling paradigm: a proof-of-concept clinical trial. Clin Pharmacol Ther 91:975–985. doi:10.1038/clpt.2011.370

15. Michelson D, Snyder E, Paradis E, Chengan-Liu M, Snavely DB, Hutzellman J, Walsh JK, Krystal AD, Benca RM, Cohn M, Lines C, Roth T, Herring WJ (2014) Safety and efficacy of sозворен during 1-year treatment of insomnia with subsequent abrupt treatment discontinuation: a phase 3 randomised, double-blind, placebo-controlled trial. Lancet Neurol 13:461–471. doi:10.1016/S1474-4422(14)70053-5

16. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660

17. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S (2001) A role for ghrelin in the central regulation of feeding. Nature 409:194–198

18. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatie MA, Bloom SR (2000) The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325–4328

19. Cowley MA, Smith RG, Diano S, Tschop M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mender P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661

20. Hashimoto H, Otsubo H, Fujihara H, Suzuki H, Ohbuchi T, Yokoyama T, Takei Y, Ueta Y (2009) Centrally administered ghrelin potently inhibits water intake induced by angiotensin II and hypovolemia in rats. J Physiol Sci 60:19–25

21. Hashimoto H, Fujihara H, Kawasaki M, Saito T, Shibata M, Otsubo H, Takei Y, Ueta Y (2007) Centrally and peripherally administered ghrelin potently inhibits water intake in rats. Endocrinology 148:1638–1647

22. Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan JL, Wang QP, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kangawa K, Nakazato M (2003) Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506–1512

23. Boss C, Roch-Brisinbar C, Steiner MA, Treiber A, Dietrich H, Jenck F, von Raumer M, Sifferlen T, Brotschi C, Heidmann B, Williams JT, Aissaaou H, Siegrist R, Gattfelf J (2014) Structure-activity relationship, biological, and pharmacological characterization of the proline sulfonamide ACT-462206: a potent, brain-penetrant dual orexin 1/orexin 2 receptor antagonist. Chem Med Chem 9:2486–2496. doi:10.1002/cmdc.201402258

24. Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. Academic Press, San Diego

25. Guan XM, Yu H, Palyha OC, Mckee KK, Feighner SD, Srinathingshihi DJ, Smith RG, Van der Ploeg LH, Howard AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Brain Res Mol Brain Res 48:23–29

26. Mitchell V, Bouret S, Beauvillain JC, Schilling A, Perret M, Kordon C, Eipelbaum J (2001) Comparative distribution of mRNA encoding the growth hormone secretagogue receptor (GHS-R) in Microcebus murinus (Primate, lemurian) and rat forebrain and pituitary. J Comp Neurol 429:469–489

27. Yoshimura M, Uezono Y, Ueta Y (2015) Anorexia in human and experimental animal models: physiological aspects related to neuropeptides. J Physiol Sci 65:385–395. doi:10.1007/s12576-015-0386-3

28. Yoshimura M, Hagimoto M, Matsuruta T, Ohkubo J, Ohno M, Maruyama Y, Ishikura T, Hashimoto H, Kakuma T, Yoshimatsu H, Terawaki K, Uezono Y, Toyohira Y, Yanagihara N, Ueta Y (2014) Effects of food deprivation on the hypothalamic feeding-regulating peptides gene expressions in serotonin depleted rats. J Physiol Sci 64:97–104. doi:10.1007/s12576-013-0296-1

29. Bonnavaux P, Mickelsen LE, Fujita A, de Lecca L, Jackson AC (2016) Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. J Physiol. doi:10.1113/JP271946

30. Waterson MJ, Horvath TL (2015) Neuronal regulation of energy homeostasis: beyond the hypothalamus and feeding. Cell Metab 22:962–970. doi:10.1016/j.cmet.2015.09.026

31. Coggins J, Brown LM, Zigmian JM, Kemp CJ, Strader AD, Benoit SC, Woods SC, Mangiaracina M, Geary N (2007) Estradiol-dependent decrease in the orexigenic potency of ghrelin in female rats. Diabetes 56:1051–1058

32. Yamada C, Saegusa Y, Nahata M, Sadakane C, Hattori T, Takeda H (2015) Influence of aging and gender differences on feeding behavior and ghrelin-related factors during social isolation in mice. PLoS One 10:e0140094. doi:10.1371/journal.pone.0140094

33. Iwasa T, Matsuzaki T, Munkhzaya M, Tungalagsuvd A, Kuhnsingji DJ, Smith RG, Van der Ploeg LH, Howard AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor (GHS-R) in Microcebus murinus (Primate, lemurian) and rat forebrain and pituitary. J Comp Neurol 429:469–489

34. Zhou L, Ghee SM, Chan C, Lin L, Cameron MD, Kenny PJ, See RE (2012) Orexin-1 receptor mediation of cocaine seeking in male and female rats. J Pharmacol Exp Ther 341:801–809. doi:10.1124/jpet.111.187567

35. Winsky-Sommerer R, Yamanaka A, Diano S, Borok E, Roberts AJ, Sakurai T, Kilduff TS, Horvath TL, de Lecca L (2004) Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. J Neurosci 24:11439–11448