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ENDOGENOUS GHRELIN REGULATES EPISODIC GH SECRETION BY AMPLIFYING GH PULSE AMPLITUDE: EVIDENCE FROM ANTAGONISM OF THE GHS-R1a RECEPTOR

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Short title: Effect of a GHS-R1a antagonist on GH secretion and food intake

Key words: GHS receptor, BIM-28163, GH, food intake

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Abstract

Ghrelin was purified from rat stomach as an endogenous ligand for the growth hormone secretagogue (GHS) receptor. As a GHS, ghrelin stimulates GH release, but it also has additional activities, including stimulation of appetite and weight gain. Plasma GH and ghrelin secretory patterns appear unrelated whereas many studies have correlated ghrelin variations with food intake episodes. In order to evaluate the role of endogenous ghrelin, GH secretion and food intake were monitored in male rats infused subcutaneously (6 µg/h during 10 h) or intracerebroventrically (5 µg/h during 48 h) with BIM-28163, a full competitive antagonist of the GHS-R1a receptor. Subcutaneous BIM-28163 infusion significantly decreased GH area under the curve during a 6 hour sampling period by 54% and peak amplitude by 46%. Twelve hours after the end of treatment these parameters returned to normal. Central treatment was similarly effective [-37% and 42% for AUC and –44% and 49% for peak amplitude on the first and second days of infusion, respectively]. Neither peripheral nor central BIM-28163 injection modifies GH peak number, GH nadir or IGF-1 levels. In this protocol, food intake is not strongly modified and water intake is unchanged. Subcutaneously infusion of BIM-28163 did not change plasma leptin and insulin levels evaluated at 12:00 h and 16:00 h. On the contrary, central BIM-28163 infusion slightly increased leptin and significantly increased insulin concentrations. Thus, endogenous ghrelin, through GHS-R1a, acts as a strong endogenous amplifier of spontaneous GH peak amplitude. The mechanisms by which ghrelin modifies food intake remain to be defined, and may involve a novel GHS receptor.

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Introduction

The secretory pattern of growth hormone (GH) is mainly driven by two counter-regulatory neurohormones, GH-releasing hormone (GHRH) and somatostatin (SRIH). However, the existence of a third factor regulating GH rhythmicity was suggested 20 years ago when met-enkephalin analogues without opiate-like activity were found to exert potent GH-releasing activity (1). Since then, numerous synthetic GH secretagogues (GHS) have been developed and shown to act through a specific G-protein coupled receptor : GHS-R1a (2). GHS-R1a is distinct from the GHRH receptor and it is mainly expressed in the hypothalamus and pituitary gland. In 1999, a 28 amino acid acylated peptide was identified as an endogenous ligand for the GHS-R1a and termed ghrelin (3). It has been demonstrated that the acylation is essential for GHS-R1a binding. Ghrelin is produced mainly by A-like cells in oxyntic glands of the stomach and it is secreted into the bloodstream (4). In addition to the gastro-intestinal tract, ghrelin expression, at either the mRNA or protein level or both, has been identified in a number of tissues, including the hypothalamus (5). In this brain structure, low levels of ghrelin have been measured in the arcuate nucleus (3, 6) which is involved in the control of GH secretion and food intake. As expected ghrelin stimulates GH release (3, 4, 7-9) but also exerts multiple activities, including increased feeding (9) and weight gain (10). The circulating levels and patterns of ghrelin and GH appear weakly related (11) whereas many studies have established strong correlations between ghrelin variations and food intake episodes. Fasting increases circulating ghrelin levels which decrease after food intake (11-13). Ghrelin gene deletion in mice impairs neither growth nor appetite (14, 15) though deleting the GHS-R gene does abolish both ghrelin and synthetic GHS effects on these two functions (16). Thus, the role of endogenous ghrelin in the regulation of pulsatile GH secretion and food intake is not yet clear. The aim of the present study was to define this role by examining the

effects of treating rats with a novel analog of ghrelin, BIM-28163, recently developed as a full competitive antagonist of the GHS-R1a (17).

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Materials and Methods

1) In vitro experiments :

Binding Assay

The cDNA of the human GHS-R1a was cloned by polymerase chain reaction (PCR) using human brain RNA (Clontech, Palo Alto, CA, USA) as the template. The PCR product was cloned into the pCR2.1 vector using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and subcloned into the mammalian expression vector pcDNA 3.1 (Invitrogen). The plasmid was transfected into the Chinese hamster ovary cell line, CHO-K1 (ATCC, Manassas, VA, USA), by the calcium phosphate method. Single cell clones stably expressing the human GHS-R1a were obtained by selecting transfected cells using 0.8 mg/ml G418 containing selection media (Life Technologies, Carlsbad, CA, USA).

Membranes were prepared for radioligand binding studies by homogenization of CHO-K1 cells expressing the human recombinant GHS-R1a in 20 ml of ice-cold 50 mM Tris-HCl with a Brinkman Polytron (Westbury, NY, USA) (setting 6, 15 sec). The homogenates were washed twice by centrifugation (39,000 g for 10 min), and the final pellets were resuspended in 50 mM Tris-HCl, containing 2.5 mM $MgCl_2$, and 0.1% BSA. For assay, aliquots (0.4 ml) were incubated with 0.05 nM [^{125}I]h-ghrelin (~2000 Ci/mmol), with and without 0.05 ml of unlabeled competing test peptide. After a 60 min incubation (4 °C), the bound [^{125}I]h-ghrelin was separated from the free by rapid filtration through GF/C filters (Brandel, Gaithersburg, MD, USA), which had been previously soaked in 0.5% polyethyleneimine/0.1% BSA. The filters were then washed three times with 5-ml aliquots of ice-cold 50 mM Tris-HCl and 0.1% bovine serum albumin, and the bound radioactivity trapped on the filters was counted by

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gamma spectrometry (Wallac LKB, Gaithersburg, MD, USA). Specific binding was defined as the total [^{125}I]h-ghrelin bound minus that bound in the presence of 1000 nM unlabeled h-ghrelin (Bachem, Torrence, CA, USA).

Intracellular Calcium Mobilization Assay

CHO-K1 cells, expressing the GHS-R1a, were harvested by incubating in a 0.3% EDTA/phosphate buffered saline solution (25 °C), and washed twice by centrifugation. The washed cells were resuspended in Hank's - buffered saline solution (HBSS) for loading of the fluorescent calcium indicator Fura-2AM. Cell suspensions of approximately 10^6 cells/ml were incubated with 2 mM Fura-2AM for 30 min at 25 °C. Unloaded Fura-2AM was removed by centrifugation twice in HBSS, and the final suspensions were transferred to a spectrofluorometer (Hitachi F-2000) equipped with a magnetic stirring mechanism and a temperature-regulated cuvette holder. After equilibration to 37 °C, the h-ghrelin analogues were added for measurement of intracellular calcium mobilization. The excitation and emission wavelengths were 340 and 510 nm, respectively.

2) In vivo experiments

Acute in vivo GH stimulation

Male Sprague-Dawley rats (250g) were anesthetized and fit with a jugular-right atrial cannula 18 h prior to the experiment. To determine basal hormone levels blood samples were withdrawn into heparinized syringes at time -10 and 0. To demonstrate antagonist activity, immediately after the time 0 blood sample, the rats were injected with either BIM-28163 (1000 µg/kg) or vehicle via the indwelling cannula. Ten minutes later, a blood sample was withdrawn and the rats were immediately injected with either h-ghrelin (10 µg/kg) or

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vehicle via the cannula. Subsequent blood samples were withdrawn 10, 20 and 40 minutes post-injection of h-ghrelin or vehicle. Plasma was separated and stored until assay for GH.

Chronic in vivo treatments

Four weeks before sampling experiments, adult male Sprague Dawley rats weighing 100–125 g (Charles River Laboratories, Inc., L'arbresle, France) were housed individually in transparent plastic containers placed in a soundproof room with controlled temperature (22-24 °C) and illumination (12 h light, 12 h dark schedule with lights off at 19:00 h). They had free access to food and water and were regularly handled and weighed, to minimize stress effects. Guidelines of the National Institute of Health *for the Care and Use of Laboratory Animals* [1996 (7th ed.) Washington, DC: National Academy Press, aka National Research Council Guide.] were followed.

SC treatment

Six days before the experiment, an osmotic minipump (Alzet Model 2002, flow rate 0.5 µl/h for 14 days; Charles River, France) was connected to a polyethylene catheter and implanted subcutaneously in the back of the animals under ether anaesthesia. The catheter had previously been filled with a combination of saline and BIM or saline. This resulted in delivery of saline during 144 hours followed by BIM-28163 (6 µg/h) or saline during 10 hours. On the day of the experiment (d0) SC treatment started at 9:00 h and rats received IP injection of 300 µg/0.2 ml BIM-28163 or 0.2 ml NaCl at 10:00 h.

ICV treatment

Six days before the experiment, a chronic ICV cannula (Alzet Brain Infusion Kit II, Charles River, France) was inserted into the lateral ventricle of the brain under pentobarbital

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anesthesia (60 mg/kg of body weight i.p, Centravet, Plancoet, France). The following coordinates were used: anterior -0.8 mm to the bregma; lateral -1.5 mm to the midline; height -3.8 mm to the bregma, with the incisor bar set at -3.3 mm below the interaural line. The cannula was secured to the skull of the animal with stainless steel and dental cement and connected to an osmotic minipump (Alzet Model 2002; flow rate 0.5 µl/h for 14 days Charles River) implanted under the dorsal skin of the animal. The flow moderator of the pump was connected to the polyethylene catheter containing 72 µl saline, which was administered during the first 6 days in all animals. A small air bubble was inserted to separate the saline solution from an additional 24 µl of BIM-28163 (5 µg/h) or saline to be delivered for an additional 48 h. Treatment started at 8:00 h on d0 until 8:00 h on d2.

Blood sampling schedule

Experiments were performed on freely moving rats. Two days before data collection, an indwelling cannula was inserted into the right atrium under ether anesthesia as previously described (18). Two hours before the sampling and recording period, the distal extremity of the cannula was connected to a polyethylene catheter filled with 25 IU/ml heparinized saline. Blood samples were withdrawn every 20 minutes and centrifuged immediately. Red blood cells were resuspended in saline and reinjected every hour to attenuate hemodynamic modifications. Plasma was stored at -20 °C until assayed for GH.

Feeding behaviour

Experiments on food and water intake were performed on a separate group of animals which underwent the same treatments. Food and water intake were measured continuously with a PC-controlled monitoring system (Feeding/Drinking Monitor, Technical & Scientific

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Equipment, Bad Homburg, Germany). Animals were acclimated to this new environment for 1 week before actual monitoring.

3) Hormonal determinations

Plasma GH concentrations were measured by EIA as previously described (19). Values are reported in terms of rGH-RP2, with sensitivity of 5 ng/ml and intra- and interassay coefficients of variation below 7%.

Plasma insulin, total leptin and IGF-1 levels were determined with RIA kits following manufacturer instructions (Linco, Saint Charles, Missouri ; DSL, Webster, Texas, USA, respectively). The sensitivity was 0.2 ng/ml, 0.5 ng/ml and 160 ng/ml respectively. For all assays, the intra- and interassay coefficients of variation were below 10%.

4) Statistical analysis

GH pulse analysis was performed using the Cluster program (20) with the *t* value set to 2 to maintain false positive rates under 1%. Cluster size was set to two prepeak and two postpeak nadir values. False positive error for peak detection was 7%.

Areas under the GH response curves (AUC) were calculated by mean of trapezoidal analysis.

Values are given as means ± SEM and statistical analysis was performed by ANOVA and paired *t* test using the JMP IN 5.1 software (SAS Institute Inc, North Carolina, USA).

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Results

1) In vitro experiments

BIM-28163 was found to displace labelled h-ghrelin binding to the human GHS-R1a with a IC_{50} (Ki) of 8.1 $\pm$ 1.3 nM. Human ghrelin, under the same conditions, had an IC50 of 1.6 $\pm$ 0.3 nM.

In the calcium mobilization assay, h-ghrelin induced concentration-dependent calcium mobilization in cells stably transfected with the human GHS-R1a between the concentrations of 0.1 and 100nM, with an EC_{50} of 5.4 $\pm$ 1.4nM. In contrast, BIM-28163 had no effect on calcium mobilization, even when tested at concentrations as high as 10^{-6} M (data not shown). When added in combination, BIM-28168 attenuated the ability of h-ghrelin to induce calcium mobilization in a dose-dependent manner, with complete inhibition achieved with a ratio of 100:1 BIM-28163:h-ghrelin (Figure 1), indicating that BIM-28163 acts as a full, competitive antagonist of ghrelin at the GHS-R1a.

Neither [^{125}I]h-ghrelin binding nor ghrelin-induced mobilization of intracellular calcium was observed in the parent CHO-K1 cell line.

2) In vivo experiments

Acute in vivo GH stimulation

Ghrelin induced GH stimulation was maximal 10 minutes post injection (data not shown). As illustrated on Figure 2, injection of h-ghrelin (10 µg/kg) alone into freely-moving, unanesthetized rats induced a significant increase in plasma GH, whereas injection of BIM-

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28163 (1000 µg/kg) was without effect. When BIM-28163 was injected 10 minutes prior to h-ghrelin, the h-ghrelin-induced increase in GH secretion was completely abolished.

Chronic in vivo treatments

BIM-28163 was infused either SC (at the rate of 6 µg/h during 10 h after an IP bolus of 300 µg) or ICV (at the rate of 5 µg/h during 48h) to unanaesthetized, freely-moving rats and blood samples were collected every 20 minutes for 6 hours the first day, one hour after the beginning of infusion, and the following day during the same period. Individual profiles are illustrated on Figure 3. BIM-28163 administration always resulted in a rapid decrease in spontaneous GH release. Throughout BIM-28163 treatment, total GH, calculated as area under the curve, was significantly lower in treated animals compared with their respective controls (table 1). The decrease in total GH corresponded to a similar decrease in GH peak amplitudes. Basal values and GH pulse number were not modified (Table 1). ICV infusion was similarly effective on the first and second day of treatment. Fifteen hours after the end of SC treatment, pulsatile GH secretion was similar to that of controls.

Neither SC nor ICV treatment modified plasma IGF-1 levels, sampled at 15:00 h [SC treatment: BIM-28163 = 779 ± 84 ng/ml (n=5) vs. saline = 855 ± 78 ng/ml; ICV treatment: BIM-28163 = 977 ± 94 ng/ml (n=7) vs. saline = 1105 ± 133 ng/ml (n=7) on the first day, and BIM-28163 = 1199 ± 96 ng/ml (n=7) vs. saline =1162 ± 113 ng/ml (n=7) on the second day]. SC infusion of BIM-28163 changed neither plasma leptin nor insulin levels evaluated at 12:00 h and 16:00 h. In contrast, ICV BIM-28163 infusion slightly increased leptin and significantly increased insulin concentrations (Table 2).

Food intake was modestly increased only during the light-on period on the second day after ICV infusion of BIM-28163 (Figure 4) and water consumption remained unchanged after either peripheral or central BIM-28163 infusion.

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Discussion

In this study, either SC or ICV infusion of a GHS-R1a antagonist, BIM-28163, was demonstrated to decrease spontaneous GH secretion without causing major changes in food intake. This h-ghrelin analog is a full competitive antagonist at the GHS-R1a. Indeed, BIM-28163 binds to the h-GHS-R1a receptor and can displace h-ghrelin binding, but BIM-28163 does not activate GHS-R1a and completely and dose-dependently inhibits the ability of ghrelin to stimulate calcium mobilization in h-GHS-R1a transfected cells and GH secretion in vivo. Morever BIM-28163 blunts the GH releasing effect of ghrelin but not the GHRH induced GH rise (17). Thus, our results indicate that ghrelin, acting through the GHS-R1a, appears to be an endogenous regulator of spontaneous GH secretion but not necessarily of food intake.

Interestingly, antagonism of the GHS-R1a in freely-moving male rats does not impair the pulsatile pattern of GH secretion; however, it significantly lowers pulse amplitude. These results suggest that endogenous ghrelin acts to amplify the basic pulsatile pattern of GH established by the interplay of hypothalamic GHRH and somatostatin (21). Indeed, it has been established that concomitant administration of ghrelin and GHRH produces an increase in GH secretion significantly greater than that induced by either factor alone (22). The observation that antagonism of the GHS-R1a receptor with BIM-28163 lowers spontaneous GH pulse amplitude and total GH secretion without altering the basic pulsatile pattern agrees well with the observation of (23) that treatment of elderly men with MK677, a non-peptide ligand and agonist of the GHS-R1a, results in a significant increase in GH secretion without changing the underlying pulsatile pattern. Previously, repetitive injections of [D-Lys_3]-GHRP6, a compound reported to be a GHS-R1a antagonist, did not significantly reduce

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plasma GH levels (24); however, a very low dose of [D-Lys$_3$]-GHRP6 was used (750 ng, less than one nanomole) considering the in vitro IC_{50} (3.2 x 10^{-6} M) of this particular analog (25) and its minimum effective dose on *in vivo* food intake (200 nM) (26). On the other hand, GH secretion was reduced in female (but not in male) transgenic rats expressing an antisense GHS-R mRNA under the control of the tyrosine hydroxylase promoter, thus selectively attenuating GHS-R protein expression in the arcuate nucleus (27). Moreover, in *ghsr*-null mice serum IGF-1 levels and body weight are reduced compared with wild type littermates suggesting decreased GH secretion (16). Taken together, these data suggest that endogenous ghrelin (or another, yet unidentified, ligand of GHS-R1a) acts as an amplifier of GH pulsatility. At the present time, a strict correlation has not been established between GH and ghrelin secretory episodes (11); however, only total ghrelin was monitored and it cannot be excluded that a stronger correlation occurs between GH and acylated ghrelin which represents only a minor proportion of total plasma ghrelin and has a very short half life (28).

In the subchronic experimental paradigm used herein, total plasma IGF-1 levels were not modified by BIM-28163 infusion. This lack of effect is in keeping with the data obtained on male transgenic rats expressing an antisense GHS-R mRNA in the arcuate nucleus (27) as well as another study in which chronic ICV administration of ghrelin for seven days did not affect IGF-1 levels (29). Moreover, 95% of IGFs circulate bound to IGFBPs and have a half life of 12-15 hours (30, 31); thus, it cannot be excluded that a longer treatment might affect IGF-1 levels.

Interestingly, plasma leptin slightly and insulin concentrations were significantly elevated by ICV BIM-28163 treatment, but not by peripheral administration. The change in plasma leptin concentrations may represent triggering of counter regulatory mechanisms as leptin has been reported to suppress feeding; although the overt effect of ghrelin on feeding in the

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present study was minimal. Surprisingly, the effect on insulin secretion was only observed following ICV administration of BIM-28163. Expression of the GHS-R1a has been described in the pancreas, yet direct interaction with BIM-28163 via peripheral administration produced no significant effect on insulin secretion. Results from both *GHS-R* (16) and *ghrelin* null (14) mice also suggest that ghrelin exerts only a minimal role in regulating insulin or leptin levels, as these parameters remain largely unchanged in both animal models. Indeed the relationship between ghrelin and insulin secretion is far from clear as, depending on the dose, model and species, ghrelin has been reported to both stimulate and suppress insulin secretion (32). The present results may suggest a central mechanism for stimulation of insulin secretion, as well as provide evidence that this analog does not cross the blood brain barrier; however, further knowledge is clearly needed to fully interpret the present results on leptin and insulin secretion.

The fact that BIM-28163 treatment, either ICV or SC, does not change feeding behaviour is surprising since both GH secretagogues and exogenous ghrelin do stimulate food intake via GHS-R1a (16). Moreover, suppression of feeding has been reported in mice after ICV injections, using two other antagonists: [D-Lys_3]-GHRP6, an analog of one of the synthetic GHSs or [D-Arg,D-Phe,D-Trp,Leu]-substance P an analog of substance P (26). Results with these compounds may be difficult to interpret as the first also binds to four of the melanocortin receptors (33) and the second antagonizes many peptide receptors (34, 35). A recent report indicated that the GHS-R1a displays a high constitutive activity and that [D-Arg,D-Phe,D-Trp,Leu]-substance P is a full inverse agonist, rather than a true antagonist (36). One might therefore hypothesize that the GHS-R1a involved in GH regulation and in food intake may have different levels of constitutive activity and, subsequently, different responses to inverse agonists and antagonists. Alternatively, it cannot be excluded that another, as yet unknown, ghrelin receptor may exist. Structural variants of GHS-R have been

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found in human, swine, chicken and fish genome (37-39). A truncated version of the GHS receptor, derived from alternative gene splicing, GHS-R 1b, and not yet observed in the rat, cannot be the novel ghrelin receptor since it binds neither ghrelin nor any of the various GHS molecules. However, GHS-R 1b, or another receptor, may interact with GHS-R1a, perhaps through receptor heterodimerization as shown in fish (39), thereby playing a counter regulatory role in controlling either GH secretion or food intake.

Another potential explanation of the discrepant results using GHS-R1a antagonists may rely on the central or peripheral location of the ghrelin systems involved. Central ghrelin probably stimulates food intake and GH secretion through neural mechanisms involving neuronal circuitry (40). An important site for GHS-R1a mediation of GH secretion is the hypothalamic arcuate nucleus (41), where GHS-R mRNA is expressed on both GHRH and NPY-expressing neurons (21). However, peripheral ghrelin can also modulate food intake and GH secretion via vagal afferent fibers (42). Blockade of the vagal afferent, either through vagotomy or through perivagal capsaicin application, totally blocks peripheral ghrelin effects on feeding while it only attenuates the stimulation of GH secretion.

In summary, this study shows that pharmacological dissociation of the effects of endogenous ghrelin on GH secretion and feeding is possible. It will remain to be determined whether other effects of this interesting peptide, such as those on adiposity or cardiac function can also be separately affected. At any rate, the present data support the feasibility of creating ghrelin analogs with selective actions on either GH or feeding behavior.

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Figure Legends

Figure 1: Dose-related attenuation of the ability of h-ghrelin to stimulate calcium mobilization in CHO-K1 cells stably transfected with the human GHS-R1a by BIM-28163, a competitive, though non-activating, ligand for GHS-R1a.

Each bar and bracket represents the mean + SEM of 3 replicate experiments. *** : p<0,001 vs ghreline; °°° : p<0,001

Figure 2: Acute blockade by BIM-28163 of h-ghrelin stimulation of GH secretion in vivo

Data are presented as peak GH levels observed 10 minutes after the injection of either vehicle, h-ghrelin (10 µg/kg), BIM-28163 (1000 µg/kg), or the combination. *** :p<0.001 vs vehicle

Figure 3: Representative GH secretory patterns during a 6-h sampling period in freely moving male rats that received subchronic SC (left panel) or ICV (right panel) administration of either BIM-28163 (open circles) or saline (closed squares).

In the SC group, rats were infused at d0 with saline or BIM-28163 (6 µg/h) from 9:00 h to 19:00 h and received an IP injection of saline or BIM-28163 (300µg) at 10:00 h. In the ICV group, rats were infused with saline or BIM-28163 (5 µg/h) for 48h from 8:00 h at d0.

Blood samples were collected every 20 min.

Figure 4: Effect of subchronic SC (left panel) or ICV (right panel) administration of BIM-28163 (open symbols) or saline (closed symbols) on food intake.

Upper panel: Cumulative food intake during the 48 h post infusion.

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Lower panel: Food intake measured during both the diurnal (7:00 h-19:00 h) and nocturnal period (19:00 h-7:00 h) before, during and after treatment.

Table 1 : Cluster analysis of GH pulsatility parameters in BIM-28163-treated rats.

In the SC group, rats were infused at d0 with saline or BIM-28163 (6 µg/h) from 9:00 h to 19:00 h and received an injection of saline or BIM-28163 (300 µg) at 10:00 h. In the ICV group, rats were infused with saline or BIM-28163 (5 µg/h) for 48 h from 8:00 h at d0.

Data are mean ± sem. ** : $p<0.01$; * : $p<0.05$ vs saline

Table 2 : Effect of subchronic BIM-28163 administration on insulin and leptin plasma levels.

SC administration: Hormones were measured in freely moving male rats at 12:00 h and 16:00 h on day 0 (d0: 3 h and 7 h after the start of the treatment) and at the same time on day 1 (d1: 17 h and 21 h after the end of the treatment),

ICV administration: Hormones were measured in freely moving male rats at 12:00 h and 16:00 h on d0 (4 h and 8 h after the start of the treatment) and at the same time on d1 (28 h and 32 h after the start of the treatment),

Data are mean ± sem.** : $p<0.01$; * : $p<0.05$ vs saline

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Route of administration	Sampling day	Treatment	AUC	Number of peaks	Peak amplitude ng/ml	Nadir ng/ml
SC	d0	Saline N = 7	12863 ± 1467	1.7 ± 0.2	69 ± 11	23 ± 1
		BIM-28163 N = 7	5948 ± 1320 **	1.7 ± 0.7	37 ± 6 *	24 ± 7
	d1	Saline N = 7	16617 ± 1174	2.5 ± 0.5	101 ± 13	26 ± 4
		BIM-28163 N =6	13958 ± 2114	2.6 ± 0.5	81 ± 9	23 ± 4
ICV	d0	Saline N = 7	13085 ± 1435	2.0 ± 0.4	70 ± 10	23 ± 1
		BIM-28163 N = 7	8235 ± 1163 *	1.6 ± 0.5	38 ± 5 *	21 ± 2
	d1	Saline N = 7	18681 ± 1497	3.0 ± 0.5	114 ± 11	31 ± 5
		BIM-28163 N = 5	10926 ± 489 **	2.2 ± 0.2	59 ± 3 **	19 ± 1

Table 1

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Route of administration	Sampling day	Treatment	Insulin ng/ml		Leptin ng/ml	
			12:00 h	16:00 h	12:00 h	16:00 h
SC	d0	Saline N = 5	1.53 ± 0.09	1.77 ± 0.08	4.56 ± 0.27	3.48 ± 0.40
		BIM-28163 N = 5	1.78 ± 0.23	1.90 ± 0.24	4.89 ± 0.66	3.78 ± 0.86
	d1	Saline N = 5	1.83 ± 0.13	1.30 ± 0.10	3.72 ± 0.31	2.60 ± 0.25
		BIM-28163 N =5	1.65 ± 0.12	1.81 ± 0.08	3.94 ± 0.66	2.88 ± 0.35
ICV	d0	Saline N = 7	1.76 ± 0.22	2.02 ± 0.23	3.74 ± 0.34	3.31 ± 0.24
		BIM-28163 N = 7	2.70 ± 0.35 *	3.07 ± 0.32 **	4.56 ± 0.24	4.10 ± 0.36
	d1	Saline N = 7	1.92 ± 0.26	2.27 ± 0.34	3.56 ± 0.23	2.61 ± 0.16
		BIM-28163 N = 5	2.86 ± 0.46 *	3.17 ± 0.70	4.78 ± 0.69	3.60 ± 0.64

Table 2

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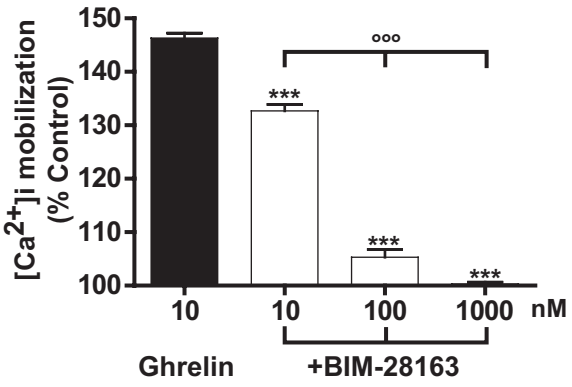

Figure 1

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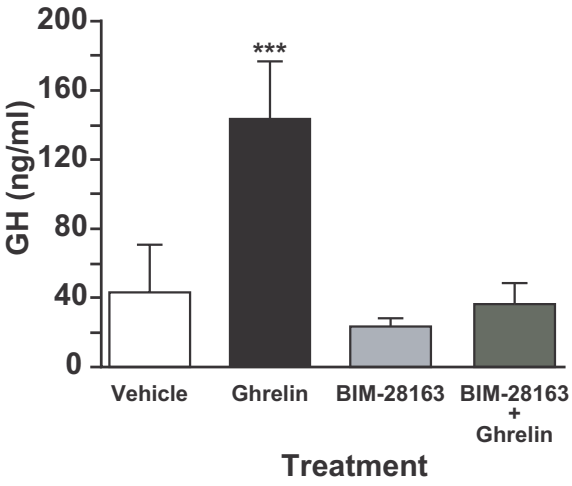

Figure 2

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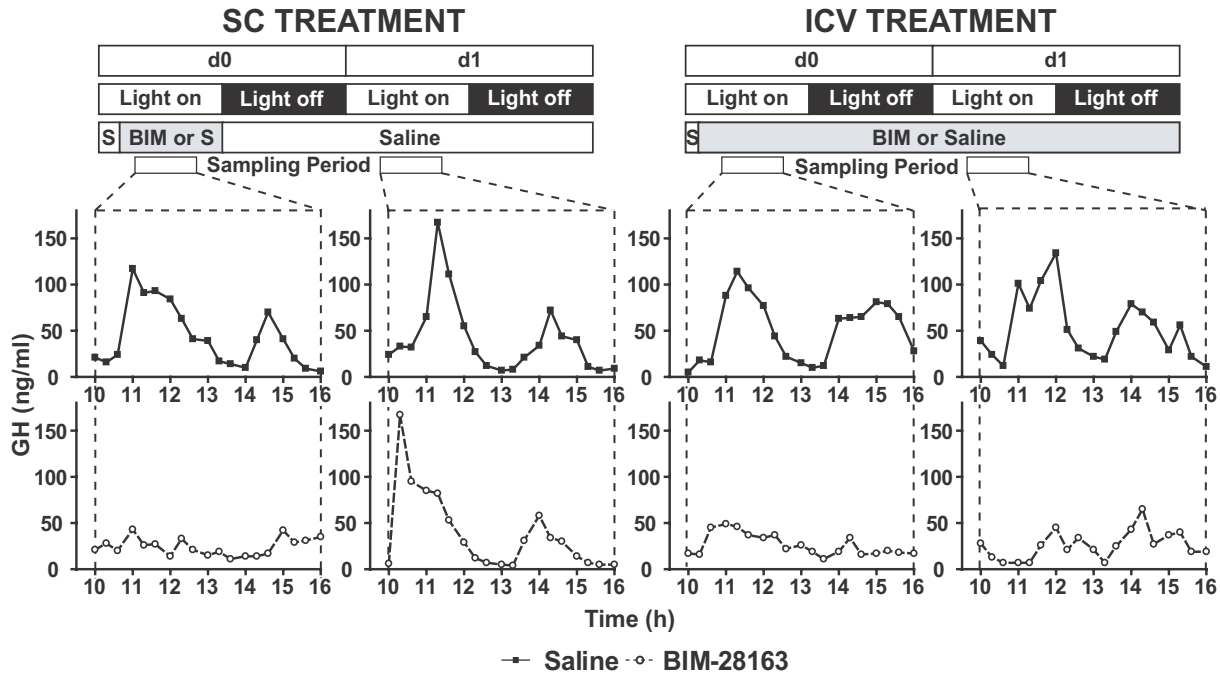

Figure 3

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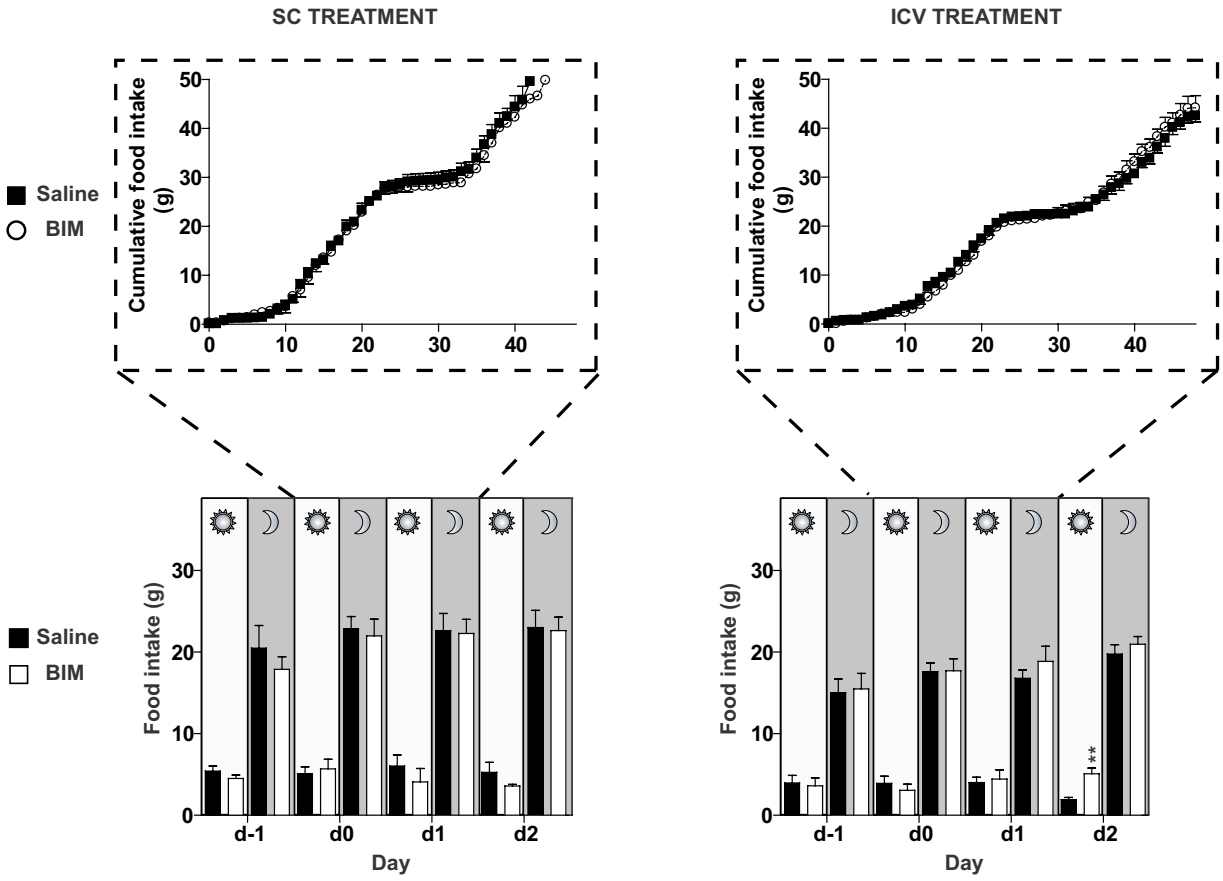

Figure 4