In Vivo Characterization of High Basal Signaling from the Ghrelin Receptor

Pia Steen Petersen, David P. D. Woldbye, Andreas Nygaard Madsen, Kristoffer L. Egerod, Chunyu Jin, Manja Lang, Maria Rasmussen, Annette G. Beck-Sickinger, and Birgitte Holst

Department of Neuroscience and Pharmacology (P.S.P., A.N.M., K.L.E., C.J., M.R., B.H.), Laboratory for Molecular Pharmacology, University of Copenhagen, DK-2200 Copenhagen, Denmark; Laboratory of Neuropsychiatry, Department of Neuroscience and Pharmacology (D.P.D.W.), and Rodent Metabolic Phenotyping Center (P.S.P., A.N.M., C.J., B.H.), University of Copenhagen & Mental Health Centre, DK-2100 Copenhagen, Denmark; and Faculty of Bioscience, Pharmacy, and Psychology (M.L., A.G.B.-S.), Institute of Biochemistry, University of Leipzig, 04103 Leipzig, Germany

The receptor for the orexigenic peptide, ghrelin, is one of the most constitutively active 7TM receptors known, as demonstrated under *in vitro* conditions. Change in expression of a constitutively active receptor is associated with change in signaling independent of the endogenous ligand. In the following study, we found that the expression of the ghrelin receptor in the hypothalamus was up-regulated approximately 2-fold in rats both during 48-h fasting and by streptozotocin-induced hyperphagia. In a separate experiment, to probe for the effect of the high basal signaling of the ghrelin receptor *in vivo*, we used intracerebroventricular administration by osmotic pumps of a peptide [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P. This peptide selectively displays inverse agonism at the ghrelin receptor as compared with an inactive control peptide with just a single amino acid substitution. Food intake and body weight were significantly decreased in the group of rats treated with the inverse agonist, as compared with the groups treated with the control peptide or the vehicle. In the hypothalamus, the expression of neuropeptide Y and uncoupling protein 2 was decreased by the inverse agonist. In a hypothalamic cell line that endogenously expresses the ghrelin receptor, we observed high basal activity of the cAMP response element binding protein, an important signaling transduction pathway for appetite regulation. The activation was further increased by ghrelin administration and decreased by administration of the inverse agonist. It is suggested that the high constitutive signaling activity is important for the *in vivo* function of the ghrelin receptor in the control of food intake and body weight. (***Endocrinology* 150: 4920–4930, 2009)**

Ghrelin is a gastrointestinal hormone secreted mainly from the endocrine cells of the stomach, exerting important functions on the regulation of appetite, food intake, and energy homeostasis (1–3). The most well-described site of action for ghrelin is the appetite-regulating center in the hypothalamus where the highest density of ghrelin receptors is detected (2, 4). Ghrelin stimulates receptors expressed on neuropeptide Y (NPY)/agouti-related peptide (AgRP)-producing neurons in the arcuate nucleus of the hypothalamus (5–7). These neurons constitute the sensory branch of the hypothalamic control center for energy homeostasis, stimulating food intake through release of the potent appetite-inducing hormones NPY and AgRP. Importantly, ghrelin is the only known systemic hormone that stimulates the NPY/AgRP neurons, balancing against a multitude of inhibitory inputs from metabolic hormones and transmitters such as cholecystokinin, insulin, leptin, peptide YY, α-MSH, and

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Abbreviations: AgRP, Agouti-related peptide; AUC, area under the curve; CB, cannabinoid; CRE, cAMP response element; CREB, CRE binding protein; CTA, conditioned taste aversion; icv, intracerebroventricular; IP, inositol phosphate; MC, melanocortin; NPY, neuropeptide Y; QPCR, real-time quantitative PCR; STZ, streptozotocin; 7TM, seven-transmembrane; UCP2, uncoupling protein 2.

γ-aminobutyric acid (8, 9). Recently, it has also been shown that ghrelin acts on neurons in the ventral tegmental area, a region important for motivational aspects of multiple behaviors, including feeding. Ghrelin administration directly into this area has been shown to increase dopamine neuronal activity, synapse formation, and dopamine turnover in the nucleus accumbens and to result in an increase in food intake (10–13). The secretion of ghrelin in humans increases in the hours before each meal and decreases rapidly after meal initiation, indicating a role for ghrelin as a meal initiator or preparator (14, 15).

The ghrelin receptor is known from *in vitro* studies to signal with approximately 50% of its maximal capacity in the absence of the agonist, ghrelin, as shown in a number of signal transduction pathways, including inositol phosphate (IP) turnover, and serum-responsive element (SRE)- and cAMP response element (CRE)-induced transcriptional activity (16–18). Other receptors involved in appetite regulation, for example, the cannabinoid (CB)-1 receptor and the melanocortin (MC)-4 receptor, are also characterized by high basal signaling (19, 20). However, the *in vivo* function of this ligand-independent signaling has until recently been rather speculative (21). For instance, it has been based on identification of naturally occurring mutations in both the MC4 receptor and the ghrelin receptor, which selectively eliminate the constitutive activity without affecting the function of endogenous ligands (22–24). In the ghrelin receptor, this type of mutation segregated with development of short stature, highlighting a physiological importance of the high basal signaling (22, 25). However, more careful analysis of the receptor signaling in the hypothalamus is not possible in a human model.

An alternative approach to visualizing the *in vivo* function of high basal signaling is to administer a selective inverse agonist. This has been done elegantly for the H3 receptor, where the inverse agonist enhanced the activity of the histaminergic neurons. In contrast, the neutral antagonist, which had no effect on it own, was able to suppress the effect of the inverse agonist (26).

The peptide [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (Bachem, Bubendorf, Switzerland) is more than 50-fold more potent as an inverse agonist than as an antagonist for the ghrelin receptor (27). In the present study, we administered [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P at a stable and constant low concentration directly into the ventricular system of the brain using an osmotic pump. These conditions enabled only the inverse agonist properties of the ligand to be studied. The purpose was to reveal whether the constitutive activity of the ghrelin receptor has a function *in vivo*, independent of the ghrelin-induced activation.

Materials and Methods

Peptide synthesis

The inverse agonist peptide and the control peptide were synthesized by automated solid phase peptide synthesis as described previously (28). The purity of all peptides was more than 95% according to analytical HPLC, and the observed masses as investigated by MALDI-ToF (matrix-assisted laser desorption/ionization-time of flight; Applied Biomics, Inc., Hayward, CA) mass spectrometry were in full agreement with the calculated masses.

Animals

Male Wistar rats weighing 275–300 g were used. Rats were housed individually in plastic cages in a temperature- and humidity-controlled environment with a 12-h light and 12-h dark cycle. Standard pellet chow and water were available *ad libitum*. The animal studies were conducted in accordance with institutional guidelines and approved by the Animal Experiments Inspectorate in Copenhagen, Denmark.

Streptozotocin (STZ) administration to rats

Animals were randomly assigned to administration of a single dose of STZ (65 mg/kg, ip) or vehicle. The animals were killed 4 d after STZ/vehicle administration, as described previously (29). Another cohort of rats was followed for 14 d where body weight, blood glucose, water, and food intake were measured regularly.

Surgery

A 28-gauge stainless steel cannula was stereotaxically implanted into the right lateral ventricle (0.8 mm anterior to bregma, 1.2 mm lateral to the midline, and 5 mm ventral to cranium externa). Surgery was carried out under ketamin/xylazin anesthesia (25 mg/ml S-ketamin and 20 mg/ml xylazin in the ratio 8:1, injected ip). After placement of supporting bolts, the cannula was secured by a foundation of dental cement (Poly-F-Plus; Dentsply DeTrey GmbII Konstanz, Germany). One week later, a miniosmotic pump (model 2001, Alzet; DURECT Corp., Cupertino, CA) was implanted sc in the neck region under ketamin/xylazin anesthesia (s-ketamin 25 mg/ml and xylazin 20 mg/ml in the ratio 8:1, ip). The cannula was connected via a plastic catheter to the pump filled with 0.1 mM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (inverse agonist at the ghrelin receptor), 0.1 mM [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P (inactive control peptide), or with the vehicle solution containing PBS and 1% BSA. Rats were randomly divided into the three groups. The miniosmotic pump delivered continuous infusion over 6 d with a flow rate of 1 μl/h. During the infusion period, body weight and food consumption were measured every 12 h at 0900 h and 2100 h.

At the end of the experiment, the rats were euthanized, and the connection between pump and cannula was checked. The brain was immediately removed, the hypothalamus dissected and quickly frozen in liquid nitrogen, and samples were stored at −80 C for subsequent quantitative PCR analysis.

Conditioned taste aversion (CTA)

A separate group of 14 male Wistar rats was used in this experiment. A 28-gauge stainless steel cannula was stereotaxically implanted as described above. One week before the experiment, animals were offered two drinking bottles with tap water to accustom them to the experimental procedure. On the day of the experiment, the rats were fasted at 0800 h and offered a 0.1% saccharin solution at 1300 h, 4 h before compound dosing and 5 h before lights off. To ensure that all rats had tasted the solution, all animals were observed to drink the saccharin solution. After removal of the saccharin solution the rats were randomized into three groups and received a 5-μl intracerebroventricular (icv) infusion of vehicle (PBS 0.1%) or 1.4 nM concentration of [D-Arg^1, D-Phe^1, D-Trp^{7,9}, Leu^{11}]-substance P (inverse agonist at the ghrelin receptor; aiming at a concentration in the cerebrospinal fluid of 20 nM). Immediately after icv infusion, all rats received an ip injection of saline or LiCl (80 mg/kg) and were offered free access to food and water (two bottles). After the randomization process, a standard two-bottle taste aversion assay was performed (30). Preference for tap water *vs.* saccharin solution was measured for two 12-h periods beginning at 72 and 96 h after conditioning [saccharin preference ratio: (saccharin intake in ml)/total fluid intake]. Treatments and bottle placements were randomized to minimize the effects of positional preference.

Real-time quantitative PCR (QPCR)

QPCR was performed using the Mx3000P (Stratagene, La Jolla, CA) as previously described (29). The SYBR Premix Ex Taq (Takara, Shiga, Japan) was used for standard SYBR green-based QPCR and for evaluation of the primers. Cycle threshold values were obtained using Stratagene Mx3000P software, and the difference in cycle threshold (ΔCt) method was used to calculate the relative fold change of RNA levels compared with a calibrator sample that was included in each round of QPCR to normalize between runs. The data were normalized by setting the maximum expression value equal to one, thus showing the relative expression of the gene. β-Actin was used as a reference gene. RNA from tissue was extracted using the SV Total RNA Isolation System (Promega, Madison, WI) followed by cDNA synthesis using the ImProm-II Reverse Transcriptase (Promega). The sequence of the primers used for measuring gene expression was: NPY, forward (f)-TGGACTGACCCTCGCTCTAT and reverse (r)-TGTCTCAGGGCTGGA-TCTCT; AgRP, fGCTGCA-GAAGGCAGAAGC, rGACTCGTGCAGCCTTACACA; CB1, fTGCT-TGCGA-TCATGGTGTAT, rTGTCTCAGGTCCTT-GCTCCT; Y1 Receptor (Y1R), fCGGCGTTCAAGGACAA-GTAT, rCGTTGATTCGTTTGGTCTCA; insulin, fGTCAAA-CAGCACCTTTGTGG, rCTCCAG-TGCCAACTGA; and β-actin, fTTCTACAATGAGCTGCGTGTG, rGGGGTGTT-GAAGGTCTCAAA.

Western blot

The immortalized hypothalamic cell line was generated as described (31). The cell lines were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 20 mM glucose, and penicillin/streptomycin and maintained at 37 C with 5% CO_2. Cells were seeded out in 80% confluence, and the next day the cells were incubated with buffer, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P, or ghrelin for 10 min. The reaction was stopped by removing the medium and washing twice with ice cold PBS. Due to the low expression level of ghrelin receptors in the hypothalamic cell line, the phosphorylated proteins (Qiagen, Valencia, CA) were isolated and the cells were lyzed in sample buffer according to the manufacturer's protocol, and separated on 10% SDS-PAGE according to Laemmli (32). After 4 d of STZ treatment, the hypothalamus was dissected and frozen at −80 C. The frozen hypothalamus was homogenized with a mechanical homogenizer and 500-μl lysis buffer (1% SDS; 10 mM Tris-HCl, pH 7.5), and debris was cleared by centrifugation. Proteins were transferred onto nitrocellulose, and Western blot analysis was carried out using mouse monoclonal antiphospho-CREB (CRE binding protein) antibody (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), primary anti-NPY (FL-97; 1:300 dilution; Santa Cruz Biotechnology), or β-actin (1:10,000 dilution; Sigma) antibody in Tris-buffered saline with Tween 20 and 2% BSA overnight at 4 C. Blots were probed with antimouse horseradish peroxidase-conjugated secondary antibodies, visualized using enhanced chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ) and quantified by densitometry. CREB phosphorylation and NPY expression were normalized according to the loading of protein and by expressing the data as a ratio of phospho-CREB/NPY over β-actin. Results were expressed as the percentage of the value obtained in nonstimulated mock-transfected cells or vehicle-treated rats.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using the appropriate unpaired Student's *t* test or Mann-Whitney *U* test. Differences between data were considered statistically significant when the test resulted in a *P* value less than 0.05.

Results

Regulation of the expression level of the ghrelin receptor

Changes in receptor expression levels are particularly interesting to study for receptors displaying a high degree of constitutive activity because this will be directly associated with changes in receptor signaling independent of the presence of the endogenous agonist ligand. Here we observed an 87% ($P = 0.004$; n = 8) increase in hypothalamic mRNA expression for the ghrelin receptor after 48-h fasting of rats (Fig. 1, *black column*). Importantly, this was shown by QPCR using specific primers exclusively detecting the full-length seven-transmembrane (7TM) splice variant of the ghrelin receptor, called ghrelin receptor 1a. As expected, the NPY expression was increased 49% in the fasted rats (Fig. 1, *dark gray column*). In contrast, the expression level of the Y1R for NPY, which does not display constitutive activity, showed similar expression levels in the fed state compared with those after 48-h fasting (Fig. 1, *light gray column*). Thus, in contrast to the constitutively active ghrelin receptor in the hypothalamic NPY system, it is not the receptor but the ligand that is regulated by fasting.

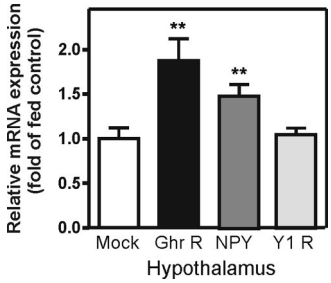

FIG. 1. Gene expression in fed and fasted rats**.** The relative expression of ghrelin receptor 1a (Ghr R; *black*), NPY (*dark gray*), NPY Y1R (*light gray*) in the hypothalamus after 48 h fasting was measured by QPCR using β-actin as the reference gene. QPCR was performed in duplicates and triplicates in two separate experiments for each animal. The insulin expression was significantly decreased in STZ-treated rats compared with fed rats (**, $P = 0.002$). Data are expressed as mean with SEM as error bar. Mann-Whitney *U* test was used for statistical analysis.

We also studied hypothalamic ghrelin receptor expression in rats with uncontrolled diabetes induced by high-dose STZ treatment. Fasting and diabetes represent two different catabolic conditions, even though hyperphagia is observed during diabetes. The STZ-induced hyperphagia is described to be caused partly by the lack of insulin and leptin but also by an increase in the plasma level of ghrelin (33, 34). The diabetic state induced by STZ treatment was verified by a strong increase in blood glucose (Fig. 2A), a decrease in body weight (Fig. 2B), and an increase in water and food intake (Fig. 2, C and D). We also observed an increase in the total plasma of ghrelin 1 and 2 d after STZ treatment. In addition, insulin expression in pancreas was strongly decreased (data not shown). Interestingly, we found an 87% increase ($P = 0.02$, n = 8) in ghrelin receptor expression in the hypothalamus already 4 d after STZ (65 mg/kg) administration (Fig. 2F, *black column*). Surprisingly, we did not observe an increase in NPY expression at this time point after STZ treatment (Fig. 2F, *dark gray column*). However, Western blot analysis using an antibody against the NPY peptide shows that after 4 d of STZ treatments, the NPY level is increased (Fig 2F, *inset*). Thus, the protein level of NPY is increased at a time point where the mRNA level is not increased and where the hyperphagia is not apparent.

In conclusion, we observed that the expression of the ghrelin receptor in the hypothalamus was regulated under various conditions, which demonstrated that the constitutive activity of the ghrelin receptor found under *in vivo* conditions could have physiological consequences independent of the endogenous ligand, ghrelin.

Intracerebroventricular administration of a selective inverse agonist for the ghrelin receptor

In vitro experiments have previously shown that the high basal signaling of the ghrelin receptor is inhibited by administration of the synthetic peptide [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P, *i.e.* it functions as an inverse agonist (18, 27). This peptide has the very interesting property of being a high potency inverse agonist (IC_{50} ~

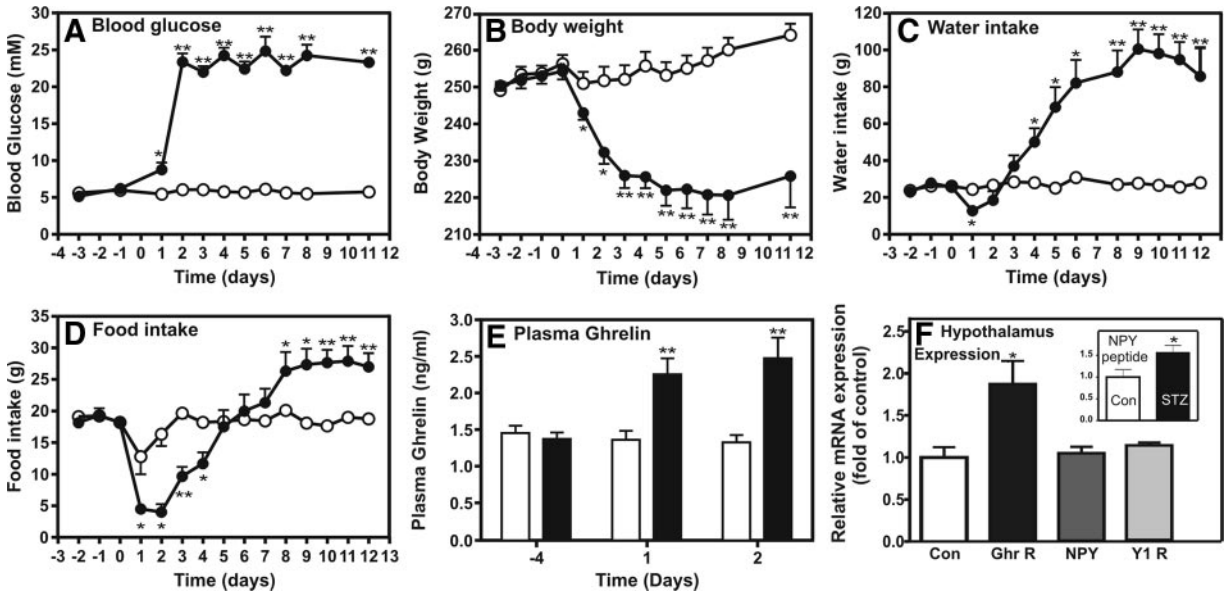

FIG. 2. STZ treatment as model for hyperphagia in rats**.** The changes in blood glucose (A), body weight (B), water intake (C), food intake (D) and nonfasting plasma ghrelin (E) were investigated after STZ-induced diabetes by single ip injection of 65 mg/kg STZ at d 0 (*black circles* or *column*) or vehicle treatment (*white circle* or *column*). In a separate cohort of STZ- or vehicle-treated animals, the effects on the relative gene expression of hypothalamus ghrelin receptor (Ghr R; *black column*), NPY (*dark gray column*), and NPY Y1R (*light gray column*) were measured by QPCR (F) using β-actin as a reference gene. QPCR was performed in duplicates or triplicates in two independent experiments for each animal. In another separate cohort of STZ- or vehicle-treated animals, the effect on NPY peptide expression was studied (F, *inset*). All data are expressed as mean SEM, with n = 6–8 for all groups. *, $P < 0.05$; **, $P < 0.001$ *vs.* vehicle group determined by ANOVA, followed by Fisher's *post hoc* analysis. Con, Control.

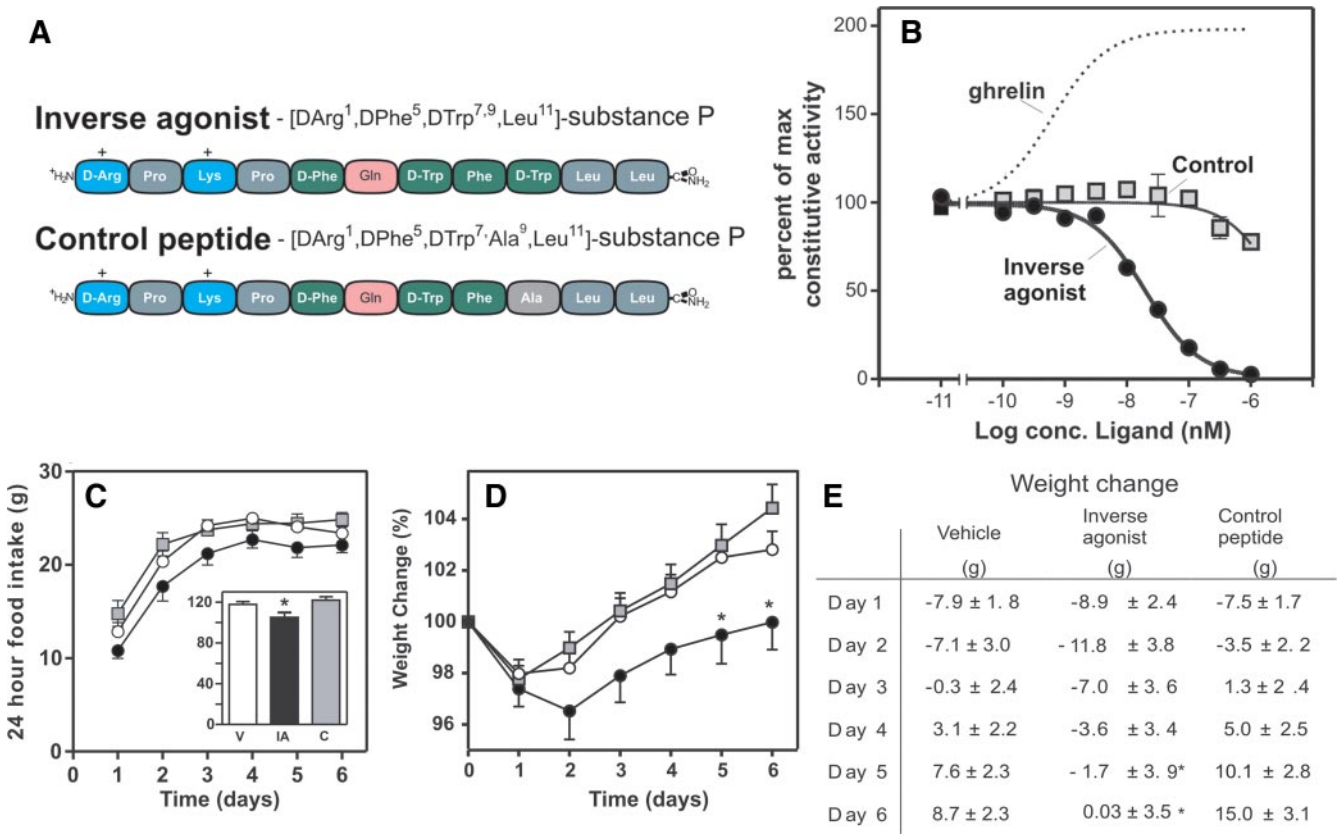

FIG. 3. Effects of the inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P and the control peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P *in vitro* and *in vivo*. Panel A, Amino sequence of the ghrelin receptor inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P and the inactive control peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P. The aromatic amino acids are *green*, the hydrophobic amino acids are *gray*, the hydrophilic noncharged amino acids are *purple*, and the positively charged amino acid is *blue*. Panel B, Dose response curves of the natural ligand ghrelin (*dotted line*), [D-Arg^1, D-Phe^5 D-Trp^{7,9}, Leu^{11}]-substance P (*filled circles*), and the control peptide (*gray squares*) measuring IP turnover in COS7 cells transiently transfected with the ghrelin receptor. Panel C, Food intake for every 24 h. The inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P-treated group is shown as *filled circles* (n = 19), the vehicle-treated group as *open circles* (n = 25), and the control peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P-treated group is in *gray squares* (n = 9). The *inset* shows the AUC for the three groups, vehicle-treated (V) in *white column*, the inverse agonist (IA) in *black column*, and the control peptide (C) in *gray column* (*, $P = 0.02$ *vs.* vehicle). Panel D, Changes in body weight from implantation of the osmotic pump at different time points during the infusion period of vehicle (*open circle*; n = 25), the active inverse agonist [D-Arg^1, D-Phe^5 D-Trp^{7,9}, Leu^{11}]-substance P (*filled circle*; n = 19) or control peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P (*gray square*; n = 9). The initial body weight at time point zero is set to 100%. On d 5 (*, $P = 0.03$) and 6 (*, $P = 0.04$), the difference in weight change between the inverse agonist-treated group and the vehicle-treated group reached significance. Panel E, Body weight change in grams from d 1. On d 5 and 6, the difference in body weight gain of the inverse agonist-infused rats compared with the vehicle group reached significant levels (*, $P = 0.03$; *, $P = 0.04$). Data are mean ± SEM, and Student's *t* test was used for statistical analysis. Conc., Concentration.

20 nM) while being a low potency antagonist ($IC_{50} \sim 1000$ nM) as demonstrated also by Schild-type analysis (18) (Fig. 3B). The dose-response curve of ghrelin, as shown in Fig. 3 as a *dotted line*, has the highest potency with an EC_{50} at 0.2 nM. In the present study, we took advantage of the 50-fold higher potency of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P as an inverse agonist compared with the antagonist on the ghrelin receptor by using it as a tool compound to probe for effects associated with the high constitutive activity of the ghrelin receptor *in vivo*. Using osmotic minipumps, we continuously infused [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P into the lateral ventricle of the brain of rats at sufficiently low concentrations to obtain inverse agonism without antagonism. The experimental dose was estimated based on a pharmacokinetic model aiming at a concentration in the cerebrospinal fluid from 7.5–20 nM at steady-state conditions and assuming a distribution volume of 350 μl with an elimination after first-order kinetics with a half-life between 2 and 5 min (the cerebrospinal fluid is replaced within 10–25 min) (35). Under these circumstances, the administered dose should be 0.1 nmol/h to obtain a steady-state concentration of approximately 7–20 nM.

As a control for potential nonspecific or toxic side effects caused by this hydrophobic peptide tool compound, we administered in parallel a chemically similar control peptide that differs from the original peptide only by a single amino acid substitution (Fig. 3A). In contrast to the inverse agonist, this peptide had only very little effect on the ligand-independent signaling even at 1 μM concentra-

tion (27). The dose-response curves of both peptides were analyzed by an assay for accumulation of IP in COS-7 cells transiently transfected with ghrelin receptor 1a.

Effect on food intake and body weight

The pumps containing either vehicle, the inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P or the control peptide P [D-Arg[1], D-Phe[5], D-Trp[7], Ala[9], Leu[11]]-substance P, were implanted sc in the rats and connected to a previously positioned icv cannula. Food intake and body weight were measured daily in the following 6 d. In all three groups of rats, the 24-h food intake was lower the first 2 d after implantation of the osmotic minipump, presumably due to surgical intervention stress, and then assumed a steady-state from d 3 (Fig. 3C). However, at every time point during the treatment period, compared with that of the other two groups, the daily food intake was lower for the group treated with the inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P. Thus, the accumulated food intake over all 6 d was significantly lower for the group treated with the ghrelin receptor inverse agonist [area under the curve (AUC) = 105 g] compared with the vehicle-treated group (AUC = 118 g; $P = 0.04$) and the group treated with the control peptide (AUC = 122 g; $P = 0.039$) (Fig. 3C, *inset*).

As shown in Fig. 3D, conceivably as a result of the postoperative stress and decrease in food intake, all three groups displayed a decrease in body weight the day after implantation of the pumps. However, the group treated with the inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P continued to decrease in body weight the following day (d 2; Fig. 3D, *black circle*) and continued to display lower body weight compared with the two other groups throughout the infusion period. The maximum weight loss was 11.8 ± 3.8 g at d 2 for the group treated with the inverse agonist, compared with 7.9 ± 1.8 g and 7.5 ± 1.7 g respectively for the vehicle and control peptides. After 5 d of continuous infusion, the rats treated with the inverse agonist still had lower body weight than before the start of the experiment (−1.7 ± 3.9 g), which was significantly lower than the vehicle-treated group (+7.6 ± 2.3 g; $P = 0.03$). After 6 d of treatment, the differences in body weight gain between the inverse agonist-treated group (+0.03 ± 3.5 g) and the vehicle- (+8.7 ± 2.3; $P = 0.04$) or control peptide-treated rats (+15 ± 3 g; $P = 0.01$) were also significant (Fig. 3, D and E).

After 6 d of inverse agonist treatment, plasma glucose under nonfasted conditions was measured, and the inverse agonist-treated rats had slightly increased plasma glucose (18 ± 1 *vs.* 14 ± 1 for vehicle and 16 ± 2 for control peptide). This was in contrast to what was observed for peripherally administered ghrelin receptor antagonist.

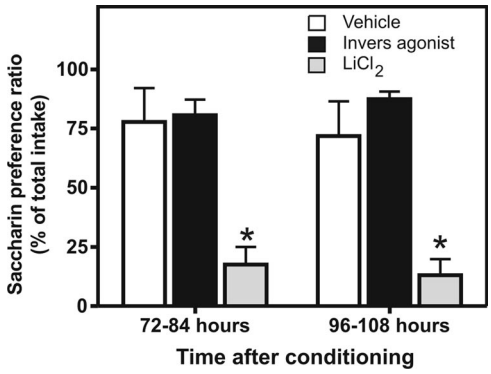

FIG. 4. CTA. The inverse agonist at the ghrelin receptor does not trigger CTA in a two-bottle CTA paradigm. Central administration of [D-Arg[1], D-Phe[1], D-Trp[7,9], Leu[11]]-substance P ghrelin (*black bars*) or vehicle (*white bars*) does not cause CTA 72 or 96 h after conditioning. As a positive control, ip injection of 80 mg/kg $LiCl_2$ (*gray bars*) resulted in a robust CTA at 72 and 96 h. All data are expressed as mean SEM, with n = 4–5 for all groups. *, $P < 0.05$ *vs.* vehicle group as determined by ANOVA, followed by Fisher's *post hoc* analysis. Invers, Inverse agonist.

CTA

To investigate whether the anorectic effect of the ghrelin receptor inverse agonist was due to malaise or induction of nonspecific general behavioral suppression, the ability of a single icv injection of [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P to elicit a CTA response was examined. As seen in Fig. 4, CTA was not induced by the inverse agonist [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]-substance P in the relevant concentrations, whereas the positive control, LiCl, elicited a robust taste aversion with decreased preference for saccharin compared with water.

Effect on gene expression

After the infusion period, the hypothalamus was dissected and mRNA expression of selected transmitters and receptors involved in appetite regulation and energy homeostasis were measured by QPCR. NPY expression was decreased by 19% in the inverse agonist-treated group compared with the vehicle-treated group ($P = 0.04$; Fig. 5A). In contrast, the AgRP expression was not significantly changed in the inverse agonist-treated group compared with the two other groups (Fig. 5B). However, the expression of the NPY Y1R was increased 26% ($P = 0.02$; Fig. 5C) in the inverse agonist-treated group, presumably as a compensatory response to the sustained decrease in NPY level.

The expression of the orexigenic CB1 receptor was not significantly different among the groups, although there was a tendency toward an up-regulation in the inverse agonist-treated rats ($P = 0.07$; Fig. 5D). Because this receptor, just like the ghrelin receptor, signals with high basal activity in the absence of a ligand, an increased expression may have functional consequences. It has been demonstrated that an intact CB signaling pathway is re-

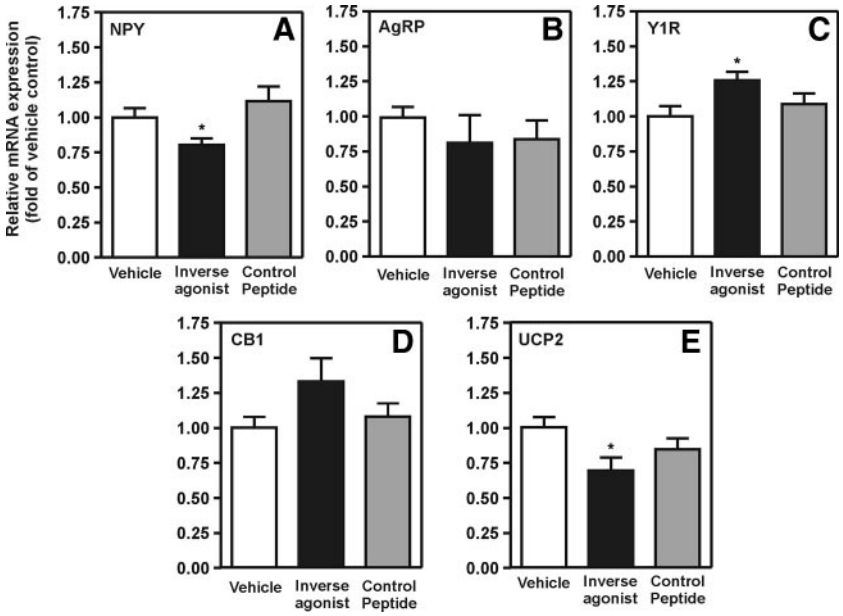

FIG. 5. Regulation of NPY, AgRP, Y1R, CB1, and UCP2 gene expression in response to treatment with ghrelin receptor inverse agonist in rats. The gene expression is measured by QPCR in a hypothalamus isolated from rats infused with vehicle (*white columns*; n = 9), inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (*black columns*; n = 6), or control peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P (*gray columns*; n = 9). A, Expression of hypothalamic NPY was significantly lower in rats infused with inverse agonist compared with rats treated with vehicle (*, $P = 0.04$). B, AgRP expression was unchanged. C, NPY receptor Y1R expression. Y1R was up-regulated in rats infused with inverse agonist compared with vehicle (*, $P = 0.02$). D, CB1 expression showed a tendency to be up-regulated by the inverse agonist compared with vehicle ($P = 0.07$). E, Compared with vehicle-treated rats, expression of UCP2 was down-regulated in the inverse agonist-infused rats (*, $P = 0.03$). Data shown are mean and SEM and statistical analysis was done using Mann-Whitney *U* test.

quired for ghrelin-induced food intake (36), and it is therefore possible that the slightly increased CB1 receptor expression level is compensatory to the decreased ghrelin receptor-mediated signaling.

It has recently been shown that ghrelin increases uncoupling protein 2 (UCP2) expression in the NPY/AgRP neurons and that the ghrelin-induced modulation of the respiration is dependent on UCP2 (37). In accordance with these results, we found that the UCP2 expression was decreased 31% by the inverse agonist compared with the vehicle-treated groups ($P = 0.03$; Fig. 5E).

Phosphorylation of CREB in hypothalamus

The signal transduction pathway that links ghrelin receptor signaling to modulation of NPY expression is not fully understood. We have previously demonstrated that the ghrelin receptor, in heterologous expression systems, activates CREB both in a ligand-dependent and -independent manner (18). Published observations indicate that leptin-induced suppression of NPY activity in the arcuate nucleus of the hypothalamus is mediated through CREB signaling (38). Encouraged by this, we investigated whether the change in ghrelin receptor signaling that results in modulation of NPY expression is also associated with changes in CREB-activated transcriptional activity. In the hypothalamic mouse cell line N42 (31), the expression of the ghrelin receptor was 20-fold lower than observed for the adult hypothalamus (QPCR data not shown). Despite the relatively low receptor expression level in the N42 cells it was possible to detect a ghrelin-induced increase in CREB phosphorylation (2.4 ± 0.2-fold) using 10 nM ghrelin (Fig. 6). Interestingly, 50 nM inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P decreased the basal signaling to 0.51 ± 0.08-fold of the basal signaling (Fig. 6). In contrast, β-actin was not affected by the treatment with either inverse agonist or ghrelin (Fig. 6).

Discussion

In the present study, we characterized the *in vivo* importance of the high basal signaling of the ghrelin receptor, previously observed *in vitro*. Increased gene expression level of the ghrelin receptor was observed during fasting and STZ-induced hyperphagia. More direct evidence came from treatment with a ghrelin receptor inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P that decreased food intake, body weight, and gene expression of NPY and UCP2 in the hypothalamus. Importantly, conditional taste aversion experiments demonstrated no acute toxic effect. It is suggested that the ghrelin receptor-induced modulation of the NPY expression level is mediated by CREB phosphorylation. A high basal level of CREB phosphorylation was observed in a hypothalamic cell line, and it was decreased by treatment with the inverse agonist and increased by treatment with ghrelin.

Ghrelin receptor expression level

The ghrelin receptor is characterized by a high basal signaling through both Gαq and Gα12/13-mediated pathways, independent of the presence of a ligand (18, 22, 39). Increasing receptor expression will accordingly increase activation of the downstream signaling pathways. Interestingly, a number of physiological conditions have been reported to be associated with changes in the expression level of the ghrelin receptor. For example, it has previously been reported by semiquantitative PCR (40) and by *in situ* hybridization (41) that the ghrelin receptor expression in

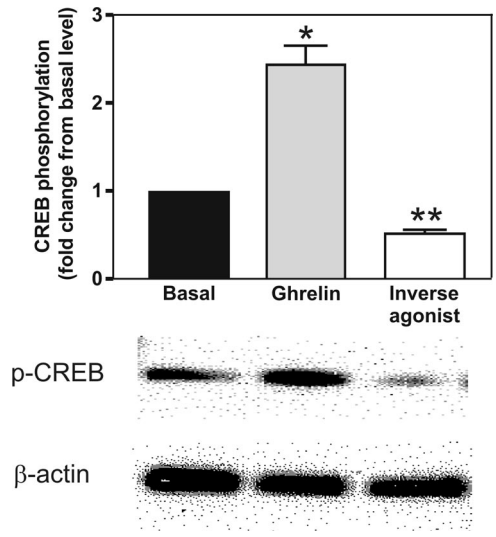

FIG. 6. Level of CREB phosphorylation (p-CREB) in cultured mouse hypothalamic cells in response to ghrelin or inverse agonist treatment. Western blot showing CREB phosphorylation in cultured mouse hypothalamic cells (N42 cell line). Cells were harvested after incubation in normal growth media (basal; *black column*), after incubation in media containing ghrelin (*gray column*) or in media containing [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P (inverse agonist; *white column*). A representative blot of three independent experiments is shown (*bottom*), and the mean of the quantification of the bands in the three experiments is shown at the *top*. Cells incubated in media containing ghrelin showed an increase in CREB phosphorylation compared with basal (*, $P = 0.02$), and the inverse agonist-treated cells showed a decreased CREB phosphorylation (**, $P = 0.009$). Data are mean and SEM. Student's *t* test was used for statistical analysis.

the arcuate nucleus is up-regulated during fasting, a finding we confirmed in the present study (Fig. 1; and Refs. 40 and 41). Similar up-regulation of the ghrelin receptor expression has been observed for Zucker rats, which are characterized by deficient leptin signaling and the concomitant hyperphagia (42). Correspondingly, Zucker rats are characterized by increased sensitivity to ghrelin and other ghrelin receptor agonists as shown by c-*fos* activation (42). Thus, the signaling of leptin/insulin seems to counterbalance the function of ghrelin signaling at the hypothalamic level (43).

It is suggested that the increased expression of the ghrelin receptor observed during hyperphagia induced by destruction of the pancreatic β-cells after STZ treatment contributes to the increased appetite. It has previously been shown that, within the first 2 d after STZ treatment, both plasma insulin and leptin were severely decreased, providing a stimulus for increased food intake (33). In contrast, plasma ghrelin levels increased acutely 24 h before initiation of hyperphagia, consistent with the possibility of a role for ghrelin in appetite stimulation and preparation of the body for its next meal (33, 44). Furthermore, the increase in the ghrelin receptor expression level corresponds very well with the observation that STZ treatment renders the animals more sensitive to ghrelin-induced food intake (33). The use of STZ treatment as a model for hyperphagia has some limitations. STZ toxicity can target not only the pancreatic β-cells but also other tissues such as the liver, kidney, and the adipocytes, which may complicate the impact on and subsequent evaluation of the associated metabolic state.

Importantly, the ghrelin receptor is up-regulated under the same conditions that increase the plasma level of its ligand, ghrelin. In contrast, the expression levels of most nonconstitutively active receptors are not regulated acutely, but in chronic conditions they may be regulated inversely to their ligand. This is exemplified by the NPY Y1R, which was not affected by fasting or STZ treatment but slightly up-regulated after 6 d of decrease in NPY expression.

Inhibition of high basal signaling as opposed to inhibition of ghrelin-induced signaling

The highly regulated expression of the ghrelin receptor indicates an *in vivo* function for its ligand-independent signaling. More direct evidence requires pharmacological or genetic ablation of the high basal signaling. To this end, we used the inverse agonist [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P, which has previously been demonstrated to inhibit the basal signaling of the ghrelin receptor *in vitro* with high potency and efficacy, as a pharmacological tool. This substance P analog constitutes a valuable tool because it acts as a selective inverse agonist without antagonist function in a wide concentration range. The appropriate concentration was obtained by administration through an osmotic minipump. Thus, only the high basal signaling was decreased, whereas the ghrelin-induced stimulation was unaffected (18).

Based on the important function of ghrelin in food intake and energy expenditure, the pharmaceutical industry has over the last few years engaged in development of antagonist compounds active at the ghrelin receptor in an attempt to obtain antiobesity drugs (45, 46). The most potent and efficacious antagonists have been administered in rodent models of obesity, and significant decreases in acute and chronic food intake, accompanied by decreased body weight, have been observed (45). Such studies suggest that blocking the ghrelin-induced modulation of energy expenditure and food intake leads to decreased body weight. The most surprising finding of the present study is that an inverse agonist, which does not affect the ghrelin-induced food intake, was able to inhibit the food intake and prevent the body weight gain observed in the control groups. Furthermore, the expression level of NPY was significantly decreased after 6 d of treatment. This is in agreement with the well-recognized mechanism of ghrelin to directly stimulate the orexigenic NPY/AgRP neurons

and indirectly, through release of γ-aminobutyric acid, inhibit the anorexigenic proopiomelanocortin/cocaine-amphetamine-regulated transcript (POMC/CART) neurons. The decrease in NPY expression was accompanied by a similar decrease in UCP2 expression, which is consistent with the recent observation by Andrews *et al.* that UCP2 activity is essential for ghrelin-mediated changes of the metabolism (37).

Because ghrelin is the only stimulatory hormone acting on these orexigenic neurons counterbalancing a multitude of inhibitory inputs, it is hypothesized that the constitutive activity of the ghrelin receptor uniquely provides a high setpoint of signal transduction on which the various inhibitory factors may act (43). In this respect, it is important that the ghrelin receptor signals with high ligand-independent activity through CRE-mediated transcriptional activity, as shown in a hypothalamic cell line. Control of CRE activity has been shown *in vivo* to be a signal transduction pathway for leptin in NPY/AgRP neurons in the hypothalamus and may represent part of a common signaling pathway for the physiologically opposing actions of ghrelin and leptin (38).

High basal signaling of 7TM G protein-coupled receptors

It has been appreciated for several years that 7TM G protein-coupled receptors are capable of constitutive signaling (47). Introduction of mutations in crucial areas may lead to spontaneous conformational changes of the receptor into the active state in the absence of agonist. When such mutations occur in humans, they can be responsible for diseases; for example, *pubertas precox* is caused by an activating mutation in the LH receptor, and nonautoimmune hyperthyroidism is caused by activating mutations in the TSH receptor (47). In contrast, the physiological importance of spontaneous activity in wild-type receptors has only been described in a few cases (21, 48). Many 7TM receptors display increased basal activity only after overexpression in a heterologous expression system. Even then, only a minor elevation in basal signaling is observed in comparison to ligand-induced signaling (48). However, the physiological importance of the constitutive activity has been demonstrated for the virally encoded 7TM receptor ORF-74. In transgenic overexpression studies, it was shown that both the ligand-induced and the constitutive signaling were important for development of *karposi sacoma*-like lesions in mice (49). The physiological importance of the constitutive activity of other 7TM receptors could be investigated using rather complicated genetic approaches of knock in techniques, but such studies have to the best of our knowledge not yet been published. Human disease causing mutations identified in the MC4 and the ghrelin receptor, which selectively eliminated the high basal signaling and not the ligand-induced signaling, has been described and segregated with obesity and short stature, respectively (22, 23). However, a more thorough analysis of the molecular mechanism is complicated in human models.

Recently, it was shown that the endogenous antagonist and inverse agonist AgRP is important for chronic feeding behavior in mice with a neural-specific knockout of the POMC, indicating that the inverse agonist function of AgRP and accordingly the constitutive activity of the MC4 receptor are of physiological importance (21). The physiological importance of the histamine H3 receptor has also been demonstrated *in vivo* in an elegant study that takes advantage of the existence of a large number of synthetic ligands, including both selective inverse agonist and neutral antagonists (26). It was shown that the inverse agonist enhanced the activity of the histamine neurons, whereas the neutral antagonist, which had no effect on its own, was able to suppress this effect from the inverse agonist (26). In the present study, we used a similar approach and took advantage of our extensive analysis of the peptide ligand [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-substance P (18, 27), which we found to be more than 50-fold more potent as an inverse agonist than as an antagonist. The chemically very similar peptide [D-Arg^1, D-Phe^5, D-Trp^7, Ala^9, Leu^{11}]-substance P served as a convincing negative control peptide as it does not act as an inverse agonist even at high micromolar concentrations.

Although the constitutive activity of 7TM receptors is a well-accepted phenomenon known from *in vitro* studies (48), the *in vivo* function has mainly been suggested by indirect evidence. However, the development of more advanced pharmacological and genetic tools (50) may reveal that constitutive activity of many wild-type 7TM receptors has important physiological relevance in more than just appetite regulation, where the most substantial evidence exists today (21, 26).

Perspective

The present study is one of very few demonstrating the physiological function of the high basal signaling displayed by a G protein-coupled 7TM receptor in the absence of a ligand. This observation may have important implications for the drug discovery efforts targeting the ghrelin receptor. Our data indicate that the most efficient inhibition of the ghrelin system would be obtained through the combined use of an inverse agonist, to decrease the basal tone of the system set by the constitutive activity of the ghrelin receptor, and an antagonist, to decrease the meal-related fluctuations in plasma ghrelin.

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Address all correspondence and requests for reprints to: Birgitte Holst, M.D., Ph.D., Laboratory for Molecular Pharmacology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark. E-mail: holst@sund.ku.dk.

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