# Supporting Online Material for

## Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin's Effects on Food Intake

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**Supporting Online Material**

## Materials and Methods

**Reagents** Human obestatin, nonamidated obestatin (NA-obestatin), and (des1-10)obestatin were synthesized by GL Biochem Ltd. (Shanghai, China) and purified by reverse phase high-performance liquid chromatography. The peptide sequences were verified by amino acid analysis and mass spectrometry. Ghrelin, motilin, neurotensin, neuromedin U, and MTII were purchased from Phoenix Pharmaceuticals (Belmont, CA). The rabbit polyclonal antibodies against synthetic human obestatin peptide were produced by Quality Controlled Biochemicals (Hopkinton, MA).

**Immunoassays for obestatin, ghrelin, and leptin** Tissue extracts and sera were used for the measurement of obestatin and ghrelin immunoreactivities. Immuno-obestatin was determined using rabbit polyclonal antibodies at a final dilution of 1:6,000. The reaction mixture consisted of 100 µl of test samples or standards together with 100 µl of the antiserum. Samples were incubated for 24 hours at 4 °C, 100 µl of tracer (10,000–15,000 cpm) was added, and the samples further incubated for another 24 hours at 4 °C. Free and bound obestatin were separated by the solid phase second antibody method using donkey anti-rabbit IgG (Phoenix Pharmaceuticals) before counting in a γ-spectrometer (LKB, Uppsala, Sweden). Total (*n*-octanoyl and *des*-acyl) ghrelin immunoreactivity was determined using a specific radioimmunoassay (Phoenix Pharmaceuticals). No cross-reactivity was found between the obestatin and ghrelin radioimmunoassays. Circulating ghrelin and obestatin levels were measured in adult male Sprague–Dawley rats before and after fasting for 48 hours, or fasting followed by free access to food or drinking water containing 50% dextrose for 2 hours. Following long term treatment with obestatin, serum leptin levels were determined using an enzyme-linked immunosorbent assay (Phoenix Pharmaceuticals).

**Purification of obestatin** Stomach preparations (67 g) from 30 rats were minced and boiled for 5 min. in 5 volumes of water to inactivate intrinsic proteases. Before homogenization with a Polytron mixer, the solution was adjusted to 1 M acetic acid and 20 mM HCl. After centrifugation at 225,000 g for 30 min., the supernatant was concentrated to 100 ml using an evaporator before precipitation under 66% acetone. After removal of the precipitates, the volume

of the supernatant was reduced by acetone evaporation before loading onto a 10-g cartridge of Sep-Pak C18 (Waters, Milford, MA) pre-equilibrated with 0.1% trifluoroacetic acid (TFA). The Sep-Pak cartridge was washed with 10% acetonitrile/0.1% TFA, and then eluted with 60% acetonitrile/0.1% TFA. Peptides in the eluate were lyophilized, dissolved in 1 M acetic acid, and fractionated using a Sephadex G-50 gel filtration column. A portion of each fraction was used for obestatin and ghrelin radioimmunoassays. Fractions containing immuno-obestatin were further separated by ion-exchange FPLC on a UNO Q1 column (BioRad) at pH 8.1. After identification of the peak containing immuno-obestatin, the samples were subjected to mass spectrometry and *de novo* N-terminal sequencing at Pan Facility (Stanford University, CA).

**Labeling of obestatin and receptor binding** Iodination of obestatin was performed using the Iodogen (Pierce, Upland, IN) procedure. Mixtures of the peptide (20 µg) and 1 mCi [$^{125}$I] NaI were transferred to precoated Iodogen vials and incubated for 4 min. The $^{125}$I-labeled peptide was applied to a Sep-Pak C18 cartridge (Waters) before elution with 60% acetonitrile/0.1%TFA. For radioligand binding assays, rat jejunum or other tissues were washed with buffer A (20 mM Hepes, 5 mM EDTA, 1 mM dithiothreitol (DTT), 10 µM amidinophenylmethanesulfonyl fluoride, 5 mg/L leupeptin, 100 mM KCl, pH 7.5), cut into small pieces, and homogenized using a motorized homogenizer. The homogenates were centrifuged at 1,000 g for 5 min. and the supernatant was centrifuged at 300,000 g for 1 hour at 2°C. The pellets (crude membrane fractions) were resuspended with buffer A without KCl, quickly frozen under liquid nitrogen, and stored at -80°C until use. Tissue homogenates were incubated in 100 µl of phosphate buffered saline containing 0.1% bovine serum albumin for 18 hours at room temperature with varying concentrations of $^{125}$I-obestatin in the presence or absence of unlabeled obestatin at 1,000-fold excess. After incubation, the tubes were centrifuged for 10 min. at 10,000 *g*, and pellets were washed twice in ice-cold PBS before quantitation of radioactivity with a γ-spectrophotometer. Specific binding was calculated by subtracting nonspecific binding from total binding. For displacement curves, a fixed concentration of $^{125}$I-obestatin was incubated with or without increasing concentrations of obestatin or other peptides.

**Analysis of gastrointestinal functions** Eight-week-old C57BL6 male mice were housed individually in a regulated environment. Before intraperitoneal treatment with different peptides, mice were deprived of food for 16 hours with free access to water. Food intake was measured by

placing preweighed pellets in the cage and weighing uneaten pellets at 5 hours after treatment. For intracerebroventricular injection of different peptides, mice were deprived of food for 16 hours. Free-hand injections were performed at 2 hours after light onset and food intake monitored for 5 hours. To estimate gastric emptying responses, mice deprived of food for 16 hours were given food pellets for 90 min. before injection of different hormones or saline. After treatment, mice were deprived of food again and sacrificed 0.5, 1, and 2 hours later. The stomach was excised at the pylorus and cardia before weighing. Gastric emptying was calculated by subtracting the stomach weight of treated mice from those sacrificed at the time of peptide injection.

**Isometric force measurements** Strips of jejunal muscle (~1 cm in length) were cut along the longitudinal axis of the circular muscle layer and the mucosa was removed to minimize contamination with endogenous peptides. Muscle strips were mounted to a TIS8105R (Kent Scientific Corporation, Torrington, CT) isometric strain gauge and immersed in a 5 ml organ bath maintained at 37°C with oxygenated KRB [Krebs-Ringer phosphate buffer, consisting of 50 mM HEPES, 100 mM NaCl, 5 mM KCl, and 1 mM each of MgCl2, NaH2PO4, and CaCl2] as previously described (*1*). A resting force of 1.0 g was applied to intestinal muscles and a 1.5 hours equilibration period was allowed before testing of different peptides during a 5 min. period. After each test, muscle strips were washed with fresh KRB for 20 min. to allow full recovery of basal contractile activities. Maximal contraction was evaluated at the beginning and end of each experiment after treatment with 10 μM of acetylcholine chloride. Contractility data were digitized and stored in a computer using DAS Wizard$^{TM}$ (Measurement Computing, Middleboro, MA). The magnitude of contractile strength is expressed as percent of maximal contraction induced by acetylcholine.

**Pituitary cell cultures** Anterior pituitaries were removed from adult male Sprague-Dawley rats and dispersed with 0.3% collagenase, 0.1% hyaluronidase and DNase I (10 μg/ml). The cell suspension was centrifuged at 300 g for 5 min. and cells were washed twice before incubation in Dulbecco minimal essential medium (DMEM) containing 0.1% bovine serum albumin (BSA), penicillin (100 units/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), and 10% fetal calf serum. Cells were seeded onto poly-lysine-coated 24-well plates at a density of ~$3\times10^5$ cells/well. Cultures were kept in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C. After a 72 h-

incubation, cells were washed with DMEM containing 0.1% BSA, then incubated for 1 hour in serum-free medium with ghrelin or obestatin. Media was removed for growth hormone measurement by an enzyme-linked immunosorbent assay.

**Receptor activation based on cAMP production and reporter gene activity** For assessment of cAMP production mediated by recombinant GPR39, GPR39 cDNA was amplified from a human stomach cDNA library (CLONTECH, Palo Alto, CA) and subcloned into the pcDNA3.1/Zeo(+) expression vector (Invitrogen, Carlsbad, CA) before sequence confirmation. The GPR39-containing plasmid was transfected into CHO or HEK293T cells using Lipofectamine 2000 (Invitrogen). After 18 to 24 hours, cells were harvested and then cultured in the cAMP assay buffer (DMEM/F12 supplemented with 0.1% BSA and 0.25 mM 3-isobutyl-1-methylxanthine). After 30 min. of culture, cells ($2 \times 10^5$ cells/well) were treated with different peptides for 16 hours before measurement of total cAMP levels in triplicates using a specific radioimmunoassay (*2*). To monitor receptor-coupled $G_{\alpha q}$ signals by GHSR or the motilin receptor, HEK293T cells were co-transfected with a chimeric G protein expression plasmid, $G_{sq5}$ (*3*) together with the GHSR or motilin receptor expression plasmid at a ratio of 1:20. After 18–24 hours of transfection, cells were harvested and used for hormonal treatments followed by cAMP assays. For assessment of signaling mediated by the serum response element (SRE), CHO or HEK293T cells ($2 \times 10^5$ cells/well) were transfected with the SRE-Luc reporter construct (PathDetect; Stratagene) together with the GPR39 cDNA. The pCMV-ß-galactosidase plasmid also was used to monitor transfection efficiency. One day after transfection, cells were treated with different peptides for 16 hours in DMEM/F12 supplemented with 0.1% BSA. Cells were harvested in lysis buffer (200 µl) (Promega Corp.) and 30 µl of the supernatant was analyzed for luciferase activity using a luminometer (Bio-Rad Laboratories, Inc.). The ß-galactosidase activity was also determined to monitor transfection efficiency. The reporter activity is expressed as relative light unit/ß-galactosidase activity (*4*, *5*).

**Cross-linking analyses** To demonstrate direct interaction between obestatin and GPR39, HEK293T cells were transfected with the GPR39 expression vector or the empty vector. After 18 hours, cells were harvested and incubated at 23°C with $I^{125}$-obestatin (150,000 cpm/tube) with or without 300-fold excess of non-labeled obestatin. Sixteen hours later, cells were washed three times and the cross linker disuccinimidyl suberate was added to a concentration of 1 mM for 30

min. at 23°C. Cells were then extracted with the solubilization buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and the cell extracts were fractionated using SDS-polyacrylamide gel electrophoresis. Radioactive bands were then detected using a PhosphorImager (Amersham Biosciences, Piscataway, NJ).

**Quantitative real-time RT-PCR** To quantify transcript levels for GPR39 in mouse tissue, real-time RT-PCR was performed using a SmartCycler (Cepheid, Sunnyvale, CA) as described previously (*2*). Total RNA was extracted from different mouse tissues using the RNeasy kit (Qiagen Science, Valencia, CA) before reverse transcription using a Sensiscript RT kit (Qiagen Science). Real-time PCR was performed using a QuantiTect Probe PCR Kit (Qiagen Sciences) and fluorescent-labeled probes (3'-end: TAMRA, 5'-end: 6-FAM). Expression of β-actin was used for copy number normalization. Standard curves for GPR39 and β-actin transcripts were generated by serial dilutions of individual cDNAs. The primer pairs and fluorescent probes used were as follows: GPR39 forward: 5'-AGACAGACCATCATATTCCTGAGAC-3'; GPR39 reverse: 5'-AGTACGTTCTGGTCCAGTCATGTT-3'; GPR39 probe: 5'-FAM-TGCCCAATCAGATCCGACGGATCA-TAMRA-3'. β-actin forward: 5'-TCTGTGTGGATTGGTGGCTCTA-3'; β-actin reverse: 5'-CTGCTTGCTGATCCACATCTG-3'; β-actin probe: 5'-FAM-CTTGCCCACAGCCTTGGCAGC-TAMRA-3'.

**Figure Legend**

**Fig. S1** Bioinformatic prediction of conserved obestatin. Based on a computer program previously used to identify unique protein signatures (*6*), we searched for potential mono- or di-basic cleavage sites in ~200 known preprohormone sequences. Candidate regions were further checked for evolutionary conservation of the putative mature regions in diverse species. Among several candidates, we identified obestatin. Amino acid sequence of preproghrelin from 11 mammalian species are shown with the signal peptide (italicized), mature ghrelin (shaded), and the flanking obestatin (underlined). Consensus basic residues representing putative convertase cleavage sites are shown as white letters on a black background. In the consensus sequence, individual residues with complete conservation are shown in upper case. GenBank (gi) numbers for individual ghrelin genes are 37183224 (human), 34541890 (monkey), 19224664 (mouse),

11067387 (rat), 27357900 (gerbil), 47523230 (pig), 52782813 (cat), 50978704 (dog), 52782814 (goat), 57526202 (sheep), and 27806613 (cattle).

**Fig. S2** Circulating leptin and ghrelin levels in rats following long-term treatment with obestatin, ghrelin, and other peptides.

**Fig. S3** Ghrelin, but not obestatin, stimulated growth hormone secretion by cultured anterior pituitary cells. Differences between treatment groups were analyzed using ANOVA and Student's t-test.

**Fig. S4** Obestatin binding to target tissues. **A)** High affinity binding of $I^{125}$-obestatin to plasma membrane preparations of rat jejunum. Crude plasma membrane fractions were incubated with increasing doses of $I^{125}$-obestatin with or without excess of nonlabeled obestatin before determination of specific obestatin binding. **B)** Hormonal specificity of obestatin binding to rat jejunum. Peptides listed were tested separately. **C)** Specific binding of $I^{125}$-obestatin to diverse rat tissues.

**Fig. S5** Obestatin activation of GPR39 in transfected HEK293T cells. **A)** Obestatin stimulation of cAMP production. **B)** Obestatin activation of the SRE-luciferase reporter.

**Fig. S6** Cross-linking of $I^{125}$-obestatin to recombinant GPR39. HEK293 cells were transfected with GPR39-containing or empty plasmids before incubation with $I^{125}$-obestatin with or without 300-fold excess of unlabeled obestatin. Molecular weight markers are shown on the left as kilodaltons (kD). Labeled obestatin cross-linked to recombinant GPR39 migrated as a 47 kD band.

References


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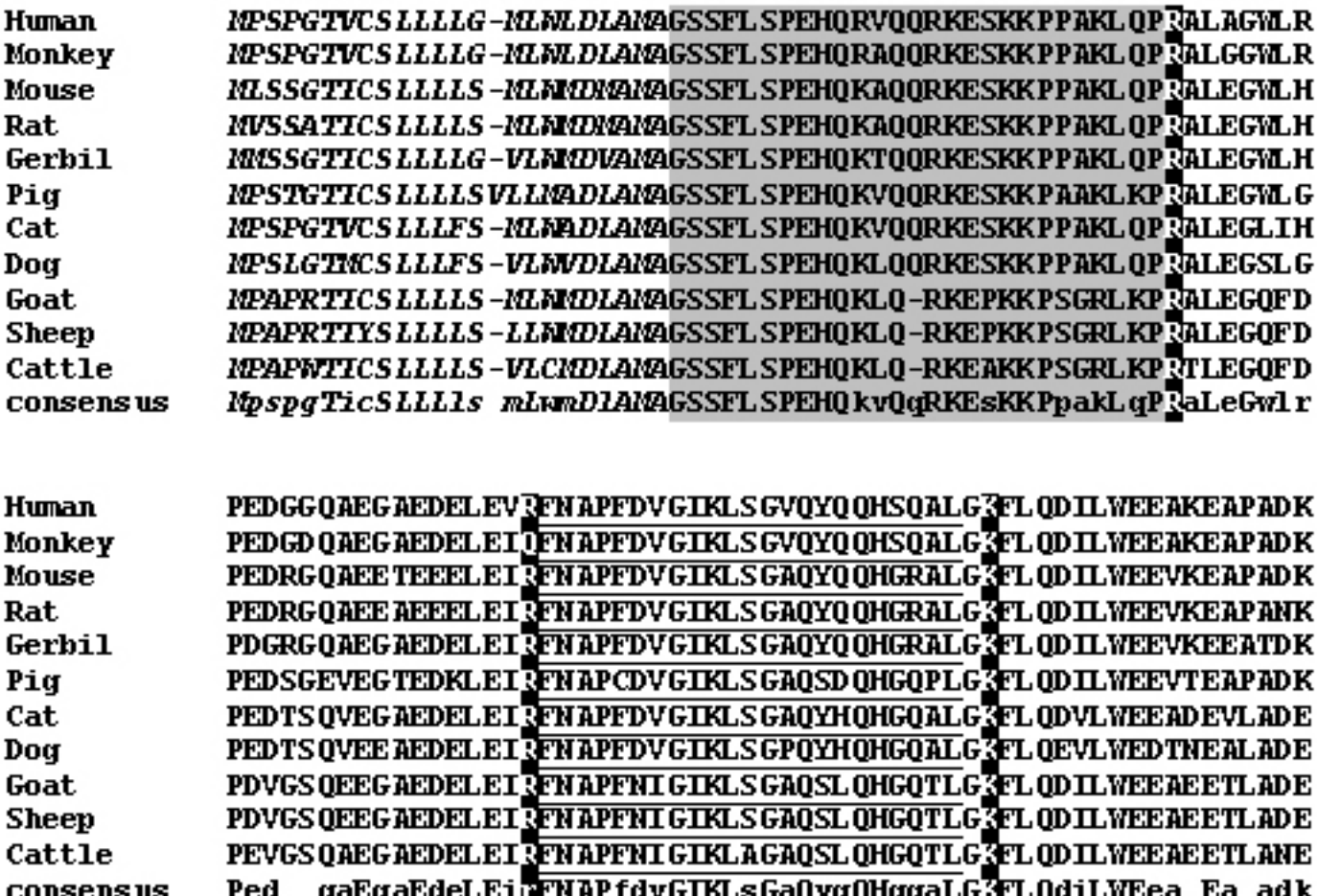
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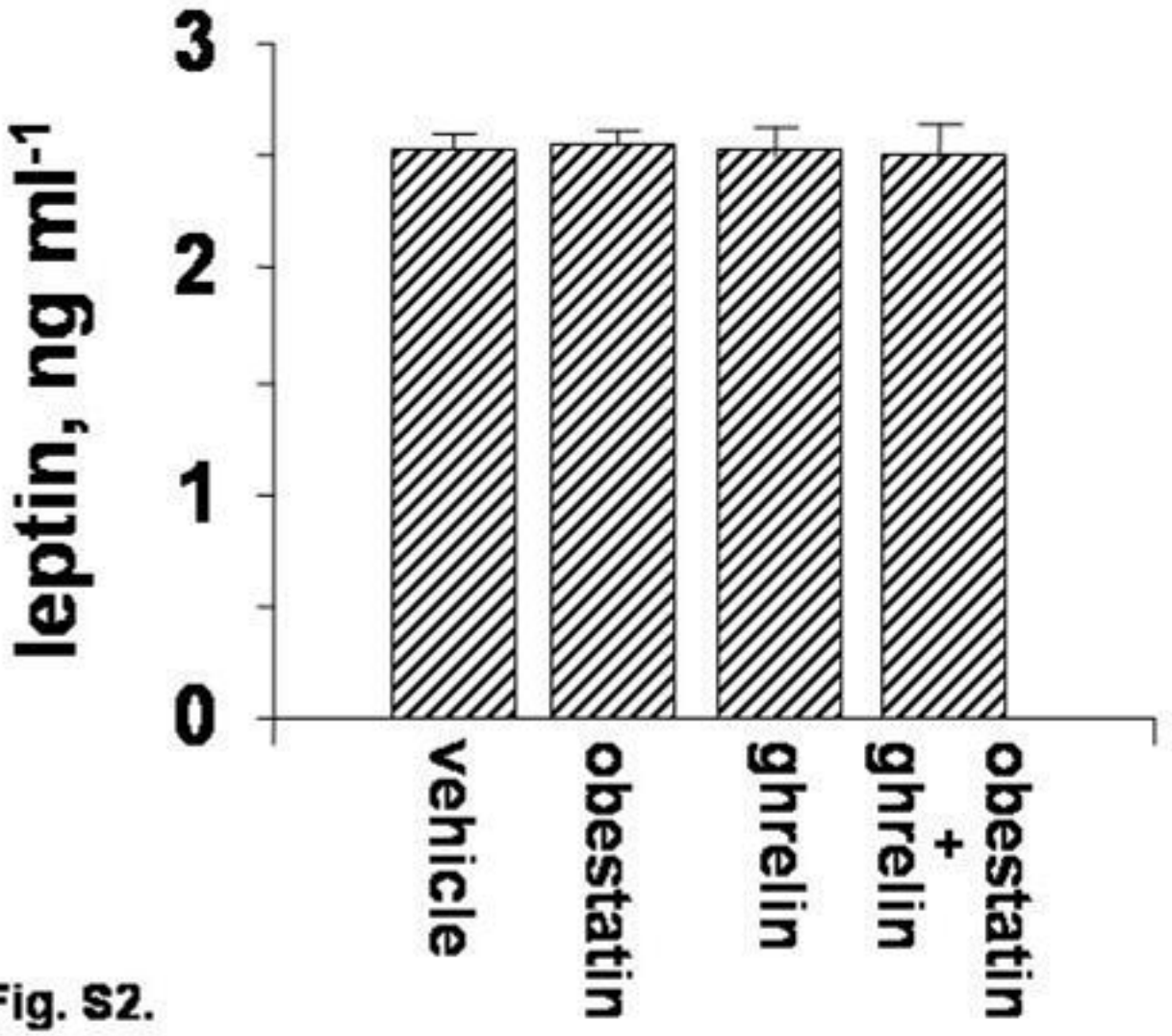
Fig.S1.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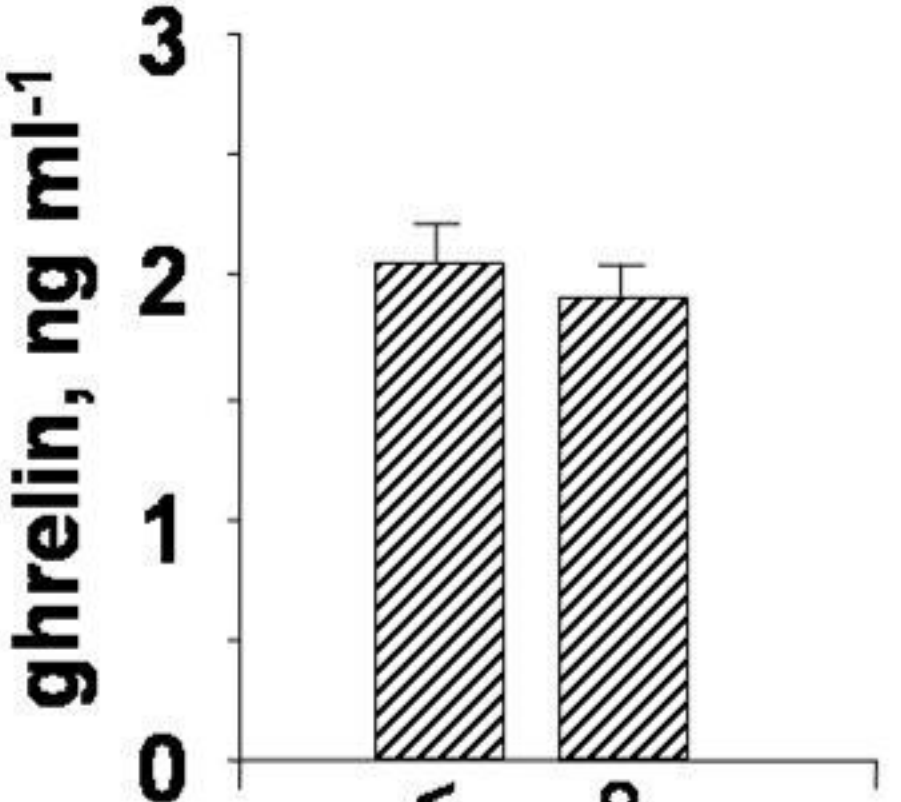
vehicle
obestatin
ghrelin
obestatin + ghrelin

Fig. S2.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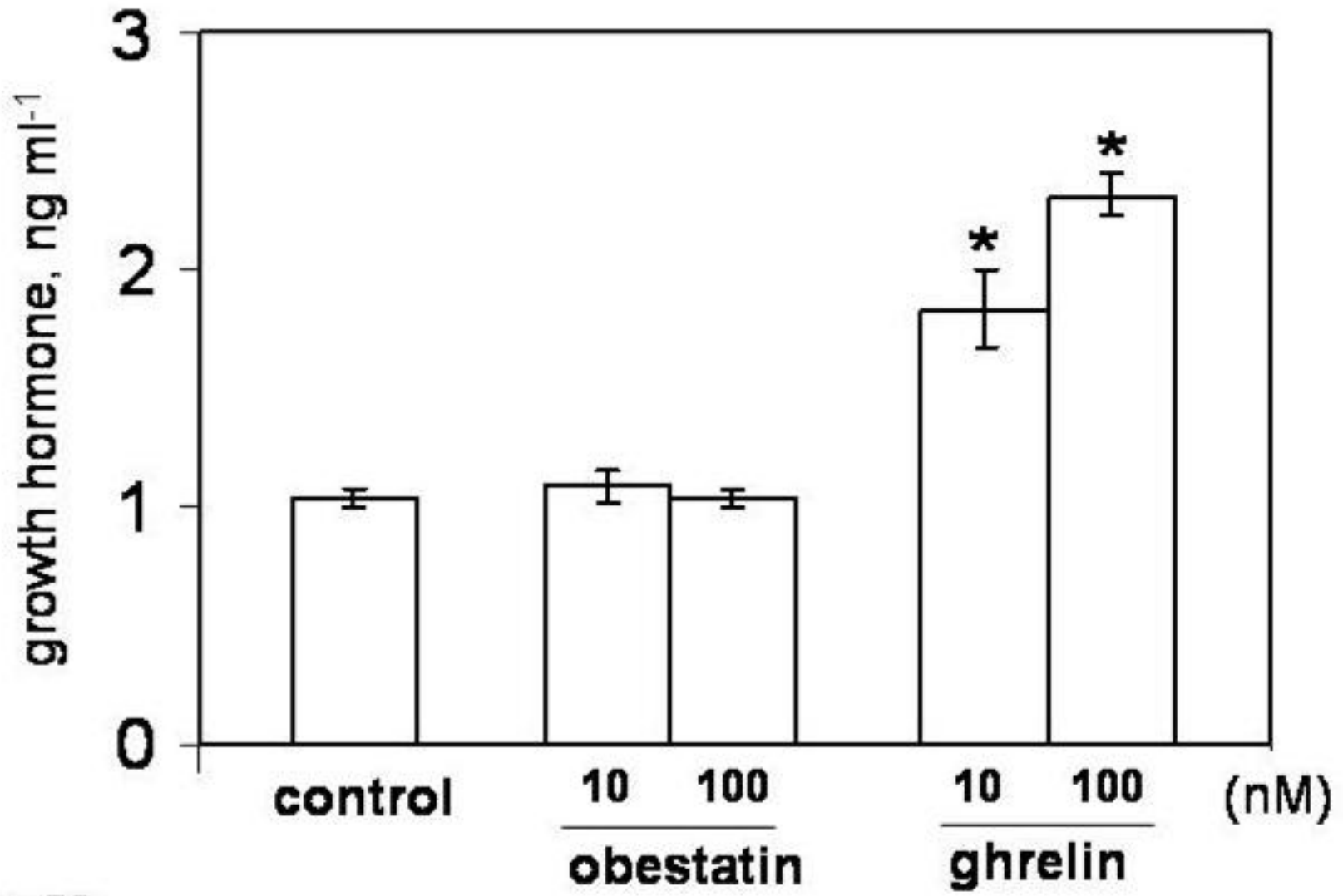
vehicle
obestatin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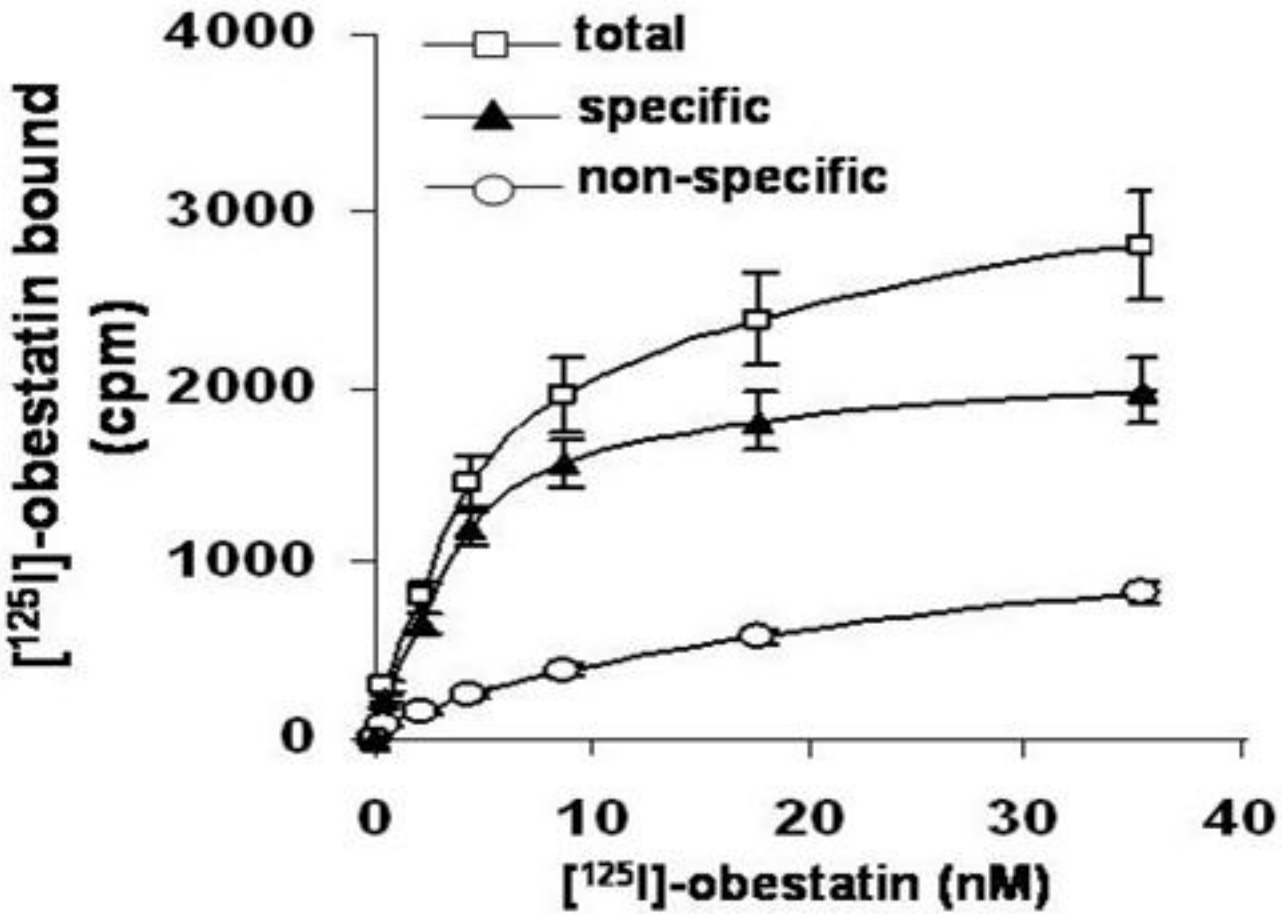
2
1
0
*
*
control
10
100
10
100
(nM)
obestatin
ghrelin

Fig. S3.

A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3000
2000
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total
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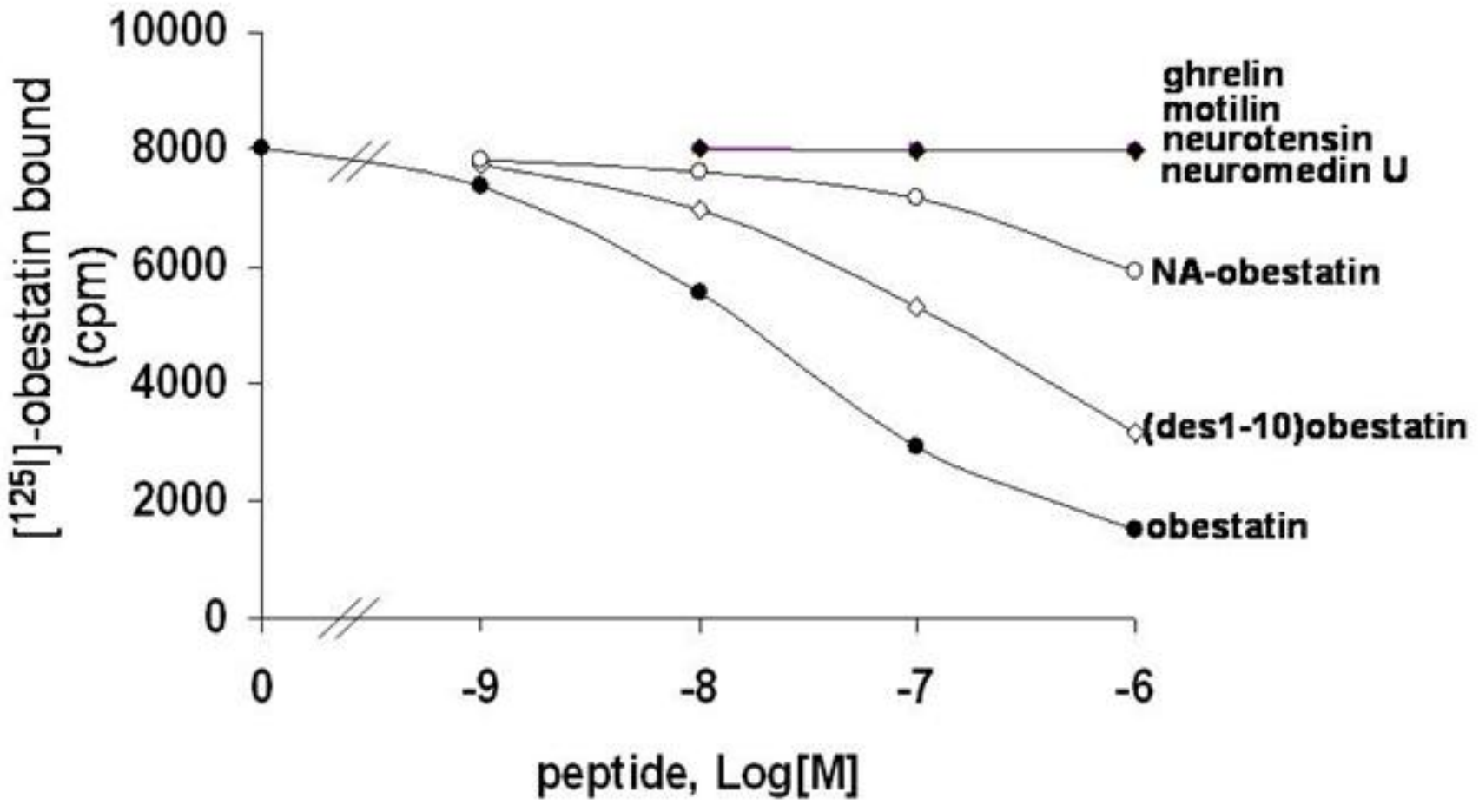
0
10
20
30
40
[125I]-obestatin (nM)

B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8000
6000
4000
2000
0
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motilin
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obestatin
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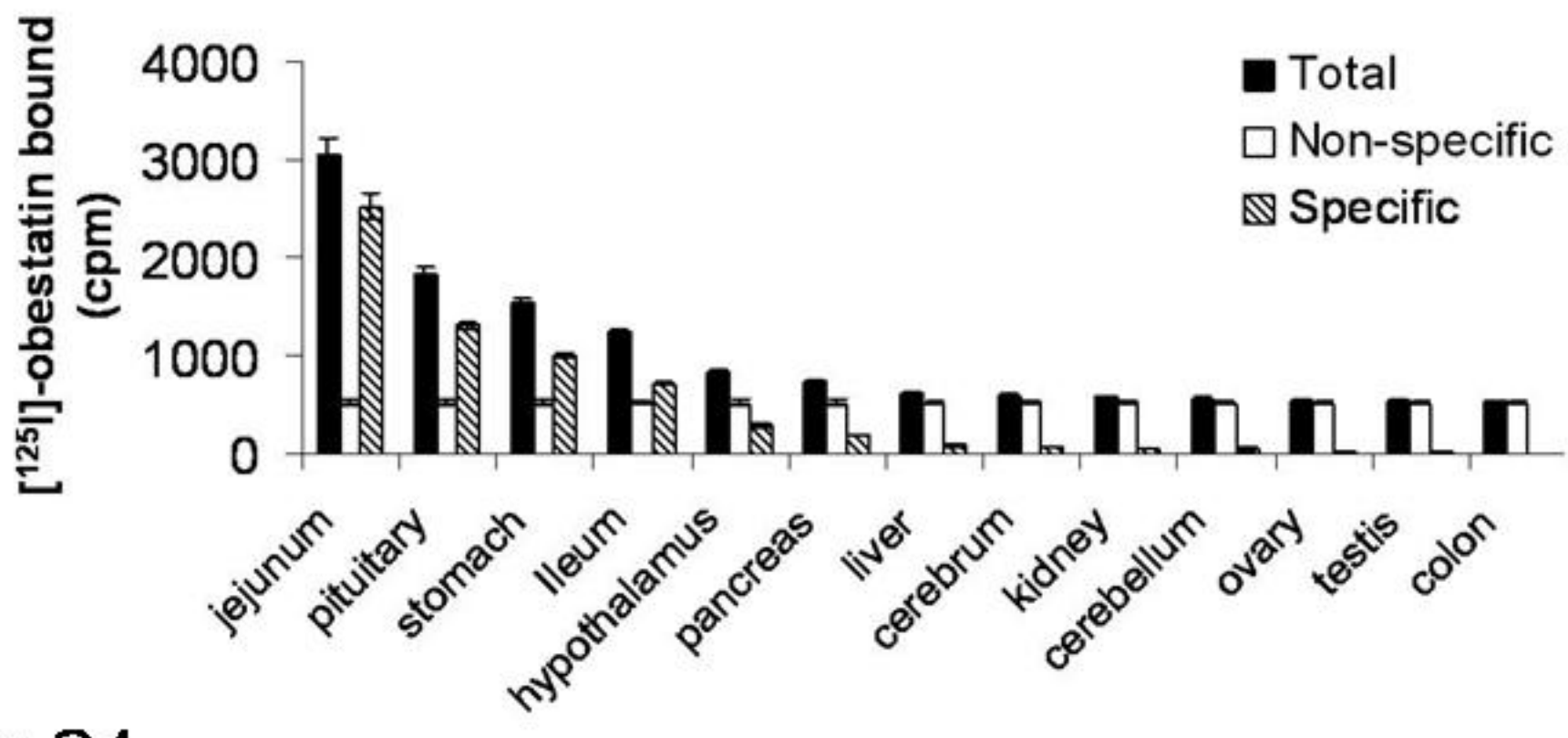
-9
-8
-7
-6
peptide, Log[M]

C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2000
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Specific
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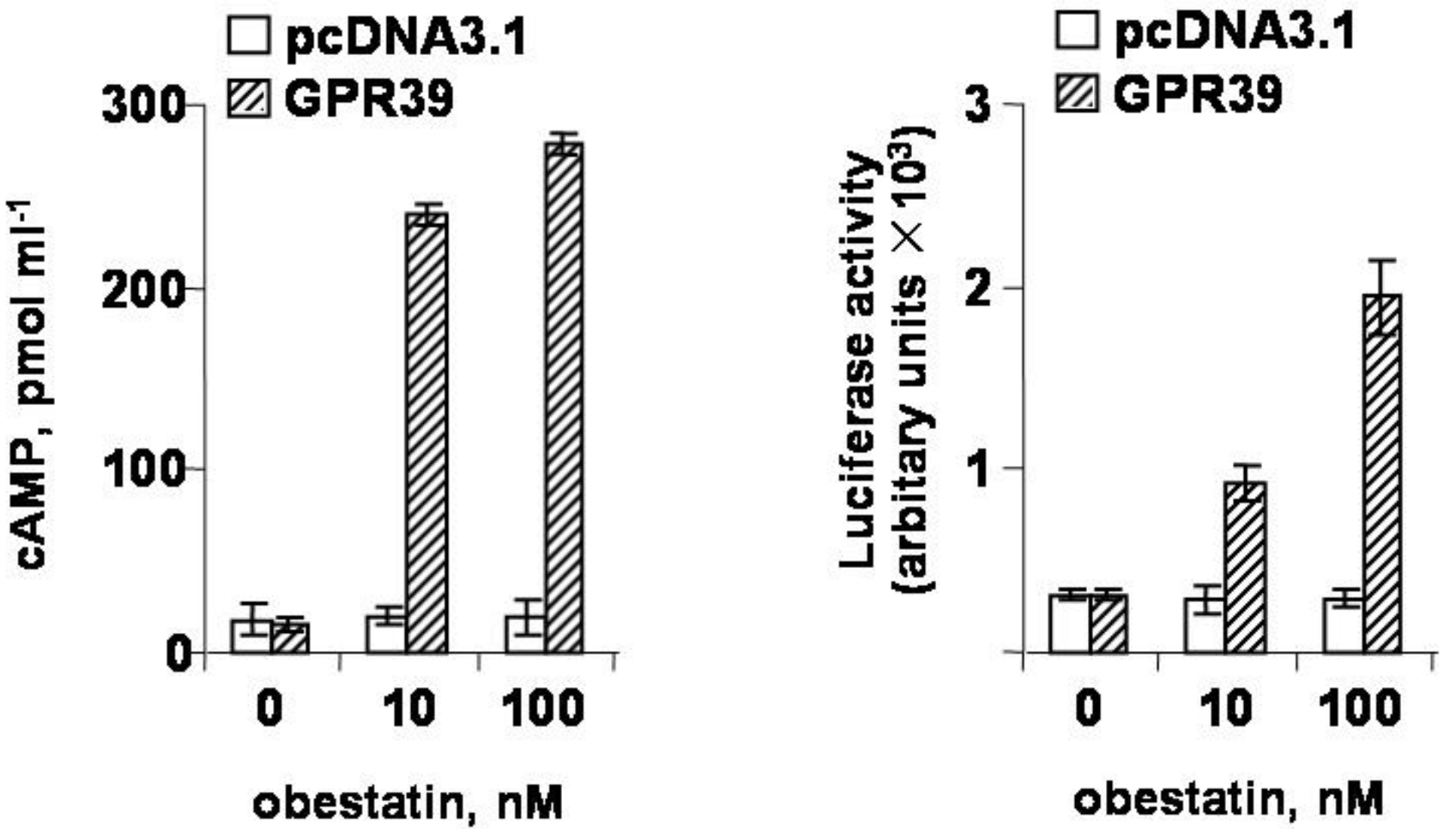
hypothalamus
pancreas
liver
cerebrum
kidney
cerebellum
ovary
testis
colon

Fig.S4.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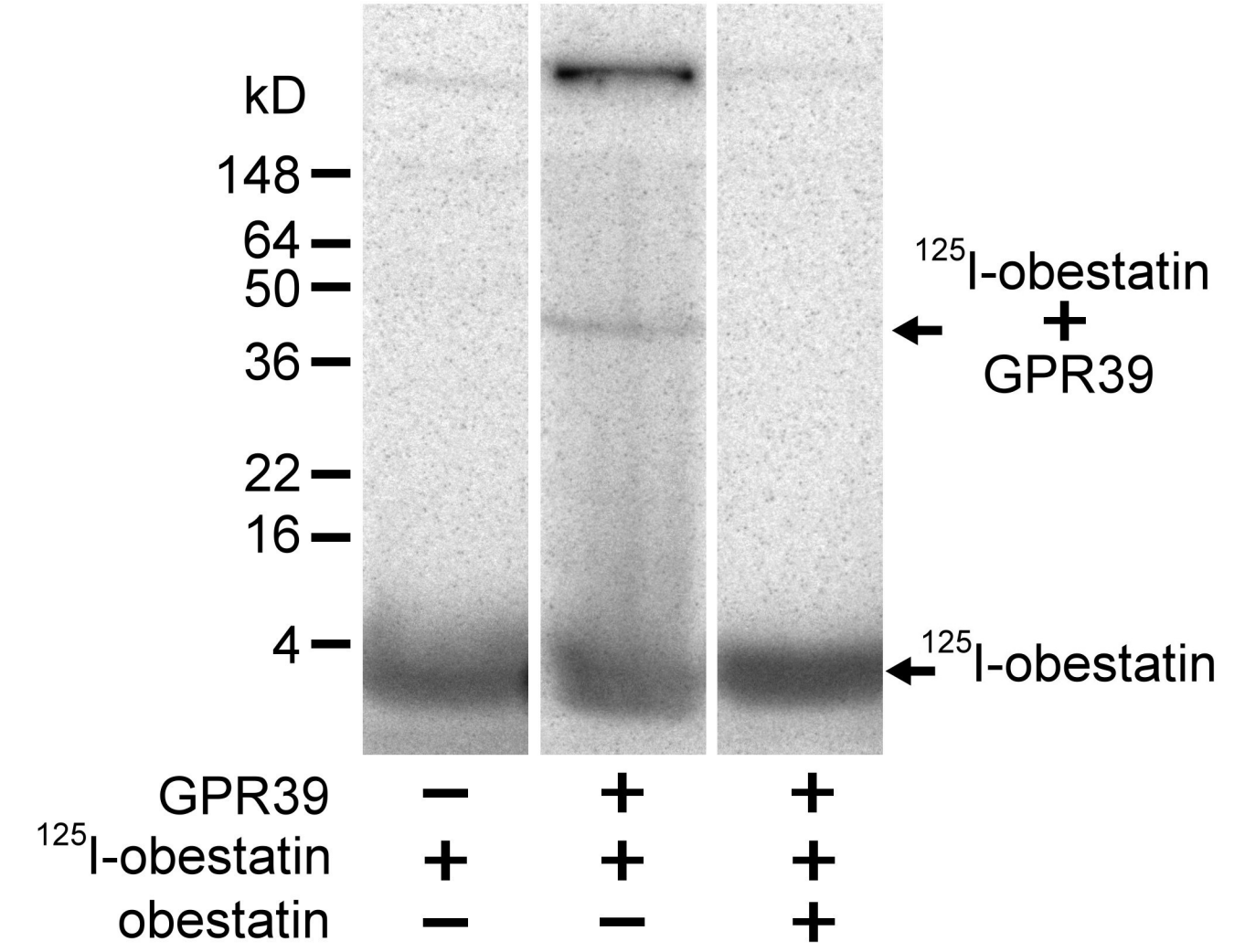
10
100
obestatin, nM

Fig. S5


kD
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36
22
16
4
125I-obestatin
+
GPR39
125I-obestatin
GPR39 − + +
125I-obestatin + + +
obestatin − − +

Fig. S6.