

Comprehensive Profiling of GPCR Expression in Ghrelin-producing Cells.

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Abstract

To determine the comprehensive GPCR expression profile in ghrelin-producing cells, and to elucidate the role of GPCR-mediated signaling in the regulation of ghrelin secretion, we determined GPCR expression profiles by RNA sequencing in the ghrelin-producing cell line MGN3-1, and analyzed the effects of ligands for highly expressed receptors on intracellular signaling and ghrelin secretion. Expression of selected GPCRs was confirmed in FACS-sorted fluorescently tagged ghrelin-producing cells from ghrelin-promoter CreERT2/Rosa-CAG-LSL-ZsGreen1 mice.

Expression levels of GPCRs previously suggested to regulate ghrelin secretion including adrenergic $\beta 1$ receptor, GPR81, oxytocin receptor, GPR120, and somatostatin receptor 2 were high in MGN3-1 cells. Consistent with previous reports, isoproterenol and oxytocin stimulated the G_s and G_q pathways, respectively, whereas lactate, palmitate, and somatostatin stimulated the G_i pathway, confirming the reliability of current assays. Among other highly expressed GPCRs, prostaglandin E receptor 4 agonist prostaglandin E2 (PGE2) significantly stimulated the G_s pathway and ghrelin secretion. Muscarine, the canonical agonist of cholinergic receptor muscarinic 4, stimulated both the G_q and G_i pathways. Although muscarine treatment alone did not affect ghrelin secretion, it did suppress forskolin-induced ghrelin secretion, suggesting that the cholinergic pathway may play a role in counterbalancing the stimulation of

1 ghrelin by G_s (e.g., by adrenaline). In addition, GPR142 ligand tryptophan stimulated ghrelin
2 secretion.

3 In conclusion, we determined the comprehensive expression profile of GPCRs in ghrelin-
4 producing cells and identified two novel ghrelin regulators, PGE2 and tryptophan. These results
5 will lead to a greater understanding of the physiology of ghrelin and facilitate the development of
6 ghrelin-modulating drugs.

7

Introduction

Gut peptide hormones play important roles in the regulation of nutrient digestion and absorption, as well as in the control of food intake, glucose, and energy metabolism. Ghrelin, a 28-amino acid peptide bearing a unique n-octanoylation modification, is secreted mainly from the stomach (1). Ghrelin mediates a wide variety of activities, including growth hormone (GH) stimulation (2), appetite stimulation(3), fat storage (4), the cardiovascular system (5), gastrointestinal motility (6), and insulin suppression (7). Plasma ghrelin levels are elevated by fasting and suppressed by feeding (8), low in obese subjects (9,10), and high in lean subjects (11), suggesting that plasma ghrelin levels are regulated by both acute and chronic energy status. However, the precise mechanisms by which ghrelin secretion is controlled have not been completely elucidated.

Recently, we established a ghrelin-producing cell line, MGN3-1 (12), from a gastric ghrelinoma derived from a ghrelin promoter-SV40 T-antigen transgenic mouse (13), and found that oxytocin, dopamine, and adrenaline stimulate *in vitro* ghrelin secretion by this cell line, whereas somatostatin and insulin suppress secretion (14).

G-protein-coupled receptors (GPCRs) have been proposed to play important roles in the regulation of ghrelin secretion (12,14-16). Taking advantage of the monoclonal nature of our ghrelin-producing cell line, we determined the comprehensive expression profile of the GPCRs

1 in MGN3-1 cells by RNA-seq, which enabled us to determine the relative expression levels of
2 these genes. In order to understand the GPCR-mediated regulation of ghrelin secretion and to
3 identify novel ghrelin regulators, we then examined the effects of ligands of the highly expressed
4 GPCRs. We also confirmed the expression of these receptors in FACS-sorted fluorescently
5 labeled ghrelin-producing cells obtained from ghrelin-promoter (GP)-CreERT2/ROSA-CAG-
6 LSL-ZsGreen mice.

Materials and methods

Animals

We designed a fusion gene consisting of 4 kb of the human ghrelin promoter (17) and CreERT2 (GP-CreERT2). Transgenic mice were developed as previously described (18). Rosa-CAG-LSL-ZsGreen1 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Heterozygous mice were used for experiments. Animals were maintained on a 12-h light/12-h dark cycle and fed with a standard diet (CE-2, 352 kcal / 100 g, CLEA Japan, Tokyo, Japan). All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Cell culture

MGN3-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 10% CO₂, as previously described (12).

RNA sequencing

Total RNA was extracted from cells using the RNeasy kit (QIAGEN, Hilden, Germany). RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library construction was performed using the TruSeq Stranded mRNA LT Sample Prep kit (Illumina, San Diego, CA). Cluster generation and sequencing were performed on a NextSeq 500

(Illumina) for MGN3-1 cells, and on a HiSeq 2000 (Illumina) for FACS-sorted ghrelin-producing cells. FASTQ files were generated using the onboard instrument software. Lead mapping and determination of the relative abundance of the genes were performed using Tophat and Cufflinks, respectively.

Batch incubation study

A batch incubation study was carried out as previously described (14). MGN3-1 cells were seeded at 7.5×10^5 cells per well and cultured for 24 h in 12-well plates. After a washing with PBS, cells were incubated at 37°C for 4 h in DMEM supplemented with 0.5% BSA and the indicated reagents, before collecting supernatants. L-tryptophan, D-tryptophan, lactate, palmitate, sodium acetate, forskolin, muscarine, sphingosine 1-phosphate, isoproterenol, serotonin, N-acetylserotonin, and melatonin were purchased from Sigma-Aldrich Japan (Tokyo, Japan); oxytocin and somatostatin from Peptide Institute (Osaka, Japan); prostaglandin D2 (PGD2) from Santa Cruz Biotechnology (Dallas, TX); estradiol, adenosine, sodium propionate, and prostaglandin E2 (PGE2) from Nacalai Tesque (Kyoto, Japan); ONO-AE3-208 from Cayman Chemical (Ann Arbor, MI); tropicamide from Tocris Bioscience (Bristol, UK); prosaptide TX14 (A) from AnaSpec (Fremont, CA); and R-spondin-1 from Peprotech (Rocky Hill, NJ).

Measurement of ghrelin concentrations in culture media

Measurement of ghrelin concentrations in culture media was performed as previously

described (12). The collected culture media were centrifuged, and the resulting supernatants were immediately applied to Sep-Pak C18 cartridges (Waters Corp., Milford, MA) pre-equilibrated with 0.9% saline. After washing cartridges with saline and 5% CH₃CN/0.1% trifluoroacetic acid (TFA), bound protein was eluted with 60% CH₃CN/0.1% TFA. Eluates were lyophilized and subjected to ghrelin RIA. Ghrelin radioimmunoassays (RIAs) were performed using an antiserum against the ghrelin C-terminus (amino acids 13–28) that detects both ghrelin and desacyl-ghrelin, as described (19,20).

Quantitative RT-PCR

Real-time quantitative PCR was performed using an StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using the appropriate primers and taqman probes (Supplemental Table 1) or Power SybrGreen. The mRNA expression of each gene was normalized to the levels of 18S rRNA.

Small interfering RNA (siRNA)

Synthetic siRNAs and a negative control were purchased from Life Technologies (Carlsbad, CA). Two types of siRNAs specific for GPR6 and GPR142 were used: GPR6,

GACACCUGUCGUGAAAUCAUCUGGA (si1) and

AAGUAAGAUCUGGAUUGCUAGUGAU (si2); and GPR142,

CUGUCAUUGGCGUUACCCUCCUGAC (si1) and

AUGCUGAUCUUCUAUGGUCCUUGUC (si2). siRNAs were delivered into MGN3-1 cells using Lipofectamine RNAi Max (Life Technologies).

Measurements of cAMP in culture medium

Intracellular cAMP concentrations were measured using the AlphaScreen cAMP assay kit (Perkin Elmer, Waltham, MA) with Envision (Perkin Elmer) on an RPN225 cAMP EIA Biotrak system (GE Healthcare Amersham Biosciences, Chalfont St Giles, UK).

Intracellular calcium measurement

MGN3-1 cells were seeded at 5×10^4 cells per well and cultured for overnight in 96-well plates. Intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) were measured using the Calcium Kit II Fluo4 (Dojindo, Kumamoto, Japan) and FDSS/ μ cell (Hamamatsu Photonics, Hamamatsu, Japan). Tropicamide and L-371,257 were purchased from Tocris Bioscience.

Immunohistochemistry

GP-CreERT2/Rosa-CAG-LSL-ZsGreen1 mice were injected with tamoxifen (0.15 mg/g BW) dissolved in corn oil three times at 2–3 day intervals, and their stomachs were resected. Formalin-fixed paraffin-embedded tissue sections were immunostained as described previously (21). Sections were incubated with anti-C-terminal ghrelin [13-28] antibody (1:2000 final dilution) (19).

Cell sorting

GP-CreERT2/Rosa-CAG-LSL-ZsGreen1 mice were intraperitoneally injected with

1 tamoxifen (0.15 mg/g BW) dissolved in corn oil three times at 2–3 day intervals. Stomachs were
2 resected and incubated in DMEM with 1.5 mg/ml type I collagenase and 0.5 mg/ml dispase
3 (Worthington, Lakewood, NJ) at 37°C for 90 min with shaking. Cells were exfoliated from the
4 epithelium of the stomach and suspended in PBS with 1 mM EGTA (Nacalai Tesque), and then
5 filtered through a 40 µm cell-strainer (BD Falcon, Bedford, MA). ZsGreen-expressing cells were
6 isolated on a MoFlo XDP cell sorter (Beckman Coulter, Pasadena, CA). RNA was extracted from
7 FACS-sorted cells using the Absolute RNA NanoPrep kit (Agilent, Santa Clara, CA). Reverse
8 transcription with amplification was performed using the SMARTer Pico PCR cDNA synthesis
9 kit (Takara Bio, Kyoto, Japan).

10 **Primary culture of gastric mucosal cells**

11 Gastric mucosal cells were obtained as described above. The cells were cultured
12 overnight in DMEM supplemented with 10% FBS before the batch incubation study. After a
13 washing with PBS, the cells were incubated at 37°C for 4 h in DMEM supplemented with 0.5%
14 BSA and the indicated reagents, before collecting supernatants.

15 **Statistical analysis**

16 All values are expressed as the means ± SE. Statistical significance of the differences in
17 mean values was assessed by ANOVA with a post-hoc test (Tukey's test) or Student's *t*-test as
18 appropriate. $p < 0.05$ were considered significant. Statistical analyses were performed using
19 Statcel 2 (OMS, Saitama, Japan).

Results

GPCR expression profile in MGN3-1 cells

To obtain complete mRNA expression profile of GPCRs in MGN3-1 cells, we conducted RNA sequencing (RNA-seq) of MGN3-1 cells (Supplemental Table 2). The results are expressed as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values, which reflect the abundance of each transcript relative to all transcripts. Previous studies suggested that adrenaline and oxytocin stimulate ghrelin secretion from MGN3-1 cells via the adrenaline β 1 and oxytocin receptors, respectively (14), whereas somatostatin and long-chain fatty acids suppress secretion through the somatostatin type 2 receptor (SSTR2) and free fatty acid receptor (FFAR) 4 (GPR120), respectively (22). The FPKM values of each of these receptors were as follows: adrenergic β 1 receptor (Adrb1), 55.5; oxytocin receptor (Oxtr), 22.7; Sstr2, 10.9; and Ffar4, 19.4. In addition, in our previous studies, histamine (histamine receptor H3: 5.06), neurotensin (neurotensin receptor 1: 1.43), and serotonin (5-hydroxytryptamine receptor 6: 1.10) had no effects on ghrelin secretion (14). Moreover, prosaptide (GPR37: 7.32) and adenosine (adenosine A2a receptor: 4.69) showed no effects on ghrelin secretion (Supplemental Figure 1G). These findings suggest that at least those receptors with FPKM values higher than 10 might influence ghrelin secretion from MGN3-1 cells. The receptors with FPKM values ≥ 10 were Gpr6, Adrb1, Gpr142, Gpr19, Gpr81, prostaglandin E receptor 4 (Ptger4), Oxtr, leucine-rich repeat

1 containing G protein-coupled receptor 4 (Lgr4), Ffar4, G protein-coupled estrogen receptor
2 (Gper) 1, cholinergic receptor, muscarinic 4 (Chrm4), Ffar2 (GPR43), and Sstr2 (family A);
3 Gpr179 (family C), latrophilin 2 (Lphn2), cadherin, EGF LAG seven-pass G-type receptor 3
4 (Celsr3), and brain-specific angiogenesis inhibitor 2 (Bai2) (adhesion receptor family); and
5 frizzled homolog 6 (Fzd6) (frizzled receptor family) (Figure 1, Supplemental Table 2). It can be
6 hypothesized that highly expressed receptors have important roles in ghrelin regulation. Therefore,
7 we investigated whether these receptors with available ligands evoke intracellular signaling
8 pathways or affect ghrelin secretion in MGN3-1 cells.

9 **G_s pathway**

10 We first tested whether known G-protein-coupled intracellular signaling pathways were
11 evoked by the stimulation of each receptor. A previous report showed that cAMP elevation by
12 forskolin or adrenaline stimulates ghrelin secretion from the ghrelin-producing cell line PG-1 cells
13 (23); we confirmed these results in our MGN3-1 cells (Figure 2A). We then evaluated the effects
14 of selected ligands on the intracellular cAMP levels in MGN3-1 cells (Figure 2B). The beta-
15 agonist isoproterenol increased intracellular cAMP levels, confirming previous results
16 demonstrating G_s activation via the β 1 receptor (14,23) (Figure 2B). The Ptger4 agonist PGE2
17 significantly stimulated cAMP elevation in MGN3-1 cells (Figure 2B, C) and also stimulated
18 ghrelin secretion (Figure 2D). The latter effect was significantly attenuated by the Ptger4

antagonist ONO-AE3-208 (Figure 2E), suggesting that PGE2 stimulates ghrelin secretion via the Ptger4/Gs pathway. **PGE2 did not affect ghrelin or GOAT mRNA levels (Figure 2F, G).** Although a relatively high concentration of PGD2 also stimulated cAMP production (Supplemental Figure 1A) and ghrelin secretion (Supplemental Figure 1B), the effects seemed to be mediated by Ptger4, as they were completely blocked by the Ptger4 antagonist ONO-AE3-208 (Supplemental Figure 1C). PGI2 did not affect cAMP levels in MGN3-1 cells (Supplemental Figure 1D).

GPR6, which is coupled with G_s (24), was the most highly expressed GPCR in MGN3-1 cells (Figure 1, Supplemental Table 2). However, we did not detect any changes in either cAMP levels or ghrelin secretion upon addition of this receptor's known ligand, sphingosine-1-phosphate (24) (Figure 2B, **H**). GPR6 has also been proposed to constitutively stimulate adenylyl cyclase (24). Again, however, we did not detect any changes in intracellular cAMP levels when we knocked down GPR6 with siRNAs (Figure 2**I, J**). Likewise, although the expression levels of Lgr4 and Gper were high in MGN3-1 cells (Figure 1), their ligands R-spondin1 and estradiol stimulated neither cAMP production (Figure 2B) nor ghrelin secretion (Figure 2**H**).

Although CGRP, GIP, secretin, and α MSH have been reported to stimulate ghrelin secretion via the G_s pathway (15), we did not observe any changes in cAMP levels in MGN3-1 cells exposed to these ligands (Supplemental Figure 1E).

G_i pathway

We next examined whether the selected ligands would suppress intracellular cAMP levels in the presence of forskolin (Figure 3A). Somatostatin significantly suppressed cAMP levels (Figure 3A, B), confirming G_i activation via SSTR2 (12). Lactate is a ligand for GPR81 that was recently shown to suppress ghrelin secretion from primary gastric mucosa cells, an effect that is blocked by pertussis toxin (15). Lactate suppressed cAMP levels in the MGN3-1 cells in a dose-dependent manner (Figure 3C), confirming activation of the GPR81/ G_i pathway by lactate. GPR120 (FFAR4), a fatty acid receptor that recognizes long-chain fatty acids, is coupled with G_q/G_i . Palmitate significantly suppressed cAMP levels in MGN3-1 cells (Figure 3D), confirming previous reports that long-chain fatty acids suppress ghrelin secretion via GPR120 (15,22,25,26).

Muscarine significantly suppressed intracellular cAMP levels in MGN3-1 cells (Figure 3A, E). We previously reported that 10^{-4} M muscarine did not alter ghrelin secretion from the MGN3-1 cells (14). In combination with forskolin, however, muscarine significantly attenuated forskolin-induced ghrelin secretion (Figure 3F).

G_q pathway

We next investigated whether selected ligands would affect $[Ca^{2+}]_i$ levels in MGN3-1 cells. We previously reported that oxytocin stimulates ghrelin secretion from MGN3-1 cells via oxytocin receptor (14). In this study, we found that oxytocin stimulated $[Ca^{2+}]_i$ elevation in MGN3-1 cells (Figure 4F), suggesting that the stimulatory effects of oxytocin are mediated by

1 G_q , $[Ca^{2+}]_i$ elevation by oxytocin was significantly attenuated by the addition of the Oxt
2 antagonist L371,257 (Figure 4M).

3 We also found that muscarine significantly stimulated $[Ca^{2+}]_i$ elevation in MGN3-1 cells
4 (Figure 4J). The effects were significantly attenuated when the cells were treated with muscarine
5 in combination with the Chrm4 antagonist tropicamide (Figure 4N), confirming that the
6 stimulatory effects of muscarine on $[Ca^{2+}]_i$ were mediated by Chrm4. Sphingosine-1-phosphate,
7 isoproterenol, lactate, PGE2, R-spondin, palmitate, estradiol, acetate, and somatostatin did not
8 affect $[Ca^{2+}]_i$ (Figure 4). In addition, the ligands for the receptors with FPKM values lower than
9 10 including histamine for histamine receptor H3 (FPKM 5.06), adenosine for adenosine A2a
10 receptor (4.69), kisspeptin for KISS1 receptor (1.79), neurotensin for neurotensin receptor 1
11 (1.43) and serotonin for 5-hydroxytryptamine receptor 6 (1.10) did not show any effects
12 (Supplemental Figure 2), supporting the FPKM threshold value.

13 A recent report showed that GPR142 is coupled with G_q , and that tryptophan is its most
14 potent ligand (27,28). Although Gpr142 had the third highest FPKM value in MGN3-1 cells, we
15 did not observe any changes in $[Ca^{2+}]_i$ (Figure 4C) or cAMP levels (Figure 2B and 5A) in cells
16 treated with tryptophan. However, tryptophan stimulated ghrelin secretion in a dose-dependent
17 manner (Figure 5B). These stimulatory effects were not observed when we used the enantiomer
18 D-tryptophan (Figure 5C). Tryptophan can be metabolized to serotonin, but neither serotonin nor

1 other inter-metabolites (n-acetylserotonin and melatonin) exerted any effects on ghrelin secretion
2 (Figure 5D), suggesting that tryptophan itself is the relevant ligand. Knockdown of GPR142 using
3 siRNA significantly attenuated the stimulatory effects of tryptophan on ghrelin secretion (Figure
4 5E, F), suggesting that this receptor mediates the effects of tryptophan on ghrelin secretion.

5 Regarding to the ghrelin production, we found no effects of tryptophan on ghrelin or GOAT
6 mRNA levels (Figure 5H, I).

7 **GPCR expression profile in FACS-sorted fluorescently tagged ghrelin-producing cells**

8 Next, we examined the expression of the aforementioned receptors in ghrelin-producing
9 cells *in vivo*. For this purpose, we developed ghrelin-promoter CreERT2 transgenic mice; by crossing
10 them with Cre-reporter, Rosa-CAG-LSL-ZsGreen1 mice, we successfully labeled ghrelin-
11 producing cells with ZsGreen in a tamoxifen-dependent manner (Figure 6A–C). Almost all the
12 ZsGreen-positive cells (96.6%) contained ghrelin-like immunoreactivity; reciprocally, 61.5% of
13 ghrelin-immunopositive cells contained ZsGreen fluorescence. We purified the ZsGreen-tagged
14 cells by FACS sorting (Figure 6D, E) and examined the mRNA expression of the aforementioned
15 receptors by RNA-seq after amplification. The FPKM values for ghrelin and GOAT were elevated
16 in ZsGreen(+) cells [ZsGreen(+) vs. (-): ghrelin, 1356.8 vs 131.0; GOAT, 0.46 vs. 0.15],
17 confirming the enrichment of ghrelin-producing cells. The FPKM values for GPR81 were higher
18 in ZsGreen(+) cells than in ZsGreen(-) cells [ZsGreen(+) vs. (-): 0.47 vs. 0.09], Ptger4 (0.65 vs.

0.13), Oxtr (0.01 vs. ND [not detected]), Ffar4 (0.34 vs. ND);, and Sstr2 (0.73 vs. ND) (Supplemental Table 3). The expression level of Adrb1 was rather low in ZsGreen(+) cells (0.09 vs. 0.63). Gpr142 and Chrm4 were not detected by RNA-seq, although we could detect their expression by qPCR (data not shown).

The effects of tryptophan and PGE2 on primary cultured gastric mucosal cells

Finally, we examined the effects of two novel ghrelin regulators, tryptophan and PGE2 on ghrelin secretion from primary cultured gastric mucosal cells. Although we confirmed the co-localization of Ptger4 and ghrelin in mouse stomach mucosa (Figure 7A), we could not find any effects of PGE2 on ghrelin secretion from primary cultured gastric mucosal cells (Figure 7B). On the other hand, tryptophan dose-dependently stimulated ghrelin secretion from primary cultured gastric mucosal cells (Figure 7B, C).

Discussion

In this study, we performed RNA-seq of total mRNA from the ghrelin-producing cell line MGN3-1 and determined the relative expression levels of GPCRs. We selected twelve GPCRs with high FPKM values whose ligands or putative ligands were available. By examining the effects of these ligands on cAMP or $[Ca^{2+}]_i$, we successfully revealed or confirmed that several ligands of highly expressed receptors exerted stimulatory or inhibitory effects on ghrelin secretion from MGN3-1 cells.

Recently, Engelstoft et al. performed qPCR array analysis of 379 GPCRs using FACS-sorted GFP-tagged ghrelin-producing cells (15). They found that 90 GPCRs were expressed and 29 of them were enriched more than 5-fold in ghrelin-producing cells relative to GFP-negative cells. In addition, using primary cultured gastric mucosal cells or gene-manipulated mice, they showed that *Adrb1*, calcitonin-related polypeptide receptor (CGRPR), gastric inhibitory polypeptide receptor (GIPR), secretin receptor (SCTR), melanocortin 4 receptor (MC4R), calcium-sensing receptor (CaSR), FFAR2, FFAR4, GPR81, and SSTRs were involved in the regulation of ghrelin secretion. Some of our findings (e.g., those concerning ADRB1, FFAR4, SSTR, and GPR81) were consistent with their observations, whereas others (concerning CGRPR, FFAR2, GIPR, SCTR, MC4R, and CaSR) were not, probably due to differences in the

experimental methods: for example, they used primary gastric mucosal cells, whereas we used immortalized ghrelin-producing cells.

The stimulatory effects of adrenaline on ghrelin secretion via *Adrb1* are well established (14,15,23,29-32). Indeed, *Adrb1* had the second highest FPKM value in our analysis, and activation of the receptor by isoproterenol increased intracellular cAMP levels in MGN3-1 cells.

These observations suggest that the sympathetic nervous system is one of the major regulators of ghrelin secretion, and may be beneficial in the context of starvation-induced hypoglycemia (33).

On the other hand, studies of cholinergic regulation of ghrelin secretion have yielded inconsistent results. Some studies reported stimulatory effects of acetylcholine on ghrelin secretion (34,35), whereas others reported no such effects (14,15,23,32). In our previous study, we observed no

effects of acetylcholine treatment alone on ghrelin secretion from MGN3-1 cells (14). In this study, however, we found that muscarinic acetylcholine receptor 4 (*Chrm4*) is expressed at high levels in MGN3-1 cells. Although we could not detect *Chrm4* expression in FACS-sorted ZsGreen(+) cells by RNA-seq, another group reported high expression of *Chrm4* in FACS-sorted GFP-tagged

ghrelin-producing cells (15). Addition of muscarine stimulated both the G_i and G_q pathways in MGN3-1 cells, indicating that both stimulatory and inhibitory pathways were simultaneously

activated by muscarine. This may explain why we observed no effect of muscarine treatment alone. However, we found that muscarine suppressed forskolin-induced ghrelin secretion,

1 suggesting that the cholinergic pathway may play a role in counterbalancing the G_s -mediated
2 stimulation of ghrelin (e.g., by adrenaline).

3 Because ghrelin-producing cells are located mainly in the stomach, various nutrients
4 have been proposed to be involved in ghrelin regulation. The suppression of ghrelin secretion by
5 long-chain fatty acids via GPR120 (FFAR4) is well established (15,22,25,26). Our current results
6 also confirm the high expression levels of Ffar4 and the inhibition of ghrelin secretion by long-
7 chain fatty acids mediated by the GPR120/ G_i pathway. Short-chain fatty acids also suppress
8 ghrelin secretion via the GPR43 (FFAR2)/ G_i pathway (15). Although we observed high
9 expression of Ffar2 in MGN3-1 cells and FACS-sorted ghrelin-producing cells, we did not detect
10 any effects of acetate or propionate on cAMP levels or **ghrelin secretion** (Figure 3A, Supplemental
11 Figure 1F). The expression levels of other fatty acid receptors, GPR40 (Ffar1), GPR41 (Ffar3),
12 and GPR119, were low in MGN3-1 cells. Engelstoft et al. found that lactate suppressed ghrelin
13 secretion via GPR81 (15). We confirmed the high expression of GPR81 in MGN3-1 cells and its
14 enrichment in FACS-sorted ghrelin-producing cells, as well as the activation of G_i pathway **and**
15 **suppression of ghrelin secretion** by lactate.

16 GPR142 is an orphan receptor, highly expressed in pancreatic beta cells, that has been
17 postulated to be coupled with G_q (27). The high expression level of GPR142 in ghrelin-producing
18 cells was also described by Engelstoft et al. (15). We confirmed the high expression of GPR142

1 in MGN3-1 cells (but not in ZsGreen(+) cells). Although the natural ligand for GPR142 remains
2 unknown, tryptophan is the most potent known agonist of this receptor (28). We found that
3 tryptophan stimulated ghrelin secretion from both MGN3-1 cells and primary cultured gastric
4 mucosal cells. Surprisingly, although tryptophan stimulated ghrelin secretion from MGN3-1 cells,
5 it evoked neither cAMP elevation nor $[Ca^{2+}]_i$ elevation. The downstream signaling pathways
6 remain to be elucidated. Tryptophan is also the ligand for calcium-sensing receptor (CaSR) (36).
7 Engelstoft et al. reported that CaSR is highly expressed in ghrelin-producing cells, and is involved
8 in the regulation of ghrelin in a manner dependent upon the Ca^{2+} level (15). In the analysis
9 reported here, the expression level of CaSR was low (FPKM 0.27) in MGN3-1 cells, and two
10 allosteric activators of CaSR, cinacalcet and AC-265347, did not evoke an increase in $[Ca^{2+}]_i$ in
11 MGN3-1 cells (Supplemental Figure 2), suggesting that CaSR-mediated signaling is unlikely to
12 be involved, at least in this cellular model.

13 Several previous reports have also documented the regulation of ghrelin secretion by
14 amino acids. Zhang *et al.*, in the only study of the effect of tryptophan on plasma ghrelin levels,
15 reported that the oral infusion of tryptophan elevates plasma ghrelin levels in pigs (37). Our
16 findings are consistent with their results. Other reports regarding the effects of amino acids on
17 plasma ghrelin levels are based on testing amino acids other than tryptophan or amino acid
18 mixtures (38-41). For example, Sugino *et al.* reported that infusion of an amino acid mixture

1 increases plasma ghrelin levels in sheep (41), and Knerr *et al.* found the same effects in healthy
2 humans (39). On the other hand, Al Massadi *et al.* reported that L-glutamine decreases ghrelin
3 secretion in an *in vitro* gastric explant model (38), and Shrestha *et al.* found that infusion of an
4 amino acid mixture into an isolated stomach reduces ghrelin release (40). The discrepancy of the
5 results between these reports and ours may reflect the differences in the amino acids tested or the
6 experimental conditions of the various studies. For example, certain amino acids can stimulate
7 other endocrine hormones, *i.e.*, insulin (42), which may indirectly affect ghrelin secretion (12,43).

8 Among the peptide hormones, somatostatin-mediated suppression of ghrelin has been
9 reported by various groups, including ourselves (12,15,26,32,44-46), and we confirmed the
10 somatostatin-mediated G_i activation in MGN3-1 cells in this study. However, a controversy
11 persists regarding the receptor subtypes responsible for the somatostatin-mediated ghrelin
12 suppression. Several groups reported that Sstr1, 3, and 5 are enriched in FACS-sorted GFP-tagged
13 ghrelin-producing cells relative to non-GFP-tagged cells (15,26,47). Silva *et al.* reported that
14 Sstr1 and 2 are expressed high level in ghrelin-producing cells in rat stomach, and that the Sstr2
15 agonist octreotide more potently inhibited ghrelin secretion than the Sstr1/5 agonist SOM230,
16 suggesting that Sstr2 is responsible for the inhibitory effect of somatostatin (48). Our results are
17 consistent with those of Silva *et al.* Sstr2 is highly expressed in MGN3-1 cells (FPKM 10.88),
18 and it was also enriched in FACS-sorted ghrelin-producing cells. By contrast, the expression

1 levels of Sstr1 (FPKM 0.27), 3 (FPKM 0.03), and 5 (FPKM 0.48) were low in MGN3-1 cells.

2 Sstr1 was not enriched in FACS-sorted ZsGreen (+) cells, and Sstr3 and Sstr5 were not detected.

3 Regarding oxytocin, we observed high expression of oxytocin receptor (FPKM 22.7),

4 but low levels of Avpr1b (FPKM 0.14) and no expression of Avpr1a and Avpr2, consistent with

5 our previous results obtained using an oxytocin receptor-specific antagonist (14). By contrast,

6 Engelstoft et al. reported that Avpr1b is enriched in GFP-tagged ghrelin-producing cells (15). In

7 our hands, oxytocin evoked $[Ca^{2+}]_i$ elevation, confirming the activation of OXTR/Gq pathway.

8 Engelstoft et al. reported that CGRP receptor, which consists of calcitonin receptor-like receptor

9 (CALCR1) and receptor activity-modifying (RAMP) protein 1, is enriched in GFP-tagged

10 ghrelin-producing cells (15). In this study, although the expression level of RAMP1 was high

11 (FPKM 21.0) in MGN3-1 cells, the level of Calcr1 was low (0.04). Other peptide hormone

12 receptors reported to be enriched in ghrelin-producing cells (15,16) were not highly expressed in

13 MGN3-1 cells: Mc4r (FPKM 0.43), Gpr (1.01), glucagon receptor (0.07), glucagon-like peptide

14 (GLP) 1 receptor (ND), GLP2 receptor (0.04), cholecystokinin (CCK) A receptor (not detected),

15 CCKB receptor (0.08), neuropeptide Y receptor Y1 (0.14), Y2 (0.12), Y5 (0.07), Y6 (0.02),

16 gastrin-releasing peptide receptor (0.08), and secretin receptor (ND). Some of them (Mc4r, Glp1r,

17 Cckar, Cckbr, Npy6r, Grpr, and Sctr) were enriched in FACS-sorted ZsGreen (+) cells, but we

could not determine whether these receptors were actually functional due to their low expression levels in our cellular model.

We found that the Ptger4 agonists PGE2 and PGD2 significantly stimulated intracellular cAMP and ghrelin secretion. Very few studies have examined the effects of prostaglandins on ghrelin secretion. Madison et al. reported that ghrelin-producing cells express prostaglandin I2 receptor (Ptgir) but not Ptger 1–4, and that administration of PGI2 to rats significantly suppresses plasma ghrelin levels (49). However, our RNA-seq data showed that Ptgir expression was low in MGN3-1 cells (FPKM 0.18), whereas Ptger4 was highly expressed (FPKM 23.02). Engelstoft et al. also reported that Ptgir in FACS-sorted ghrelin-producing cells was expressed below the noise level (15). Addition of PGI2 affected neither $[Ca^{2+}]_i$ nor cAMP levels in MGN3-1 cells (Supplemental Figure 1D and Supplemental Figure 2H). The physiological significance of PGE2-mediated ghrelin regulation is not clear, but may be related to mucosal protection of the stomach. Prostaglandins including PGE2 are produced in the gastric mucosa (50) and play a crucial role in mucosal protection (51) (e.g., it is well known that cyclooxygenase inhibitors induces mucosal damage, resulting in gastric ulcer). Because ghrelin protects against ethanol-induced gastric damage in an NO- and prostaglandin-dependent manner (52,53), PGE2-induced ghrelin secretion may contribute to mucosal protection. PGE2 also acts on gastric smooth muscle. It stimulates longitudinal muscle contraction and inhibits circular muscle contraction (54). Since ghrelin has

strong gastropromotor action (6), PGE₂-induced ghrelin secretion may have a role in the regulation of stomach movement. Although PGE₂ significantly stimulated ghrelin secretion from MGN3-1 cells, we failed to detect stimulatory effects of PGE₂ on ghrelin secretion from primary cultured gastric mucosal cells. Immunohistochemical analysis showed co-localization of ghrelin and Ptger4, but it suggested that Ptger4 was also expressed in other type of the cells. It is possible that indirect effects of Ptger4 via some humoral factors from other type of the cells might mask the direct effects of PGE₂ on ghrelin-producing cells.

We observed high expression of several orphan GPCRs in MGN3-1 cells, including Gpr142 (FPKM 47.4) and Gpr19 (FPKM46.9), as well as relatively high levels of Gpr85 (FPKM 7.37), Gpr37 (FPKM 7.31), and Gpr62 (FPKM 5.56). Among these, Gpr142 and Gpr37 are highly enriched in GFP-tagged ghrelin-producing cells (15). Although previous studies reported that Gpr27 and Gpr21 are also highly enriched, the levels of these receptors were not high in MGN3-1 cells (FPKM 0.20 and ND, respectively).

In summary, we determined the expression profile of GPCRs in the ghrelin-producing cell line MGN3-1. Combining the expression profile of GPCRs and assays for intracellular signaling pathways, we identified novel ghrelin regulators, including PGE₂ and tryptophan. These results will improve our understanding of the physiology of ghrelin and facilitate the development of ghrelin-modulating drugs.

1

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2

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3

4

Figure Legends

Figure 1. Expression levels of GPCRs with high FPKM values in MGN3-1 cells

Expression levels of GPCRs with FPKM values ≥ 10 in MGN3-1 cells.

Figure 2. G_s -mediated stimulation of ghrelin secretion from MGN3-1 cells

A. Forskolin (10^{-6} M) significantly stimulated ghrelin secretion from MGN3-1 cells. B. Effects of ligands of highly expressed GPCRs on intracellular cAMP levels in MGN3-1 cells. Sphingosine-1P (10^{-4} M), isoproterenol (10^{-4} M), tryptophan (10^{-3} M), lactate (10^{-4} M), PGE2 (10^{-7} M), oxytocin (10^{-6} M), R-spondin (10^{-4} M), palmitate (10^{-4} M), estradiol (10^{-4} M), muscarine (10^{-4} M), acetate (10^{-4} M), and somatostatin (10^{-6} M) were added to the media. C, D. PGE2 dose-dependently stimulated intracellular cAMP levels (C) and ghrelin secretion (D) from MGN3-1 cells; this effect was attenuated by the Ptger4 antagonist ONO-AE3-208 (E). n = 9; **: P < 0.01 in comparison to vehicle, #: P < 0.01 in comparison to PGE2. F, G. The effects of PGE2 (10^{-6} M) on ghrelin and GOAT mRNA levels in MGN3-1 cells. n=6. H. Sphingosine-1P (10^{-4} M), R-spondin (10^{-4} M), and estradiol (10^{-4} M) did not affect ghrelin secretion from MGN3-1 cells. I, J. Knockdown of GPR6 by siRNA1 and 2 (I) did not affect intracellular cAMP levels in MGN3-1 cells (J). n = 9; **: P < 0.01 in comparison to negative control (neg).

Figure 3. G_i -mediated suppression of ghrelin secretion from MGN3-1 cells

A. Effects of ligands of highly expressed GPCRs on intracellular cAMP levels stimulated by forskolin in MGN3-1 cells. Sphingosine-1-P (10^{-4} M), isoproterenol (10^{-4} M), tryptophan (10^{-3} M), lactate (10^{-4} M), PGE2 (10^{-7} M), oxytocin (10^{-6} M), R-spondin (10^{-4} M), palmitate (10^{-4} M), estrogen (10^{-4} M), muscarine (10^{-4} M), acetate (10^{-4} M), and somatostatin (10^{-6} M) were added to the media along with 10^{-6} M forskolin. $n = 8$; **: $P < 0.01$ in comparison to control, #: $P < 0.01$ in comparison to forskolin alone. B–D. Somatostatin (B), lactate (C), and palmitate (D) dose-dependently suppressed forskolin (10^{-6} M)-stimulated elevation of cAMP levels in MGN3-1 cells. E. Palmitate (10^{-4} M) and lactate (10^{-4} M), but not acetate (10^{-4} M) significantly suppressed ghrelin secretion from MGN3-1 cells. F, G. Muscarine dose-dependently suppressed forskolin-induced cAMP elevation (F) and ghrelin secretion from MGN3-1 cells (G). $n = 9$; **: $P < 0.01$ in comparison to control, #: $P < 0.01$ in comparison to forskolin alone.

Figure 4. G_q -mediated stimulation of ghrelin secretion from MGN3-1 cells

A–L: Effects of ligands of highly expressed GPCRs on $[Ca^{2+}]_i$ in MGN3-1 cells. The data are presented as ratios relative to the basal fluorescence intensity. M. OXTR antagonist L-371,253 dose-dependently suppressed oxytocin (10^{-7} M)-induced $[Ca^{2+}]_i$ elevation. The data are presented as differences between maximum and minimum fluorescence intensities. $n = 4$; **: $P < 0.01$ in

1 comparison to oxytocin alone. N. The Chrm4 antagonist tropicamide dose-dependently
2 suppressed muscarine (10^{-4} M)-induced $[Ca^{2+}]_i$ elevation. n = 4; **: P < 0.01 in comparison to
3 muscarine alone.

4
5 **Figure 5. Effect of tryptophan, a GPR142 agonist, on ghrelin secretion from MGN3-1 cells**

6 A. Tryptophan did not affect intracellular cAMP levels in MGN3-1 cells. B, C. Tryptophan, but
7 not D-tryptophan, dose-dependently stimulated ghrelin secretion from MGN3-1 cells. D.
8 Tryptophan metabolites including serotonin (10^{-4} M), n-acetylserotonin (10^{-4} M), and melatonin
9 (10^{-4} M) did not affect ghrelin secretion from MGN3-1 cells. E–G. Knockdown of GPR142 by
10 two different siRNAs (si1, si2) significantly attenuated tryptophan (10^{-2} M)-stimulated ghrelin
11 secretion from MGN3-1 cells. n = 9; **: P < 0.01 in comparison to vehicle; ##: P < 0.01 in
12 comparison to tryptophan; ††: P < 0.01 in comparison to negative control (neg). **H, I. The effects**

13 **of tryptophan (10^{-2} M) on ghrelin and GOAT mRNA levels in MGN3-1 cells. n=6.**

14
15 **Figure 6. FACS sorting of fluorescently tagged ghrelin-producing cells**

16 A–C. Representative images of ZsGreen-positive (A) and ghrelin-immunoreactive cells (B) in
17 stomach of GP-CreERT2/Rosa-CAG-LSL-ZsGreen1 mice after injection of tamoxifen. D, E.
18 Scatter plots of FACS analysis of stomach cells obtained from GP-CreERT2/Rosa-CAG-LSL-
19 ZsGreen 1 mice (D) and controls (E). Cells in the circled area were purified by FACS sorting.

Figure 7. Effects of tryptophan and PGE2 on the ghrelin secretion from primary cultured gastric mucosal cells.

A. Immunohistochemical analysis of Ptger4 and ghrelin in wild-type mouse stomach. B. The effects of PGE2 (10^{-5} M) and tryptophan (10^{-2} M) on ghrelin secretion from primary cultured gastric mucosal cells. C. Tryptophan dose-dependently stimulated ghrelin secretion from primary cultured gastric mucosal cells. n = 6; **: $P < 0.01$ in comparison to vehicle.

References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature*. 1999;402(6762):656-660.
2. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. Ghrelin strongly stimulates growth hormone release in humans. *J Clin Endocrinol Metab*. 2000;85(12):4908-4911.
3. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature*. 2001;409(6817):194-198.
4. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature*. 2000;407(6806):908-913.
5. Nagaya N, Uematsu M, Kojima M, Ikeda Y, Yoshihara F, Shimizu W, Hosoda H, Hirota Y, Ishida H, Mori H, Kangawa K. Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. *Circulation*. 2001;104(12):1430-1435.
6. Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima

M, Kangawa K. Ghrelin stimulates gastric acid secretion and motility in rats.

Biochem Biophys Res Commun. 2000;276(3):905-908.

7. Reimer MK, Pacini G, Ahren B. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology.* 2003;144(3):916-921.

8. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes.* 2001;50(8):1714-1719.

9. Iwakura H, Akamizu T, Ariyasu H, Irako T, Hosoda K, Nakao K, Kangawa K. Effects of ghrelin administration on decreased growth hormone status in obese animals. *Am J Physiol Endocrinol Metab.* 2007;293(3):E819-825.

10. Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes.* 2001;50(4):707-709.

11. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab.* 2001;86(10):4753-4758.

12. Iwakura H, Li Y, Ariyasu H, Hosoda H, Kanamoto N, Bando M, Yamada G, Hosoda K, Nakao K, Kangawa K, Akamizu T. Establishment of a novel ghrelin-producing cell line. *Endocrinology*. 2010;151(6):2940-2945.
13. Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T. A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. *Am J Physiol Endocrinol Metab*. 2009;297(3):E802-811.
14. Iwakura H, Ariyasu H, Hosoda H, Yamada G, Hosoda K, Nakao K, Kangawa K, Akamizu T. Oxytocin and dopamine stimulate ghrelin secretion by the ghrelin-producing cell line, MGN3-1 in vitro. *Endocrinology*. 2011;152(7):2619-2625.
15. Engelstoft MS, Park WM, Sakata I, Kristensen LV, Husted AS, Osborne-Lawrence S, Piper PK, Walker AK, Pedersen MH, Nohr MK, Pan J, Sinz CJ, Carrington PE, Akiyama TE, Jones RM, Tang C, Ahmed K, Offermanns S, Egerod KL, Zigman JM, Schwartz TW. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. *Mol Metab*. 2013;2(4):376-392.
16. Iwakura H, Kangawa K, Nakao K. The regulation of circulating ghrelin - with recent updates from cell-based assays [Review]. *Endocr J*. 2015;62(2):107-122.
17. Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, Hosoda

H, Kojima M, Kangawa K, Nakao K. Genomic structure and characterization of the 5'-flanking region of the human ghrelin gene. *Endocrinology*. 2004;145(9):4144-4153.

18. Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K. Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. *J Biol Chem*. 2005;280(15):15247-15256.

19. Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun*. 2000;279(3):909-913.

20. Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K. Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. *J Biol Chem*. 2005;280(15):15247-15256.

21. Iwakura H, Hosoda K, Doi R, Komoto I, Nishimura H, Son C, Fujikura J, Tomita T, Takaya K, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M,

Kangawa K, Imamura M, Nakao K. Ghrelin expression in islet cell tumors: augmented expression of ghrelin in a case of glucagonoma with multiple endocrine neoplasm type I. *J Clin Endocrinol Metab.* 2002;87(11):4885-4888.

22. Janssen S, Laermans J, Iwakura H, Tack J, Depoortere I. Sensing of fatty acids for octanoylation of ghrelin involves a gustatory G-protein. *PLoS One.* 2012;7(6):e40168.

23. Zhao TJ, Sakata I, Li RL, Liang G, Richardson JA, Brown MS, Goldstein JL, Zigman JM. Ghrelin secretion stimulated by β 1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice. *Proc Natl Acad Sci U S A.* 2010;107(36):15868-15873.

24. Uhlenbrock K, Gassenhuber H, Kostenis E. Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. *Cellular signalling.* 2002;14(11):941-953.

25. Gong Z, Yoshimura M, Aizawa S, Kurotani R, Zigman JM, Sakai T, Sakata I. G protein-coupled receptor 120 signaling regulates ghrelin secretion in vivo and in vitro. *Am J Physiol Endocrinol Metab.* 2014;306(1):E28-35.

26. Lu X, Zhao X, Feng J, Liou AP, Anthony S, Pechhold S, Sun Y, Lu H, Wank S. Postprandial inhibition of gastric ghrelin secretion by long-chain fatty acid

1 through GPR120 in isolated gastric ghrelin cells and mice. *Am J Physiol*
2 *Gastrointest Liver Physiol.* 2012;303(3):G367-376.

3 27. Lizarzaburu M, Turcotte S, Du X, Duquette J, Fu A, Houze J, Li L, Liu J,
4 Murakoshi M, Oda K, Okuyama R, Nara F, Reagan J, Yu M, Medina JC.
5 Discovery and optimization of a novel series of GPR142 agonists for the treatment
6 of type 2 diabetes mellitus. *Bioorg Med Chem Lett.* 2012;22(18):5942-5947.

7 28. Du X, Kim YJ, Lai S, Chen X, Lizarzaburu M, Turcotte S, Fu Z, Liu Q, Zhang Y,
8 Motani A, Oda K, Okuyama R, Nara F, Murakoshi M, Fu A, Reagan JD, Fan P,
9 Xiong Y, Shen W, Li L, Houze J, Medina JC. Phenylalanine derivatives as
10 GPR142 agonists for the treatment of type II diabetes. *Bioorg Med Chem Lett.*
11 2012;22(19):6218-6223.

12 29. Mani BK, Chuang JC, Kjalarsdottir L, Sakata I, Walker AK, Kuperman A,
13 Osborne-Lawrence S, Repa JJ, Zigman JM. Role of Calcium and EPAC in
14 Norepinephrine-Induced Ghrelin Secretion. *Endocrinology.* 2014;155(1):98-107.

15 30. Gagnon J, Anini Y. Glucagon stimulates ghrelin secretion through the activation
16 of MAPK and EPAC and potentiates the effect of norepinephrine. *Endocrinology.*
17 2013;154(2):666-674.

18 31. Gagnon J, Anini Y. Insulin and norepinephrine regulate ghrelin secretion from a

1 rat primary stomach cell culture. *Endocrinology*. 2012;153(8):3646-3656.

2 32. de la Cour CD, Norlen P, Hakanson R. Secretion of ghrelin from rat stomach
3 ghrelin cells in response to local microinfusion of candidate messenger
4 compounds: a microdialysis study. *Regul Pept*. 2007;143(1-3):118-126.

5 33. Zhao TJ, Liang G, Li RL, Xie X, Sleeman MW, Murphy AJ, Valenzuela DM,
6 Yancopoulos GD, Goldstein JL, Brown MS. Ghrelin O-acyltransferase (GOAT)
7 is essential for growth hormone-mediated survival of calorie-restricted mice. *Proc*
8 *Natl Acad Sci U S A*. 2010;107(16):7467-7472.

9 34. Shrestha YB, Wickwire K, Giraudo SQ. Direct effects of nutrients, acetylcholine,
10 CCK, and insulin on ghrelin release from the isolated stomachs of rats. *Peptides*.
11 2009;30(6):1187-1191.

12 35. Hosoda H, Kangawa K. The autonomic nervous system regulates gastric ghrelin
13 secretion in rats. *Regul Pept*. 2008;146(1-3):12-18.

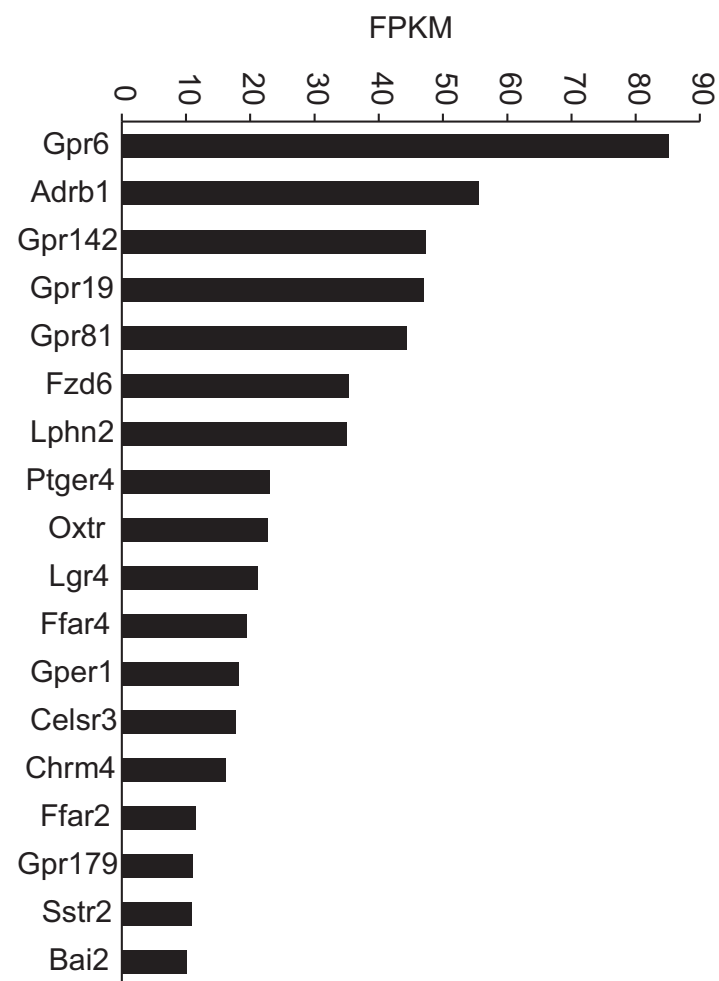
14 36. Conigrave AD, Quinn SJ, Brown EM. L-amino acid sensing by the extracellular
15 Ca²⁺-sensing receptor. *Proc Natl Acad Sci U S A*. 2000;97(9):4814-4819.

16 37. Zhang H, Yin J, Li D, Zhou X, Li X. Tryptophan enhances ghrelin expression and
17 secretion associated with increased food intake and weight gain in weanling pigs.
18 *Domest Anim Endocrinol*. 2007;33(1):47-61.

- 1 **38.** Al Massadi O, Pardo M, Roca-Rivada A, Castelao C, Casanueva FF, Seoane LM.
2 Macronutrients act directly on the stomach to regulate gastric ghrelin release. *J*
3 *Endocrinol Invest.* 2010;33(9):599-602.
- 4 **39.** Knerr I, Groschl M, Rascher W, Rauh M. Endocrine effects of food intake: insulin,
5 ghrelin, and leptin responses to a single bolus of essential amino acids in humans.
6 *Ann Nutr Metab.* 2003;47(6):312-318.
- 7 **40.** Shrestha RG, Rodriguez-Abreu C, Aramaki K. Wormlike micelles in mixed amino
8 acid surfactant/nonionic surfactant aqueous systems and the effect of added
9 electrolytes. *J Oleo Sci.* 2009;58(5):243-254.
- 10 **41.** Sugino T, Kawakita Y, Fukumori R, Hasegawa Y, Kojima M, Kangawa K, Obitsu
11 T, Taniguchi K. Effects of glucose and amino acids on ghrelin secretion in sheep.
12 *Anim Sci J.* 2010;81(2):199-204.
- 13 **42.** Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, Rull J. Stimulation of insulin
14 secretion by amino acids. *J Clin Invest.* 1966;45(9):1487-1502.
- 15 **43.** Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E, Boyadjian R.
16 Insulin regulates plasma ghrelin concentration. *J Clin Endocrinol Metab.*
17 2002;87(8):3997-4000.
- 18 **44.** Norrelund H, Hansen TK, Orskov H, Hosoda H, Kojima M, Kangawa K, Weeke

- J, Moller N, Christiansen JS, Jorgensen JO. Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. *Clin Endocrinol (Oxf)*. 2002;57(4):539-546.
45. Broglio F, Koetsveld Pv P, Benso A, Gottero C, Prodam F, Papotti M, Muccioli G, Gauna C, Hofland L, Deghenghi R, Arvat E, Van Der Lely AJ, Ghigo E. Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans. *J Clin Endocrinol Metab*. 2002;87(10):4829-4832.
46. Shimada M, Date Y, Mondal MS, Toshinai K, Shimbara T, Fukunaga K, Murakami N, Miyazato M, Kangawa K, Yoshimatsu H, Matsuo H, Nakazato M. Somatostatin suppresses ghrelin secretion from the rat stomach. *Biochem Biophys Res Commun*. 2003;302(3):520-525.
47. Sakata I, Park WM, Walker AK, Piper PK, Chuang JC, Osborne-Lawrence S, Zigman JM. Glucose-mediated control of ghrelin release from primary cultures of gastric mucosal cells. *Am J Physiol Endocrinol Metab*. 2012;302(10):E1300-1310.
48. Silva AP, Bethmann K, Raulf F, Schmid HA. Regulation of ghrelin secretion by somatostatin analogs in rats. *Eur J Endocrinol*. 2005;152(6):887-894.
49. Madison LD, Scarlett JM, Levasseur P, Zhu X, Newcomb K, Batra A, Bowe D, Marks DL. Prostacyclin signaling regulates circulating ghrelin during acute inflammation. *J Endocrinol*. 2008;196(2):263-273.

- 1 **50.** Peskar BM. On the synthesis of prostaglandins by human gastric mucosa and its
2 modification by drugs. *Biochim Biophys Acta*. 1977;487(2):307-314.
- 3 **51.** Tarnawski A, Brzozowski T, Sarfeh IJ, Krause WJ, Ulich TR, Gergely H,
4 Hollander D. Prostaglandin protection of human isolated gastric glands against
5 indomethacin and ethanol injury. Evidence for direct cellular action of
6 prostaglandin. *J Clin Invest*. 1988;81(4):1081-1089.
- 7 **52.** Sibilia V, Rindi G, Pagani F, Rapetti D, Locatelli V, Torsello A, Campanini N,
8 Deghenghi R, Netti C. Ghrelin protects against ethanol-induced gastric ulcers in
9 rats: studies on the mechanisms of action. *Endocrinology*. 2003;144(1):353-359.
- 10 **53.** Brzozowski T, Konturek PC, Konturek SJ, Kwiecien S, Drozdowicz D, Bielanski
11 W, Pajdo R, Ptak A, Nikiforuk A, Pawlik WW, Hahn EG. Exogenous and
12 endogenous ghrelin in gastroprotection against stress-induced gastric damage.
13 *Regul Pept*. 2004;120(1-3):39-51.
- 14 **54.** Bennett A, Murray JG, Wyllie JH. Occurrence of prostaglandin E2 in the human
15 stomach, and a study of its effects on human isolated gastric muscle. *Br J*
16 *Pharmacol Chemother*. 1968;32(2):339-349.
- 17



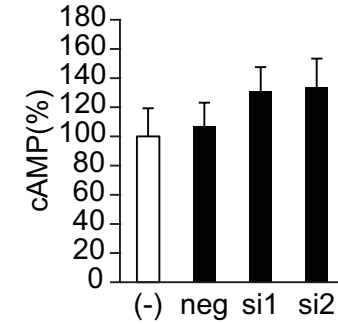
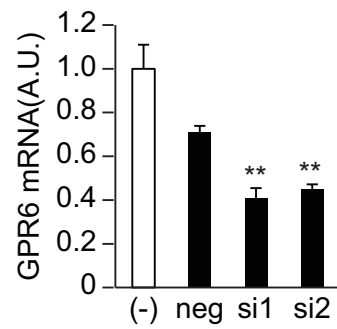
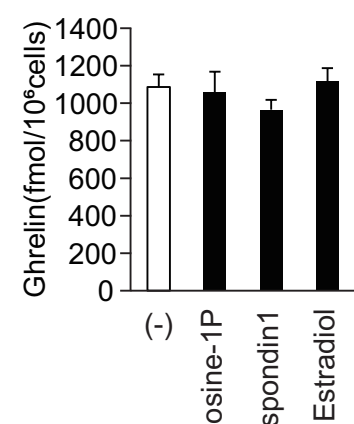
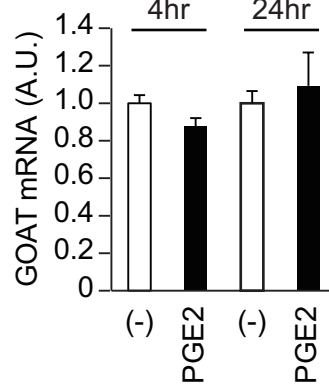
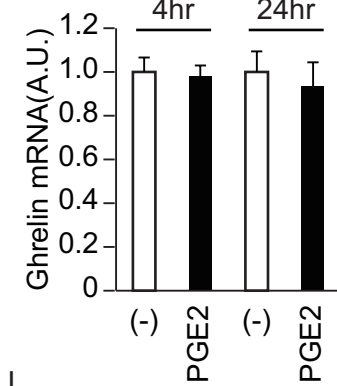
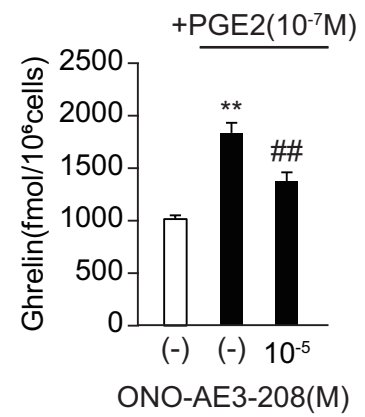
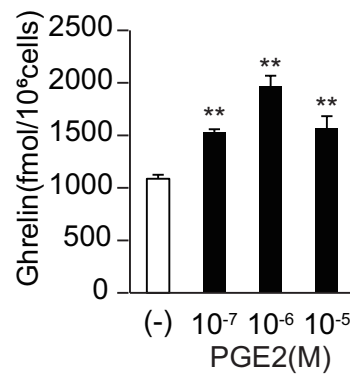
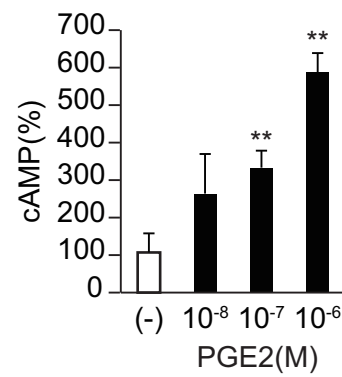
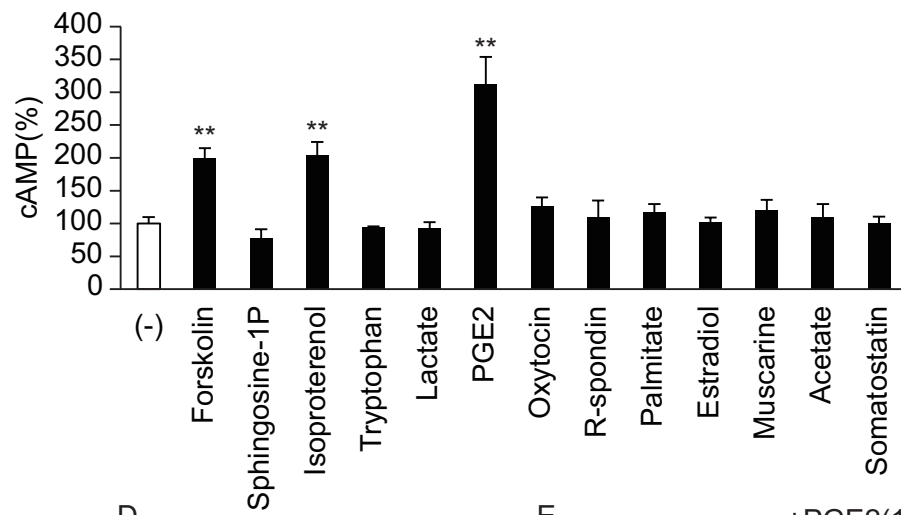
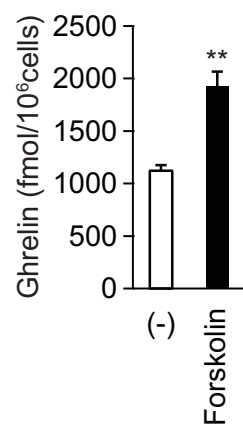


Figure 2

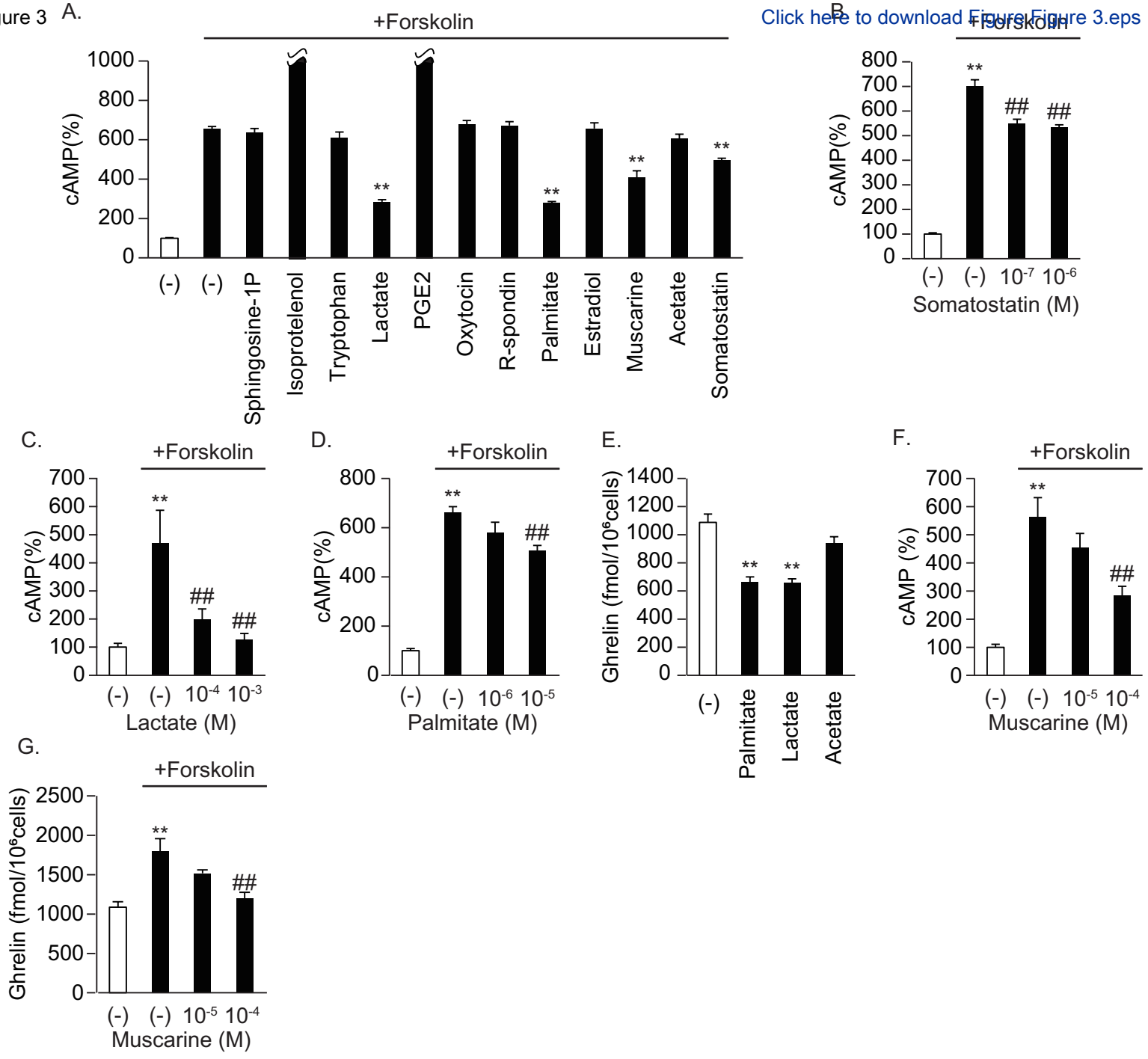


Figure 3

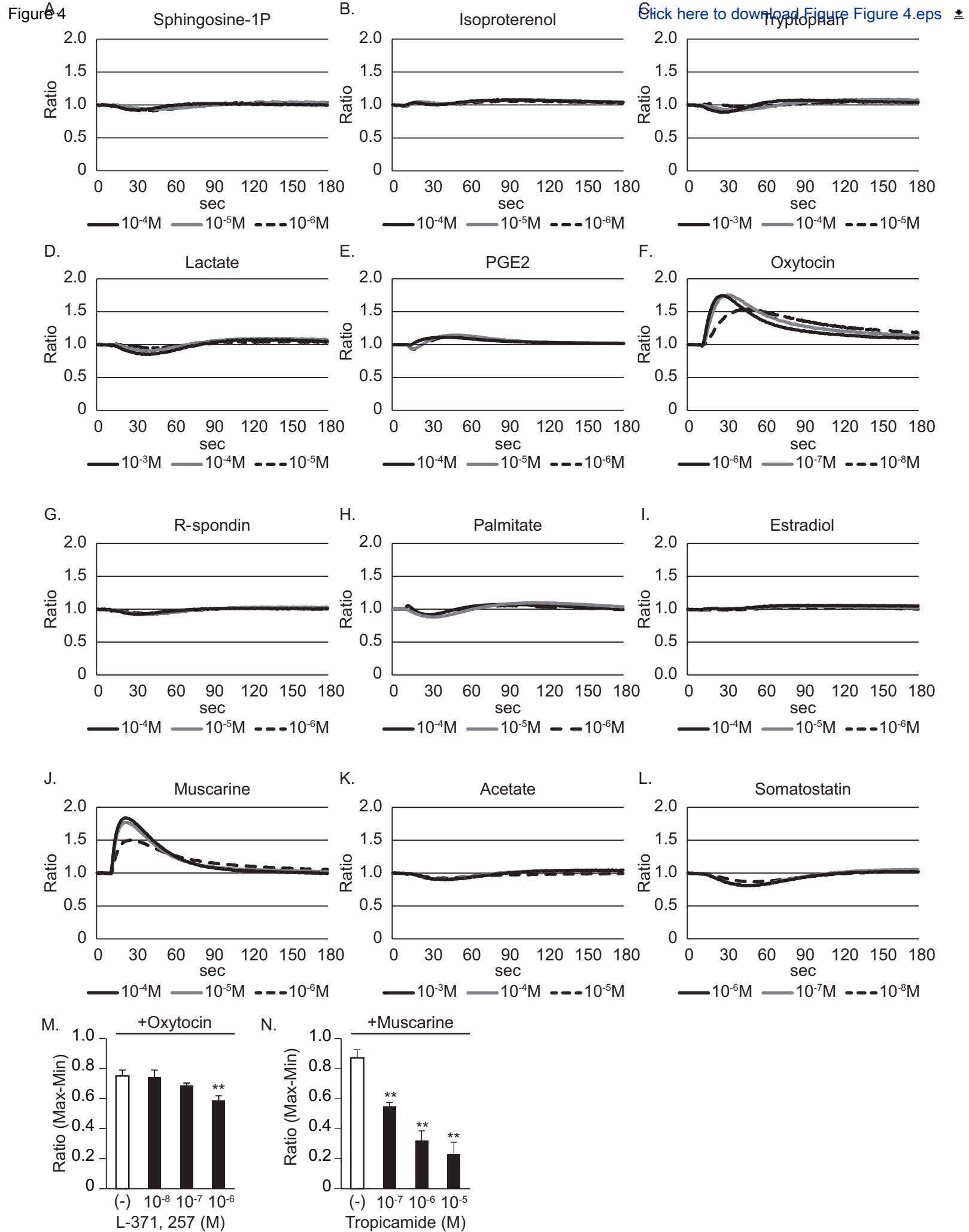


Figure 4

Figure 5

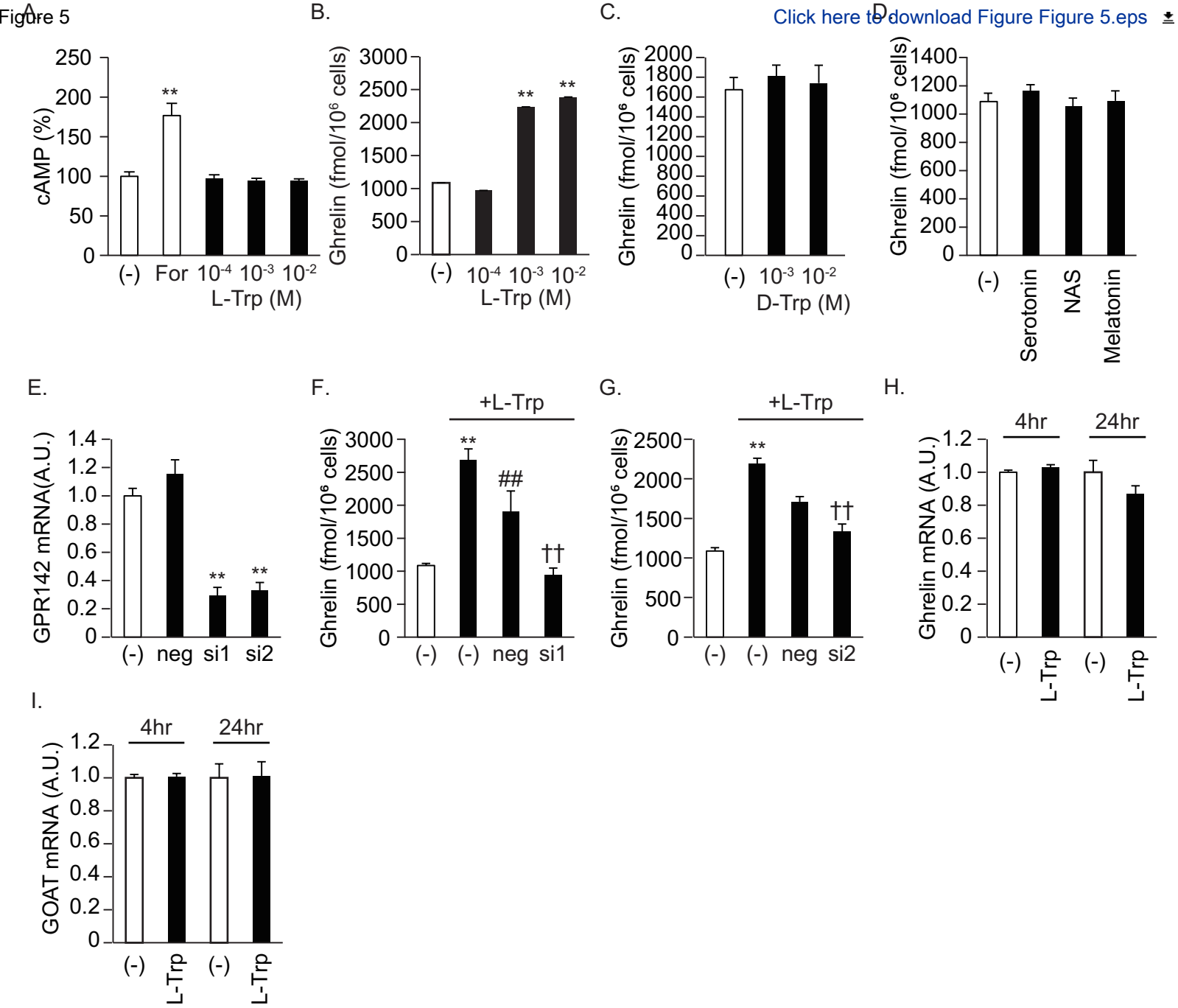
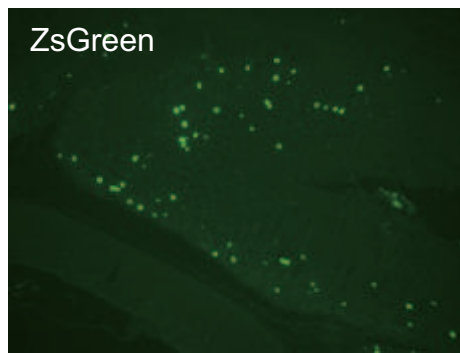
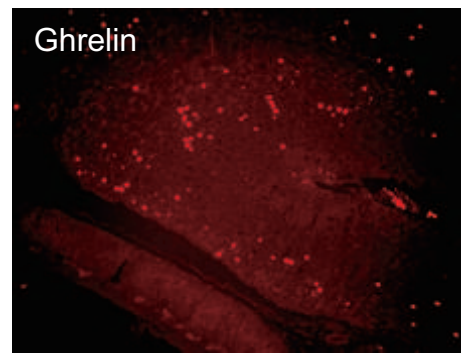


Figure 5

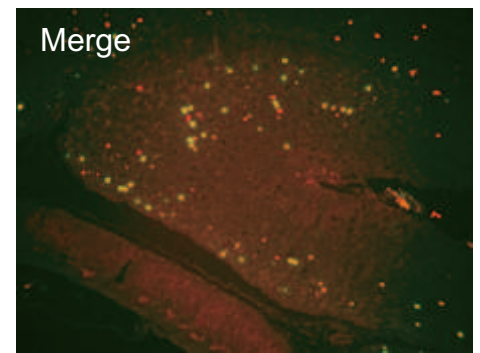
Figure 6A



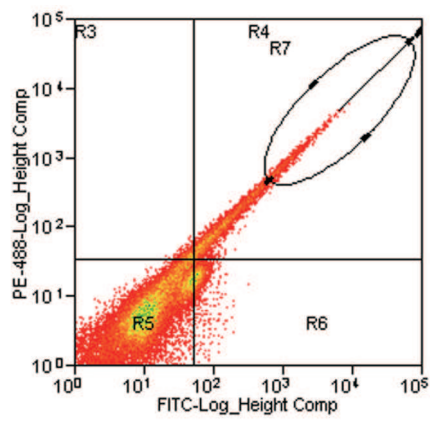
B



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D



E

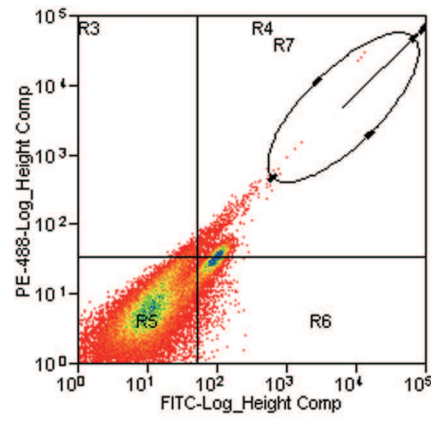


Figure 6

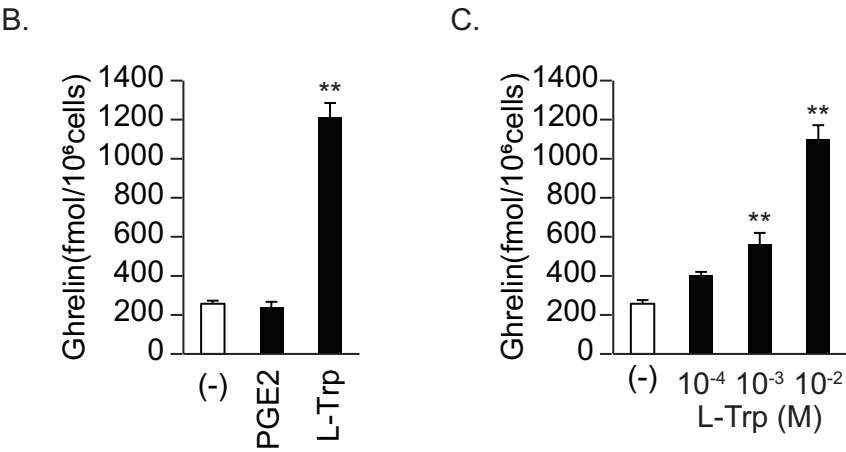
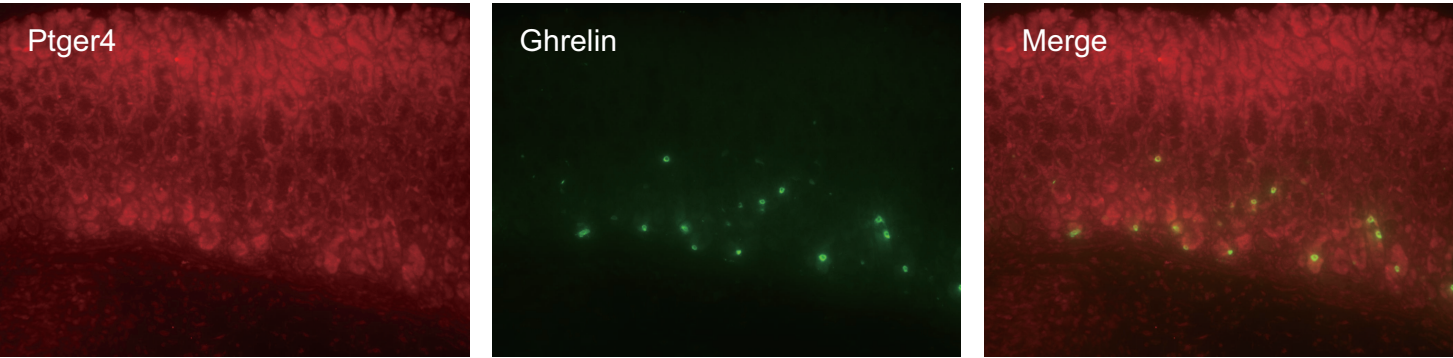


Figure 7