GPR171 is a hypothalamic G protein-coupled receptor for BigLEN, a neuropeptide involved in feeding

Ivone Gomes[a], Dipendra K. Aryal[b,1], Jonathan H. Wardman[a,c,1], Achla Gupta[a], Khatuna Gagnidze[a,2], Ramona M. Rodriguiz[b,d], Sanjai Kumar[e], William C. Wetsel[b,d,f,g,h], John E. Pintar[i], Lloyd D. Fricker[c], and Lakshmi A. Devi[a,3]

[a]Department of Pharmacology and Systems Therapeutics, Ichan School of Medicine at Mount Sinai, New York, NY 10029; Departments of [b]Psychiatry and Behavioral Sciences, [f]Cell Biology, [g]Neurobiology, and [h]Medicine (Endocrinology) and [d]Mouse Behavioral and Neuroendocrine Analysis Core Facility, Duke University Medical Center, Durham, NC 27710; [c]Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461; [e]Department of Chemistry and Biochemistry, Queens College and the Graduate Center of The City University of New York, Queens, NY 11367; and [i]Department of Neuroscience and Cell Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 00854

Edited by Huda Akil, University of Michigan, Ann Arbor, MI, and approved August 23, 2013 (received for review July 9, 2013)

Multiple peptide systems, including neuropeptide Y, leptin, ghrelin, and others, are involved with the control of food intake and body weight. The peptide LENSSPQAPARRLLPP (BigLEN) has been proposed to act through an unknown receptor to regulate body weight. In the present study, we used a combination of ligand-binding and receptor-activity assays to characterize a $G\alpha_{i/o}$ protein-coupled receptor activated by BigLEN in the mouse hypothalamus and Neuro2A cells. We then selected orphan G protein-coupled receptors expressed in the hypothalamus and Neuro2A cells and tested each for activation by BigLEN. G protein-coupled receptor 171 (GPR171) is activated by BigLEN, but not by the C terminally truncated peptide LittleLEN. The four C-terminal amino acids of BigLEN are sufficient to bind and activate GPR171. Overexpression of GPR171 leads to an increase, and knockdown leads to a decrease, in binding and signaling by BigLEN and the C-terminal peptide. In the hypothalamus GPR171 expression complements the expression of BigLEN, and its level and activity are elevated in mice lacking BigLEN. In mice, shRNA-mediated knockdown of hypothalamic GPR171 leads to a decrease in BigLEN signaling and results in changes in food intake and metabolism. The combination of GPR171 shRNA together with neutralization of BigLEN peptide by antibody absorption nearly eliminates acute feeding in food-deprived mice. Taken together, these results demonstrate that GPR171 is the BigLEN receptor and that the BigLEN–GPR171 system plays an important role in regulating responses associated with feeding and metabolism in mice.

proSAAS | NPY/AgRP | neuroendocrine peptide | deorphanization | orexigenic

Neuropeptides are the largest class of chemical messengers with more than 100 known neuropeptides that function in cell–cell signaling. A number of neuropeptides contribute to the regulation of feeding and body weight, including well-established peptides such as neuropeptide Y (NPY) and agouti gene-related peptide (AgRP). Ablation of hypothalamic NPY/AgRP neurons causes a dramatic reduction in body weight (1), and selective activation of these neurons drives a robust feeding behavior in mice (2). However, knockout of either NPY or AgRP does not significantly alter body weight or feeding (3), leading to the hypothesis that additional factors in these neurons contribute to feeding/body weight regulation (4). ProSAAS-derived peptides (Fig. 1*A*) are logical candidates for this control because they are among the most abundant peptides present in mouse hypothalamus (5–7) and are greatly enriched in the AgRP/NPY neurons of the arcuate nucleus (8). ProSAAS-derived peptides exhibit activity-dependent release from hypothalamic neurons (9). Transgenic overexpression of proSAAS leads to an increase (10) and proSAAS knockout leads to a decrease in body weight (11). Finally, immunoneutralization of two proSAAS-derived peptides, AVDQDLGPEVPPENVLGALLRV and LENSSPQAPARRLLPP (PEN and BigLEN), from the hypothalamus results in reduced feeding (8). Here we describe GPR171 as an orphan G protein-coupled receptor (GPCR) activated by BigLEN that plays a functional role in feeding and metabolism.

Results and Discussion

Characterization of a Functional Receptor for BigLEN in the Hypothalamus and in Neuro2A Cells. To characterize the BigLEN receptor, we carried out binding and signaling studies with hypothalamic membranes. Binding assays with [^{125}I]Tyr-BigLEN revealed a specific and high-affinity site (K_d ~0.5 nM) for BigLEN (Fig. 1 *B* and *C*). Comparison of [^{125}I]Tyr-BigLEN binding in mouse brain regions, pituitary, and peripheral tissues showed highest binding in the hypothalamus, moderate binding in the cortex, and low binding in the peripheral tissues (Table S1). G protein activity assays with rat hypothalamic membranes revealed that BigLEN, but not a control peptide, produced a dose-dependent increase in [^{35}S]GTPγS binding (Fig. 1*D*). Preincubation with pertussis toxin, a specific $G\alpha_{i/o}$ inhibitor, led to a significant attenuation of BigLEN-mediated [^{35}S]GTPγS binding (Fig. 1*E*), suggesting that the hypothalamic BigLEN receptor is a $G\alpha_{i/o}$-coupled receptor. Consistent with this idea, BigLEN treatment led to a significant decrease in adenylate cyclase activity (Fig. 1*F*). Neuro2A neuroblastoma cells express a BigLEN receptor with properties similar to those of the hypothalamic receptor. Membranes from

Significance

The mechanism by which vertebrate animals control their body weight is a complex process involving a variety of molecules that regulate feeding and metabolism. Some of these molecules are neuropeptides that bind to specific receptors in feeding centers of the brain. One of the most abundant peptides in brain, LENSSPQAPARRLLPP (named BigLEN), has been proposed to function as a neuropeptide involved in regulating body weight, but the receptor through which this peptide acts had not been identified. We screened candidate receptors and found one, G protein-coupled receptor 171 (GPR171), that is activated by BigLEN. Additional studies showed that the BigLEN–GPR171 system plays an important role in regulating feeding and metabolism in mice. Thus, GPR171 is a potential target for developing antiobesity drugs.

Author contributions: D.K.A., R.M.R., W.C.W., J.E.P., L.D.F., and L.A.D. designed research; I.G., D.K.A., J.H.W., A.G., K.G., and J.E.P. performed research; S.K. and J.E.P. contributed new reagents/analytic tools; I.G., D.K.A., R.M.R., and L.A.D. analyzed data; and I.G., R.M.R., W.C.W., L.D.F., and L.A.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]D.K.A. and J.H.W. contributed equally to this work.

[2]Present address: Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University, New York, NY 10065.

[3]To whom correspondence should be addressed. E-mail: lakshmi.devi@mssm.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1312938110/-/DCSupplemental.

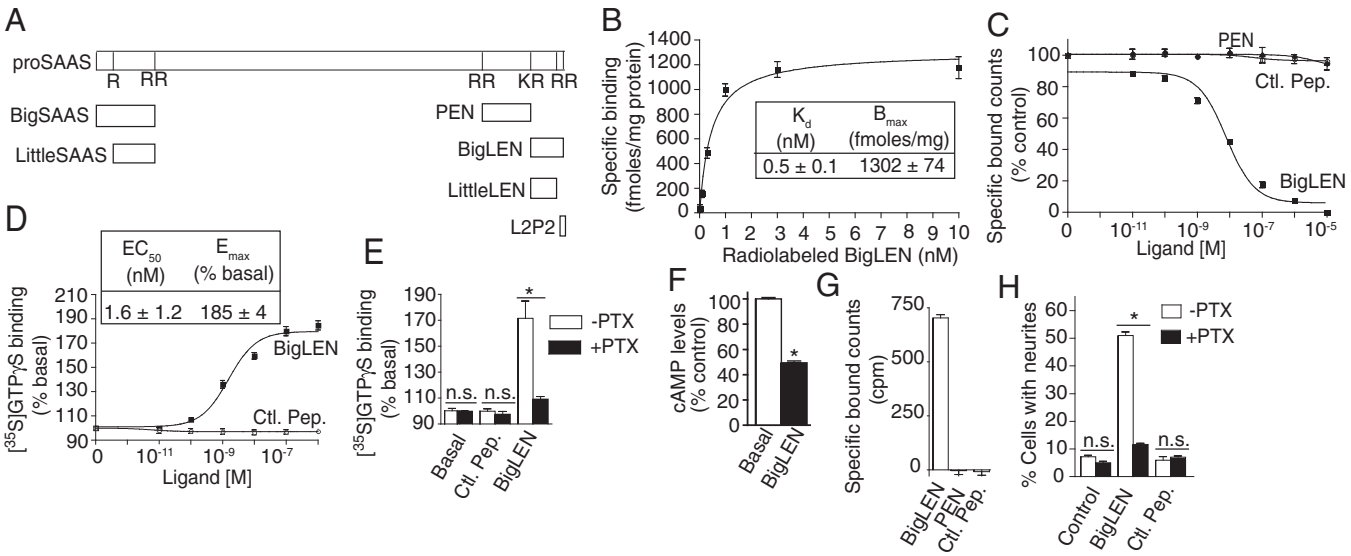

Fig. 1. ProSAAS-derived peptides bind to and activate a $G\alpha_{i/o}$-coupled receptor. (*A*) Schematic diagram of peptides derived from proSAAS processing. R, arginine; K, lysine. (*B*) Binding of [^{125}I]Tyr-BigLEN to rat hypothalamic membranes. Nonspecific binding was determined with 10 μM Tyr-BigLEN and was <10% of the total binding. Bmax, maximal binding capacity. (*C*) BigLEN, but not PEN or a control peptide (Ctl. Pep.), displaces [^{125}I]Tyr-BigLEN binding to rat hypothalamic membranes. (*D*) BigLEN, but not a control peptide, dose-dependently increases [^{35}S]GTPγS binding to rat hypothalamic membranes. Emax, maximal possible effect. (*E*) Pretreatment with pertussis toxin (PTX) blocks the BigLEN-mediated increases in [^{35}S]GTPγS binding. *$P < 0.0036$ from +PTX (unpaired *t* test). (*F*) BigLEN inhibits adenylate cyclase activity in hypothalamic membranes. *$P < 0.0001$ vs. basal response. (*G*) Specific binding of [^{125}I]Tyr-BigLEN to Neuro2A cells in the presence of BigLEN, PEN or a control peptide. (*H*) BigLEN induces neurite outgrowth in Neuro2A cells; this outgrowth is blocked by PTX pretreatment. *$P < 0.0036$ from +PTX (unpaired *t* test). Data represent means ± SE of three independent experiments performed in triplicate in *B*, *C*, and *E*–*G*, six independent experiments performed in triplicate in *D*, and four independent experiments in performed triplicate in *H*. n.s., not significant.

Neuro2A cells exhibited specific [^{125}I]Tyr-BigLEN binding (Fig. 1*G*) and signaling with BigLEN as evidenced by the dose- and time-dependent increases in ERK1/2 phosphorylation (Fig. S1 *A* and *B*). Treatment of Neuro2A cells with BigLEN leads to a robust increase in neurite outgrowth that is blocked by pertussis toxin (Fig. 1*H*), consistent with previous findings that activation of $G\alpha_{i/o}$-coupled receptors induces neuritogenesis in Neuro2A cells (12–15). Together these results suggest the presence of a $G\alpha_{i/o}$-coupled receptor in hypothalamus and Neuro2A cells that is activated by BigLEN.

Identification of GPR171 as the BigLEN Receptor. We searched a GPCR expression database for orphan GPCRs that were enriched in hypothalamus (16) and Neuro2A cells (17). Using the Allen Brain Atlas (18), we winnowed the list of candidates by examining their expression in the ventral hypothalamus and identified 50 hypothalamic orphan GPCRs. This list of candidates was matched against a list of orphan GPCRs expressed in Neuro2A cells (17). The top four orphan receptors (GPR19, GPR108, GPR165, and GPR171) were selected for further screening using a cell-based Ca^{+2}-release assay. Epitope-tagged orphan GPCRs were expressed in CHO cells along with a promiscuous chimeric $G_{16/i3}$ protein, and receptor activation-mediated increases in intracellular Ca^{+2} levels were monitored using Fluo-4NW. Of the GPCRs tested, only GPR171 produced an increase in intracellular Ca^{+2} with BigLEN (Fig. 2 *A* and *B*). This response was similar in amplitude and duration to the response to ATP, a positive control which stimulates an endogenous receptor in CHO cells. No effect was observed when the cells expressing the GPR171 receptor were treated with LittleLEN or a control peptide (Fig. 2 *A* and *B*).

GPR171 (also known as H963) is highly conserved among mice, rats, and humans (Fig. S2). It belongs to the P2Y-like family of receptors (19–22), being most similar in amino acid sequence to the free fatty acid and dicarboxylic acid receptors (Fig. 2*C*). Although it is in the P2Y-like family, GPR171 is not activated by a variety of carboxylic acids (Fig. 2*D*). Treatment of BigLEN with trypsin did not eliminate activity in the intracellular Ca^{+2} assay (Fig. 3*A*); this treatment converts BigLEN into LittleLEN-Arg and the C-terminal peptide Leu-Leu-Pro-Pro (L2P2). When L2P2 alone was tested, it was able to activate GPR171 (Fig. 3*A*) and displace radiolabeled BigLEN from rat hypothalamic membranes with an EC_{50} of 76 nM, whereas a shorter peptide, LPP did not displace radiolabeled BigLEN binding (Fig. 3*B*). L2P2 treatment caused a dose-dependent increase in GTPγS binding to hypothalamic membranes (Fig. 3*C*) and a dose-dependent decrease in adenylate cyclase activity (Fig. 3*D*). Taken together, these results indicate that BigLEN binds to GPR171 through its C-terminal region and that the four C-terminal residues are necessary and sufficient to activate GPR171.

The expression of GPR171 siRNA in Neuro2A cells led to a reduction in GPR171 protein levels (Fig. 4*A*) and a decrease in BigLEN-mediated signaling (Fig. 4*B*). GPR171siRNA had no effect on signaling mediated by LittleLEN or Deltorphin II (a δ-opioid receptor agonist) (Fig. 4*B*). Furthermore, GPR171siRNA expression decreased neurite outgrowth in Neuro2A cells under basal conditions as well as after BigLEN or L2P2 treatment (Fig. 4*C*). The expression of GPR171 shRNA (using a lentiviral expression system) also decreased GPR171 protein levels (Fig. S3*A*), leading to robust decreases in BigLEN binding as well as BigLEN- or L2P2- mediated signaling (Fig. 4 *D* and *E* and Fig. S3 *B* and *C*). In comparison, overexpression of GPR171 led to an increase in BigLEN binding (Fig. 4*D*). Pretreatment with an antibody to GPR171 (but not to an unrelated antibody) led to reduced GTPγS binding to hypothalamic membranes in response to BigLEN and L2P2 (Fig. 4*F*). Taken together, these results suggest that GPR171 is a receptor for BigLEN in both the hypothalamus and Neuro2A cells.

Distribution of GPR171 in Brain and Regulation of Expression. Immunohistochemical analysis shows that GPR171 is highly expressed in mouse hypothalamus, including the arcuate nucleus, paraventricular nucleus (PVN), and dorsomedial hypothalamus (Fig. S4 *A* and *B*); this distribution matches that of GPR171 mRNA reported in the Allen Brain Atlas (18) and GenSAT database (23). Double-labeling immunohistochemistry revealed

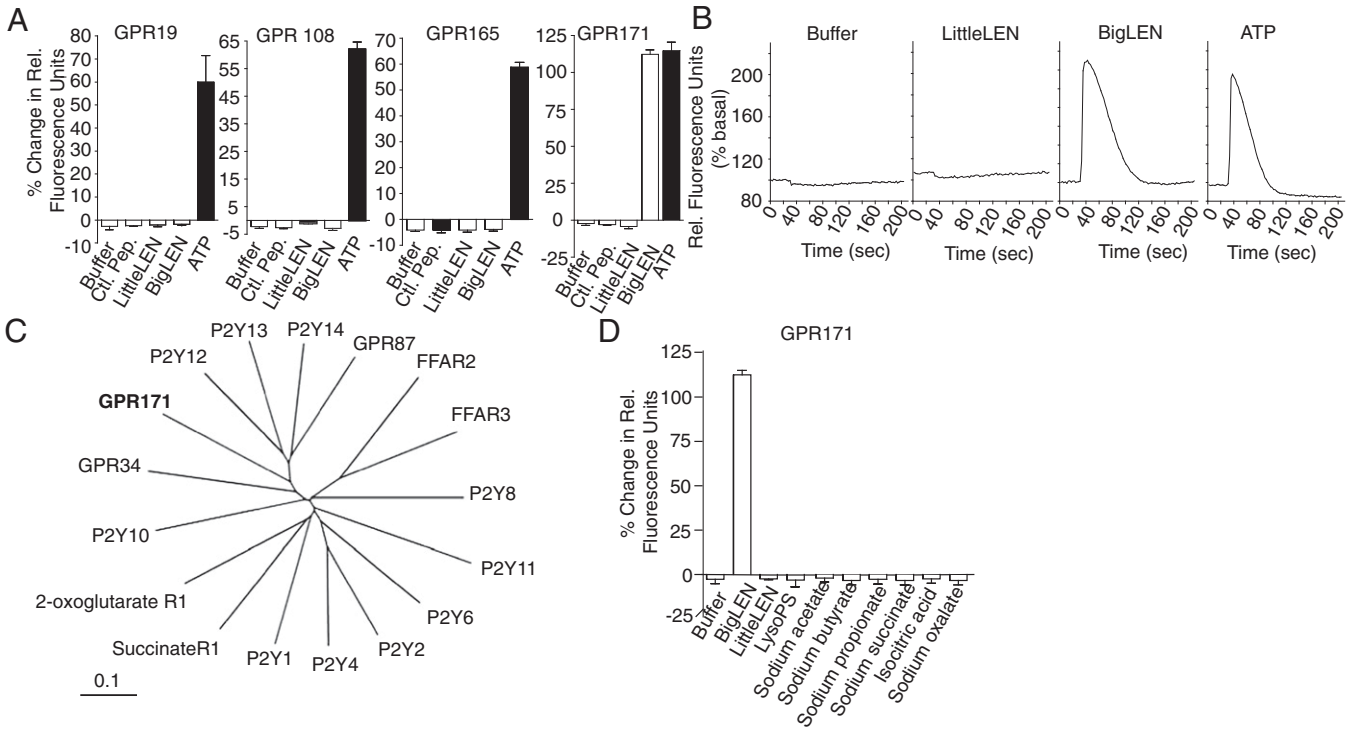

Fig. 2. Identification of GPR171 as the receptor activated by the proSAAS-derived peptide, BigLEN. (*A*) BigLEN, but not LittleLEN or a control peptide (Ctl. Pep.), increases intracellular Ca^{+2} levels in CHO cells expressing GPR171 but not in cells expressing GPR19, GPR108, or GPR165. (*B*) Representative plots from *A* of intracellular Ca^{+2} mobilization in CHO-GPR171 cells (along with a chimeric $G_{16/i3}$ protein) treated with buffer, LittleLEN, BigLEN, or ATP (1 μM). (*C*) A phylogenetic tree showing the relation of GPR171 to P2Y receptors, dicarboxylic acid receptors, free fatty acid receptors (FFARs), and other orphan GPCRs. (Scale bar, 0.1 substitutions per nucleotide.) (*D*) Intracellular Ca^{+2} mobilization in CHO-GPR171 cells treated with or without 1 μM BigLEN or ligands including dicarboxylic acids. Data in *A, B*, and *D* represent means ± SE of three independent experiments carried out in sextuplicate.

the presence of GPR171 and BigLEN within the arcuate nucleus (Fig. S4*C*) as well as in the PVN and other brain regions. The presence of GPR171 protein and mRNA in the PVN is consistent with the previous finding that medial parvocellular neurons respond electrophysiologically to exogenous BigLEN (8). The intensity of GPR171 staining in the arcuate nucleus is higher in mice lacking BigLEN (i.e., proSAAS-KO mice) than in WT controls (Fig. S4*D*). Similarly, hypothalamic membranes of proSAAS-KO mice have significantly elevated BigLEN binding (1.3 ± 0.03 pmoles/mg in WT and 1.8 ± 0.06 pmoles/mg in proSAAS-KO mice) and G protein signaling (165% in WT and 175% in proSAAS-KO mice). These results suggest a compensatory increase in GPR171 expression in the absence of BigLEN, consistent with studies of other receptor systems that found upregulation of the receptor in the absence of neurotransmitters (24). Collectively, these results support the hypothesis that the BigLEN–GPR171 system modulates the activity of neurons in the ventral hypothalamus.

Knockdown of GPR171 Affects Feeding Behaviors and Metabolism in Mice. GPR171 shRNA lentivirus injected into the third ventricle substantially reduced the levels of GPR171 mRNA in the ventral hypothalamus (Fig. 5*A*). Following a 2-wk recovery period, body weights were similar in the mice injected with control shRNA (24.9 ± 1.24 g) and mice injected with GPR171 shRNA (25.5 ± 0.72 g). At this time, mice were placed into the Comprehensive Laboratory Animal Monitoring System (CLAMS) unit for 4 d. Responses of both groups were lower during the day (0700–1900 h) than during the night (1900–0700 h) (Fig. S5 and Table S2). Both the respiratory-exchange ratio (RER) and heat production were increased in the GPR171 shRNA group. A more detailed examination of the data revealed two periods of peak responses; early night (1800–2400 h) responses were significantly higher than the late night (0300–0900 h) responses (Fig. 5 *B–F* and Table S3). During the early night, the GPR171 shRNA-knockdown mice consumed more food and water, were more active, and maintained higher levels of RER and heat production than the controls. Taken together, these results indicate that GPR171 plays a complex role in regulating feeding-associated behaviors as well as activity in mice.

The arcuate nucleus is a well-established site for the regulation of feeding behaviors (25–30). Because BigLEN and other proSAAS-derived peptides are highly expressed in the NPY neurons of the arcuate nucleus (8), we examined whether shRNA-induced knockdown of GPR171 would affect feeding-associated responses. Previous studies found that overexpression of proSAAS increased body weight in both male and female mice (10), whereas disruption of proSAAS decreased body weight in male mice (11). In the present study, no changes in body weight were observed in male mice injected with GPR171 shRNA virus. However, in the current study body weight was measured only for 2 wk after viral injection; in previous studies the mice were followed over much longer times, and it took 7–12 wk for significant body weight changes to emerge. Moreover, overexpression of proSAAS or deletion of this gene should affect not only the levels of BigLEN but also those of other proSAAS-derived peptides, whereas shRNA-induced knockdown of GPR171 should influence only BigLEN responses.

The specific involvement of BigLEN in GPR171-mediated feeding behaviors was examined. As seen in a previous study (8), administration of BigLEN antibodies to the third ventricle led to a significant decrease in acute feeding following an overnight fast (Fig. 5*G*). This decrease in feeding was augmented further in mice treated with GPR171 shRNA, with food intake reduced by >90% compared with the control mice (Fig. 5*H*). This combined effect of decreased GPR171 expression and removal of BigLEN further supports the idea that GPR171 functions as the

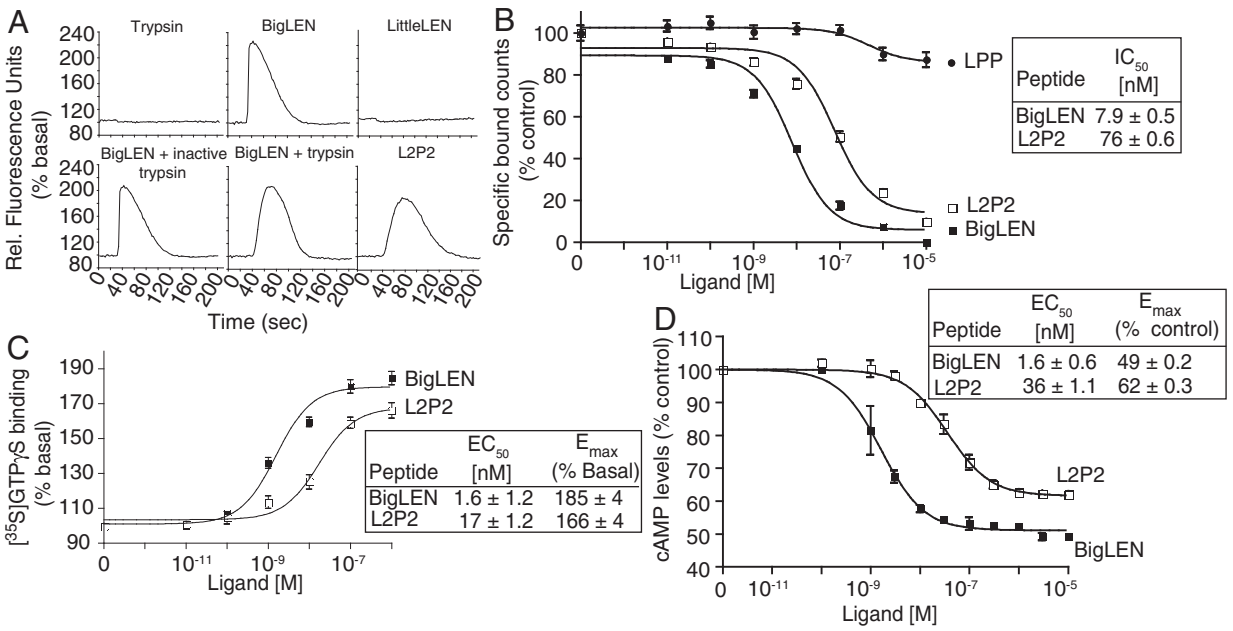

Fig. 3. BigLEN and the C-terminal peptide L2P2 exhibit activity at GPR171. (*A*) Intracellular Ca^{+2} mobilization in CHO-GPR171 cells treated with trypsin, 1 μM BigLEN with or without trypsin or inactivated trypsin, or 1 μM L2P2 shows that L2P2 is sufficient to activate GPR171. (*B*) L2P2, but not LPP, displaces [^{125}I]Tyr-BigLEN binding from rat hypothalamic membranes. (*C*) BigLEN or L2P2 dose-dependently increases [^{35}S]GTPγS binding to hypothalamic membranes. (*D*) Treatment of hypothalamic membranes with BigLEN or L2P2 (0–10 μM) decreases cAMP levels. Data represent means ± SE of three independent experiments. n = 3–6.

receptor for BigLEN in vivo and that the BigLEN–GPR171 system plays a major role in regulating behaviors associated with feeding in mice.

In summary, BigLEN appears to be a ligand for GPR171 based on the findings that (*i*) heterologous expression of GPR171 leads to high-affinity binding of and signaling by BigLEN; (*ii*) knockdown of GPR171 leads to reduced responses by BigLEN in the hypothalamus and in Neuro2A cells; (*iii*) a GPR171-specific antibody blocks BigLEN-mediated signaling; and (*iv*) GPR171 levels and activity are increased in the hypothalami of mice lacking BigLEN. Furthermore, the finding that feeding and metabolism are modulated by GPR171 knockdown in the ventral hypothalamus

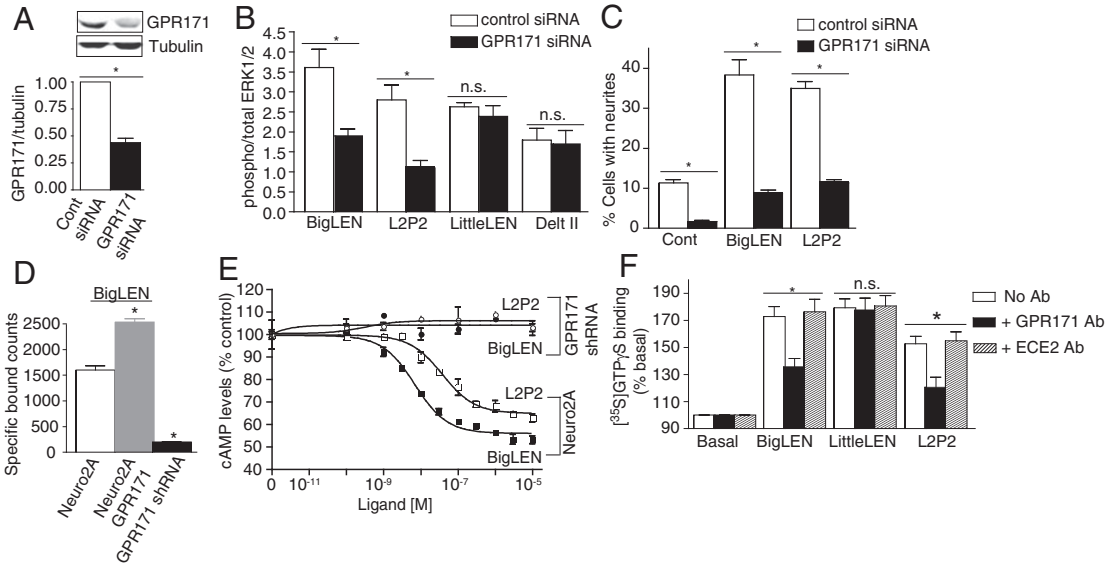

Fig. 4. Knockdown or blockade of GPR171 levels reduces signaling by BigLEN and L2P2. (*A*) Expression of GPR171 siRNA reduces GPR171 protein levels. *P < 0.0001 vs. control siRNA. (*B*) Expression of GPR171 siRNA reduces BigLEN- or L2P2-induced ERK1/2 phosphorylation but not that induced by LittleLEN or the δ-opioid receptor agonist, Deltorphin II (Delt II). *P < 0.0257 vs. control siRNA. (*C*) Expression of GPR171 siRNA reduces BigLEN- or L2P2-mediated neurite outgrowth in Neuro2A cells. *P < 0.0001 vs. control siRNA. (*D*) Overexpression of GPR171 increases but shRNA to GPR171 decreases specific [^{125}I]Tyr-BigLEN binding in Neuro2A cells. *P < 0.0001 vs. Neuro2A. (*E*) BigLEN and L2P2 do not signal in Neuro2A cells expressing GPR171 shRNA. (*F*) BigLEN- or L2P2-mediated increases in [^{35}S]GTPγS binding to rat hypothalamic membranes are blocked by pretreatment with the antibody to GPR171 (GPR171 Ab) but not by the antibody to endothelin-converting enzyme-2 (ECE2 Ab). *P < 0.05 vs. either No Ab or ECE2 Ab controls. Data represent means ± SE of three independent experiments in *A* or three independent experiments performed in triplicate in *B*–*F*. n.s., not significant.

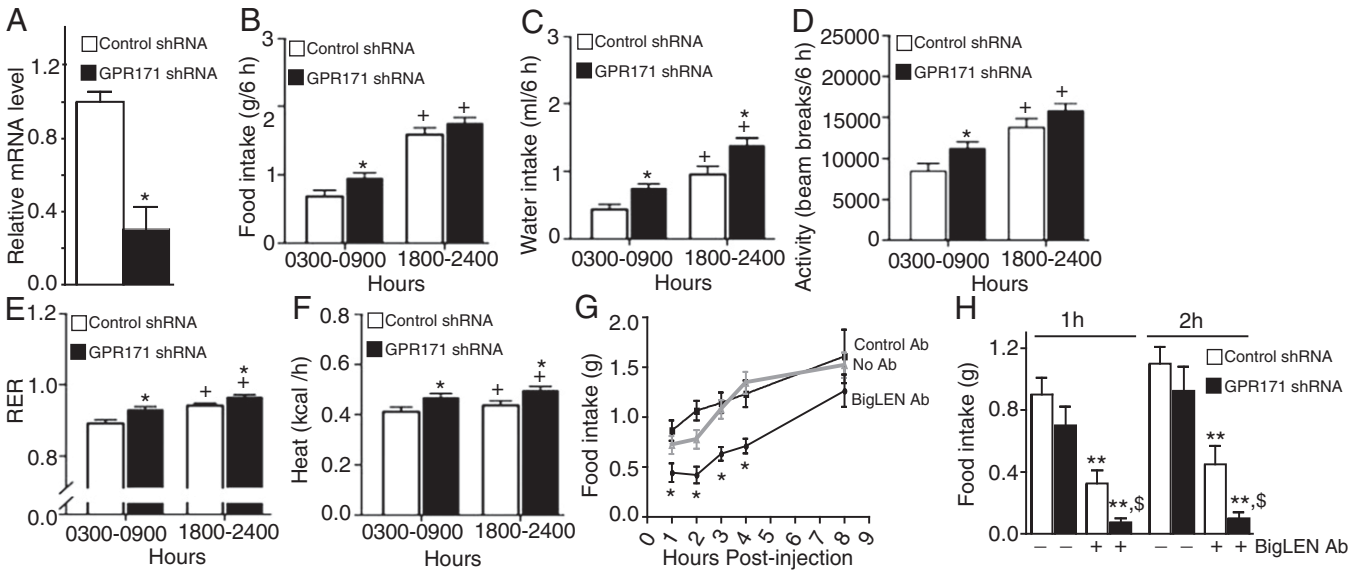

Fig. 5. Behaviors associated with shRNA-mediated knockdown of GPR171 expression. (*A*) RT-PCR analysis showing that intracerebroventricular (i.c.v.) administration of GPR171 lentiviral shRNA leads to a decrease in the levels of GPR171 mRNA in the mouse ventral hypothalamus. $^{*}P < 0.0001$ vs. control shRNA ($n = 6$ per group). (*B–F*) Feeding-associated responses at peak response times during the late (0300–0900 h) and early (1800–2400 h) night. GPR171 shRNA mice consumed significantly more food (*B*), drank more water (*C*), and exhibited more activity (*D*) than controls during late night. A significant increase in RER (*E*) and heat production (*F*) were noted in the GPR171 shRNA mice relative to controls at both night time points. Data in *B–F* represent means ± SE of 12–16 mice per treatment condition. $^{*}P < 0.05$, GPR171 shRNA vs. control for the same time point; $^{+}P < 0.05$, 0300–0900 h vs. 1800–2400 h for the same treatment. Statistical analyses are provided in Table S3. (*G*) I.c.v. administration of BigLEN antibody, but not control antibody (normal rabbit IgG), significantly reduces acute food intake up to 4 h postinjection. (*H*) GPR171 shRNA mice exhibit significantly less acute feeding following fasting than control shRNA mice. I.c.v. administration of BigLEN antibody causes a greater decrease in food intake in mice treated with GPR171 shRNA than in mice treated with control antibody. Data in *G* and *H* represent means ± SE of nine mice per treatment condition; $^{**}P < 0.01$, shRNA treatment vs. BigLEN Ab; $^{\$}P < 0.05$, control shRNA+ BigLEN Ab vs. GRP171 shRNA + BigLEN Ab.

supports a role for the BigLEN–GPR171 system in regulating these functions. This idea is supported by the finding that third ventricular injections of antibodies to BigLEN affect food intake in mice that can be augmented by GPR171 knockdown and by previous studies which found that overexpression or deletion of proSAAS produces alterations in body weight (10, 11). The identification of GPR171 as the receptor for BigLEN provides insights into these mechanisms but also may provide a target for therapeutic modulation of food intake and metabolism.

Methods

Ligand-Binding Assays. Membranes (100 μg) from WT and proSAAS-KO mouse brain regions and peripheral tissues or from cells expressing GPR171 or lentiviral shRNA GPR171 were subjected to saturation binding with 0–10 nM [^{125}I]Tyr-BigLEN as described for other peptide receptors (31–33). Details are given in *SI Methods*. For displacement assays, 3 nM [^{125}I]Tyr-BigLEN and 0–10 μM of control peptide, rat BigLEN (LENSSPQAPARRLLPP), rat PEN (AVDQDLGPEVPPENVLGALLRV), L2P2, or LLP were used.

[^{35}S]GTPγS Binding. Membranes (10 μg) were subjected to a [^{35}S]GTPγS binding assay in response to BigLEN, LittleLEN, or a control peptide (0–1 μM final concentration) as described (33, 34). Details are given in *SI Methods*. Pertussis toxin (50 ng/mL) was added 30 min before the assay. Antibody blocking was with anti-GPR171 (5 μg) or anti–endothelin-converting enzyme-2 (5 μg) for 30 min before the assay. Similar antibody-blocking studies were not carried out with the other signaling or ligand-binding assays.

ERK1/2 Phosphorylation. CHO cells expressing GPR171 were treated with 1 μM BigLEN for 0–60 min or 0–10 μM BigLEN for 2 min. Neuro2A cells transfected with siRNA or infected with shRNA were treated with 1 μM of BigLEN (2 min), LittleLEN (5 min), or Deltorphin II (5 min). PhosphoERK1/2 was measured as described (35–37). Details are given in *SI Methods*.

Determination of cAMP Levels. Membranes (2.5 μg) were pretreated with 40 μM forskolin for 30 min followed by BigLEN or L2P2 (0–10 μM) for 30 min. cAMP levels were quantified using the HitHunter cAMP detection kit (DiscoverX). Details are given in *SI Methods*.

Neurite Outgrowth Assays. The effects of BigLEN or L2P2 on neurite outgrowth were measured as described (14, 34). Pertussis toxin (15 ng/mL) treatment was overnight before ligand treatment.

Intracellular Ca^{+2} Responses. CHO cells expressing *myc*-tagged GPR19, GPR108, GPR165, or GPR171 along with $G\alpha_{16/i3}$ were treated without or with ligands (1 μM). Intracellular Ca^{+2} levels were measured using Fluo4NW as described (34, 38). Relative units represent the percent fold change over basal levels.

Homology and Phylogenetic Analysis. The sequences homologous to GPR171 and H963 were aligned using ClustalW. A phylogenetic tree was generated using ClustalX, and the dendrogram was constructed using the TreeView algorithm. Details are given in *SI Methods*.

Western Blot Analysis. Lysates (30 μg protein) were subjected to Western blot analysis using rabbit anti-GPR171 (1:1,000), mouse anti-tubulin (1:20,000), anti-rabbit IRDye 800 (1:10,000), and anti-mouse IRDye 6800 antibodies (1:10,000) as described (37). Details are given in *SI Methods*.

Immunohistochemistry. Coronal sections were immunostained with Alexa 488-labeled anti-BigLEN (1:5,000) and rabbit anti-GPR171 (1:1,000) antibodies, and staining was visualized with donkey anti-rabbit Cy3–conjugated antibodies (1:1,000) as described (8).

RT-PCR. Quantitative RT-PCR was performed using sense and antisense primers for mouse GPR171 and glyceraldehyde 3-phosphate dehydrogenase (Sigma) on cDNA prepared from total RNA isolated from the ventral hypothalami of mice expressing control or lentiviral shRNA to GPR171; cDNA was prepared as described (8). Quantitative analysis was performed using the $\Delta\Delta C_T$ method and REST software (www.gene-quantification.de) (39).

Animals and Viral Injections. Male C57BL/6J mice, 10–12 wk of age (Jackson Laboratories), were anesthetized with ketamine and xylazine, and the third ventricle (relative to bregma: anteroposterior: −1.46 mm; mediolateral: 0.00 mm; dorsoventral: −5.80 mm) was infused over 15 min with 2 μL of control (12 mice) or lentiviral shRNA (16 mice) to GPR171. Mice were housed individually in a humidity- and temperature-controlled room with a 12-h light/dark cycle (lights on: 0700–1900 h) and free access to RLD-5001 diet (Lab Diet) and

water throughout the experiment. All surgical procedures and behavioral tests were conducted according to a protocol approved by the Duke University Animal Care and Use Committee.

Behavioral Testing. Two weeks after viral injections, all animals were monitored individually for 84 consecutive h in CLAMS (Columbus Instruments). Motor activity and feeding and drinking behaviors as well as O_2 intake and CO_2 output were monitored with Oxymax software, and the RER was calculated as described (40, 41). The data from the individual parameters were examined across light and dark cycles and then were grouped into two 6-h blocks: 1800–2400 h (the first peak response period) and 0300–0900 h (the second peak response period). The results are presented as means ± SE for the different block-hours. The results for feeding, drinking, and motor activity are shown as cumulative means; RER and heat are average values. After completion of the CLAMS study, mice were killed by decapitation, and the brain was removed for biochemical assays.

Effect of BigLEN Antibodies on Feeding. Purified antibodies to BigLEN or normal rabbit IgG (5 μg/2 μL) were infused at a rate of 0.5 μL/min into the third ventricle of cannulated mice that were fasted for 12 h (during lights out), and the amount of food consumed during at various time points postinjection was measured as described (8). Previously, it had been found that BigLEN antibodies blocked feeding, whereas administration of BigLEN peptide was without effect, presumably because of the instability of the peptide in brain for the duration of the feeding study (8).

Statistics. For the analysis of the in vitro data, GraphPad Prism 4.0 was used; statistical significance was determined by *t* test or one-way ANOVA. For the analyses of CLAMS data, SPSS 20 (IBM) was used. Food intake, water consumption, motor activity, RER, and heat production were analyzed with repeated-measures ANOVA, with time as the within-subject repeated measure and treatment condition as the between-subject measure. Bonferroni corrected pair-wise comparisons were used as the post hoc test. In all cases, $P < 0.05$ was considered statistically significant. No animals were excluded from the data analysis. Details of the statistical analyses are given in *SI Methods*.

ACKNOWLEDGMENTS. We thank Miss Maribel Lim for help with RT-PCR analysis; Mr. Christian Elms for support with mouse husbandry; Mr. Theodore Rhodes for the open-field testing and for assistance with the CLAMS studies; and Drs. Raphael Rozenfeld, Sheila Collins, Emmeline Maillet, and Mariana Max for reading and providing suggestions about this manuscript. This work was supported in part by National Institutes of Health Grants NS026880 and DA019521 (to L.A.D.), DA004494 (to L.D.F.), and DA08622 (to J.E.P.), by the New Jersey Governor's Council on Autism (J.E.P.), and by Professional Staff Congress-City University of New York Award 62150-00 40 (to S.K.).

1. Luquet S, Perez FA, Hnasko TS, Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310(5748):683–685.
2. Krashes MJ, et al. (2011) Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J Clin Invest* 121(4):1424–1428.
3. Qian S, et al. (2002) Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. *Mol Cell Biol* 22(14): 5027–5035.
4. Flier JS (2006) AgRP in energy balance: Will the real AgRP please stand up? *Cell Metab* 3(2):83–85.
5. Fricker LD, et al. (2000) Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. *J Neurosci* 20(2):639–648.
6. Fricker LD (2012) *Neuropeptides and Other Bioactive Peptides: From Discovery to Function* (Morgan & Claypool Life Sciences, Princeton, NJ) pp 1–109.
7. Mzhavia N, Berman Y, Che FY, Fricker LD, Devi LA (2001) ProSAAS processing in mouse brain and pituitary. *J Biol Chem* 276(9):6207–6213.
8. Wardman JH, Berezniuk I, Di S, Tasker JG, Fricker LD (2011) ProSAAS-derived peptides are colocalized with neuropeptide Y and function as neuropeptides in the regulation of food intake. *PLoS ONE* 6(12):e28152.
9. Hatcher NG, et al. (2008) Mass spectrometry-based discovery of circadian peptides. *Proc Natl Acad Sci USA* 105(34):12527–12532.
10. Wei S, et al. (2004) Obesity and diabetes in transgenic mice expressing proSAAS. *J Endocrinol* 180(3):357–368.
11. Morgan DJ, et al. (2010) The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *J Neurochem* 113(5):1275–1284.
12. He JC, et al. (2005) The G α(o/i)-coupled cannabinoid receptor-mediated neurite outgrowth involves Rap regulation of Src and Stat3. *J Biol Chem* 280(39):33426–33434.
13. Rios C, Gomes I, Devi LA (2006) mu opioid and CB1 cannabinoid receptor interactions: Reciprocal inhibition of receptor signaling and neuritogenesis. *Br J Pharmacol* 148(4): 387–395.
14. Fricker AD, Rios C, Devi LA, Gomes I (2005) Serotonin receptor activation leads to neurite outgrowth and neuronal survival. *Brain Res Mol Brain Res* 138(2):228–235.
15. Jordan JD, et al. (2005) Cannabinoid receptor-induced neurite outgrowth is mediated by Rap1 activation through G(alpha)o/i-triggered proteasomal degradation of Rap1GAPII. *J Biol Chem* 280(12):11413–11421.
16. Regard JB, Sato IT, Coughlin SR (2008) Anatomical profiling of G protein-coupled receptor expression. *Cell* 135(3):561–571.
17. Bromberg KD, Ma'ayan A, Neves SR, Iyengar R (2008) Design logic of a cannabinoid receptor signaling network that triggers neurite outgrowth. *Science* 320(5878): 903–909.
18. Lein ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445(7124):168–176.
19. Wittenberger T, Schaller HC, Hellebrand S (2001) An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. *J Mol Biol* 307(3):799–813.
20. Gonzalez NS, Communi D, Hannedouche S, Boeynaems JM (2004) The fate of P2Y-related orphan receptors: GPR80/99 and GPR91 are receptors of dicarboxylic acids. *Purinergic Signal* 1(1):17–20.
21. Rossi L, Lemoli RM, Goodell MA (2013) Gpr171, a putative P2Y-like receptor, negatively regulates myeloid differentiation in murine hematopoietic progenitors. *Exp Hematol* 41(1):102–112.
22. Sugiyama T, Ohgami S, Nakayama D (2010) Novel lysophosphatidylserine receptor. Int Patent App No. PCT/JP2009/066348.
23. Heintz N (2004) Gene expression nervous system atlas (GENSAT). *Nat Neurosci* 7(5):483.
24. Clarke S, Zimmer A, Zimmer AM, Hill RG, Kitchen I (2003) Region selective up-regulation of micro-, δ- and κ-opioid receptors but not opioid receptor-like 1 receptors in the brains of enkephalin and dynorphin knockout mice. *Neuroscience* 122(2):479–489.
25. Livesey G, Elia M (1988) Estimation of energy expenditure, net carbohydrate utilization, and net fat oxidation and synthesis by indirect calorimetry: Evaluation of errors with special reference to the detailed composition of fuels. *Am J Clin Nutr* 47(4): 608–628.
26. McGuinness OP, Ayala JE, Laughlin MR, Wasserman DH (2009) NIH experiment in centralized mouse phenotyping: The Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse. *Am J Physiol Endocrinol Metab* 297(4):E849–E855.
27. Arora S, Anubhuti (2006) Role of neuropeptides in appetite regulation and obesity—a review. *Neuropeptides* 40(6):375–401.
28. Hillebrand JJG, de Wied D, Adan RAH (2002) Neuropeptides, food intake and body weight regulation: A hypothalamic focus. *Peptides* 23(12):2283–2306.
29. Dietrich MO, et al. (2012) AgRP neurons regulate development of dopamine neuronal plasticity and nonfood-associated behaviors. *Nat Neurosci* 15(8):1108–1110.
30. Maury E, Ramsey KM, Bass J (2010) Circadian rhythms and metabolic syndrome: From experimental genetics to human disease. *Circ Res* 106(3):447–462.
31. Gomes I, Ijzerman AP, Ye K, Maillet EL, Devi LA (2011) G protein-coupled receptor heteromerization: A role in allosteric modulation of ligand binding. *Mol Pharmacol* 79(6):1044–1052.
32. Rozenfeld R, et al. (2011) AT1R-CB_1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II. *EMBO J* 30(12):2350–2363.
33. Gupta A, et al. (2010) Increased abundance of opioid receptor heteromers after chronic morphine administration. *Sci Signal* 3(131):ra54.
34. Heimann AS, et al. (2007) Hemopressin is an inverse agonist of CB1 cannabinoid receptors. *Proc Natl Acad Sci USA* 104(51):20588–20593.
35. Gupta A, et al. (2007) Conformation state-sensitive antibodies to G-protein-coupled receptors. *J Biol Chem* 282(8):5116–5124.
36. Trapaidze N, Gomes I, Cvejic S, Bansinath M, Devi LA (2000) Opioid receptor endocytosis and activation of MAP kinase pathway. *Brain Res Mol Brain Res* 76(2):220–228.
37. Gomes I, Filipovska J, Devi LA (2003) Opioid receptor oligomerization. Detection and functional characterization of interacting receptors. *Methods Mol Med* 84:157–183.
38. Gomes I, et al. (2009) Novel endogenous peptide agonists of cannabinoid receptors. *FASEB J* 23(9):3020–3029.
39. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30(9):e36.
40. Cawley NX, et al. (2004) The carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficits. *Endocrinology* 145(12):5807–5819.
41. Thupari JN, Landree LE, Ronnett GV, Kuhajda FP (2002) C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. *Proc Natl Acad Sci USA* 99(14):9498–9502.