Published in final edited form as:

Sci Signal. ; 9(430): ra55. doi:10.1126/scisignal.aac8035.

Identification of a small-molecule ligand that activates the neuropeptide receptor GPR171 and increases food intake

Jonathan H. Wardman^{1,*†}, Ivone Gomes^{1,*}, Erin N. Bobeck^{1,*}, Jennifer A. Stockert¹, Abhijeet Kapoor², Paola Bisignano², Achla Gupta¹, Mihaly Mezei², Sanjai Kumar³, Marta Filizola², and Lakshmi A. Devi^{1,‡}

¹Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

²Department of Structural and Chemical Biology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

³Department of Chemistry and Biochemistry, Queens College, Flushing, NY 11367, USA

Abstract

Several neuropeptide systems in the hypothalamus, including neuropeptide Y and agouti-related protein (AgRP), control food intake. Peptides derived from proSAAS, a precursor implicated in the regulation of body weight, also control food intake. GPR171 is a heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) for BigLEN (b-LEN), a peptide derived from proSAAS. To facilitate studies exploring the physiological role of GPR171, we sought to identify small-molecule ligands for this receptor by performing a virtual screen of a compound library for interaction with a homology model of GPR171. We identified MS0015203 as an agonist of GPR171 and demonstrated the selectivity of MS0015203 for GPR171 by testing the binding of this compound to 80 other membrane proteins, including family A GPCRs.

Permissions Obtain information about reproducing this article: <http://www.sciencemag.org/about/permissions.dtl>

[†]Corresponding author. lakshmi.devi@mssm.edu.

^{*}These authors contributed equally to this work.

[‡]Present address: Department of Neurology, Rigshospitalet, 1429 Copenhagen, Denmark.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/9/430/ra55/DC1

Fig. S1. Binding of b-LEN-derived peptides and ligands of P2Y₁₂ or P2Y₁₄ to GPR171.

Fig. S2. Sequence alignment between mouse GPR171 and P2Y₁₂.

Fig. S3. Homology model of GPR171 superimposed with the P2Y₁₂ crystal structure.

Fig. S4. Surface receptor abundance of mutant GPR171 constructs expressed in CHO cells.

Fig. S5. Selectivity profile MS0015203.

Fig. S6. Effect of acute administration of MS0015203 on food intake in mice.

Fig. S7. Effect of GPR171 knockdown on receptor abundance in hypothalamus.

Table S1. Structures of compounds representative of each cluster.

Table S2. Displacement of radiolabeled b-LEN by a compound with chemical similarity to MS0015203.

Author contributions: L.A.D., I.G., and M.F. designed the research; J.H.W., I.G., E.N.B., A.G., J.A.S., A.K., P.B., and M.M. performed the research; J.H.W., I.G., L.A.D., and E.N.B. analyzed the data; S.K. supplied the reagents; L.A.D., I.G., and M.F. wrote the paper.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: The in-house chemical library is available at the Experimental Therapeutics Institute at Icahn School of Medicine at Mount Sinai. The homology model can be obtained from M. Filizola (Department of Structural and Chemical Biology, Icahn School of Medicine at Mount Sinai).

Reducing the expression of *GPR171* by shRNA (short hairpin RNA)–mediated knockdown blunted the cellular and tissue response to MS0015203. Peripheral injection of MS0015203 into mice increased food intake and body weight, and these responses were significantly attenuated in mice with decreased expression of *GPR171* in the hypothalamus. Together, these results suggest that MS0015203 is a useful tool to probe the pharmacological and functional properties of GPR171 and that ligands targeting GPR171 may eventually lead to therapeutics for food-related disorders.

INTRODUCTION

Neuropeptides of the neuroendocrine system have diverse roles in translating information from the periphery to the central nervous system (CNS) and modulating sensory and behavioral responses ranging from pain perception to reward processing. Furthermore, neuropeptides have roles in anxiety and depression. Neuropeptides derived from proSAAS are abundant in the brain (1). The distribution of proSAAS and proSAAS-derived peptides in the brain is consistent with neuroendocrine function. They are abundant in areas of the brain involved in feeding, such as the arcuate nucleus of the hypothalamus (2), and transgenic overexpression of proSAAS increases body weight in mice (3), whereas knockdown of proSAAS leads to a small, but statistically significant, decrease in body weight (4).

In mice, intracerebroventricular administration of antibodies to BigLen (b-LEN), a proSAAS-derived peptide, decreased food intake, suggesting an involvement of this peptide in regulation of feeding (5). Electrophysiological studies show that b-LEN induces a rapid, reversible inhibition of inhibitory glutamatergic postsynaptic currents in parvocellular neurons of the paraventricular nucleus (PVN) (5). Moreover, inhibition of post-synaptic heterotrimeric guanine nucleotide–binding protein (G protein) activity blocked b-LEN–mediated inhibition of glutamate release (5), suggesting that this peptide signals through a postsynaptic G protein–coupled receptor (GPCR). The orphan receptor GPR171 is the b-LEN receptor in the hypothalamus (6), and reducing the abundance of GPR171 in the hypothalamus by intracerebroventricular administration of lentiviral short hairpin RNA (shRNA) decreases food intake (6).

In the hypothalamus, b-LEN colocalizes with neuropeptide Y (NPY) in the arcuate nucleus (5). NPY-positive neurons in this brain region are also positive for agouti-related protein (AgRP) (7), and these neurons project to the PVN of the hypothalamus to regulate feeding behaviors (7, 8). Activation of these neurons by optogenetics or designer receptor exclusively activated by designer drugs (DREADD) increases food intake in mice (9–12). Because activation of NPY/AgRP neurons may lead to the release of b-LEN, we hypothesized that b-LEN binding to GPR171 in the PVN contributes to the regulation of feeding behaviors.

To explore the role of GPR171 in the regulation of feeding, we sought to identify small-molecule nonpeptide ligands (agonists and antagonists) for the receptor. We identified and characterized one such small-molecule agonist of GPR171, and we used this molecule to explore the pharmacological properties of GPR171 and the role for this receptor in feeding and control of body weight in mice.

RESULTS

Identifying the minimal peptide that binds GPR171

GPR171 is a class A GPCR that, on the basis of sequence homology, is most closely related to the purinergic receptors P2Y₁₂ and P2Y₁₄ (13, 14). This family of purinergic receptors is activated by various molecules including nucleotides, dicarboxylic acids, lipids, and peptides (15). Because we previously found that GPR171 is activated by a relatively long (16-amino acid) peptide (6), we sought to identify the minimal peptide required to activate the receptor. We used N-terminally truncated b-LEN– derived peptides to evaluate the minimal sequence that displaced radio-labeled b-LEN binding. The C-terminal four amino acids (LLPP, present in both the mouse and rat b-LEN sequences) were sufficient to displace b-LEN from the receptor (fig. S1A). Because this sequence is so small, small-molecule ligands may exist that can affect receptor activity.

Because GPR171 is most closely related to P2Y₁₂ and P2Y₁₄ (13), we tested whether P2Y ligands [including 2-methylthioadenosine 5′-diphosphate (2MeSADP), adenosine 5′-diphosphate (ADP), and adenosine 5′-O-2-thiodiphosphate (ADPβS), which bind to P2Y₁₂, and uridine 5′-diphosphate (UDP), UDP-galactose, and UDP-glucose, which bind to P2Y₁₄] displaced receptor-bound radiolabeled b-LEN from rat hypothalamic membranes. In addition, we tested the ability of dicarboxylic acids to displace receptor-bound radiolabeled b-LEN because of the structural similarity between dicarboxylic and P2Y receptors (16). None of the P2Y or dicarboxylic acid receptor ligands displaced b-LEN when used at concentrations of 10 μM (fig. S1B). These data suggested that differences exist in the ligand-binding pockets of GPR171 and the related receptors P2Y₁₂ and P2Y₁₄.

Homology modeling of mouse GPR171 and in silico screening of compounds

To identify small-molecule ligands for GPR171, we generated a homology model of the receptor based on the crystal structure of the agonist-bound P2Y₁₂ [Protein Data Bank (PDB) ID: 4PXZ (17)]. The model does not include the N and C termini of the receptor because no atomic coordinates for these regions are available from the crystal structure of P2Y₁₂, and small molecules do not bind at these sites in all available crystal structures of purinergic receptors (17–19). Inspection of the aligned sequences of mouse P2Y₁₂ and GPR171 with the model revealed several residues that are identical in the ligand-binding pockets (fig. S2). However, we also observed a few differences. In particular, the crystal structure of the agonist-bound P2Y₁₂ has the ligand embedded into the receptor's transmembrane bundle, interacting with 13 residues within 3.5 Å from the ligand. An overlap of this P2Y₁₂ structure with the mouse GPR171 homology model (fig. S3A) reveals that 3 of the 13 residues in the agonist-binding pocket of P2Y₁₂ [specifically S101^{3,29}, V102^{3,30}, and V190^{5,39}; superscripts refer to the Ballesteros-Weinstein generic numbering scheme for class A GPCRs (20)] differ from those of GPR171 (A91^{3,29}, C92^{3,30}, and T180^{5,39}). This observation may explain, in part, why none of the tested P2Y ligands, which bind P2Y₁₂, displaced b-LEN (fig. S1B).

We used the Molsoft ICM-Pro software (21, 22) and the GPR171 model to virtually dock 10,526 small molecules from an in-house library of chemical compounds to which we added

4470 tautomers and isomers of these compounds. The 5842 top-scoring compounds (energy score below -15 kcal/mol) were then clustered in 502 groups (table S1) on the basis of their chemical similarity (Tanimoto distance cutoff was set to 0.5). From the 236 groups with 4 or more molecules, we selected 13 molecules that formed interactions with one or more conserved residues between P2Y₁₂ and GPR171 within the identified binding pocket region and that were available through the in-house facility. These molecules—MS0012092, MS0014045, MS0015203, MS0015917, MS0014696, MS0014904, MS0017635, MS0021570_1, MS0022752, MS0020337, MS0021049, MS00123522, and MS0012102—were selected from groups containing 80, 49, 275, 103, 33, 13, 18, 4, 6, 165, 9, 7, and 12 compounds, respectively (table S1, highlighted in pink).

To assess the ability of the 13 selected small molecules to bind and activate GPR171, we applied the molecules to cells heterologously co-expressing GPR171 and a chimeric Gα subunit containing sequences of Gα₁₅ and Gα_{i3}, which, when activated, stimulates release of Ca²⁺ from intracellular stores (6), and measured intracellular Ca²⁺ signals with a fluorescent Ca²⁺ indicator. In these assays, adenosine 5'-triphosphate (ATP) served as a positive control, because the cells have endogenous purinergic receptors that respond to ATP; LittleLEN (l-LEN) served as a negative control because GPR171 does not respond to l-LEN as shown previously (6). Among the 13 molecules tested, only MS0015203 produced a response that was comparable to that of b-LEN (Fig. 1A), the known endogenous ligand for GPR171.

Compared with the binding of 2MeSADP to P2Y₁₂ in the crystal structure, the interaction of MS0015203 with the GPR171 model was predicted to make several different contacts with the receptor (fig. S3B). Whereas 2MeSADP contacts R93^{3.21}, S101^{3.29}, V102^{3.30}, and Y105^{3.33} in transmembrane domain 3 (TM3); N159^{4.60} in TM4; H187^{5.36}, V190^{5.39}, and N191^{5.40} in TM5; R256^{6.55}, Y259^{6.58}, and Q263^{6.62} in TM6; K280^{7.35} in TM7; and K179 in extracellular loop 2 (fig. S3A, right), MS0015203 is predicted to make hydrogen bonds with Y99^{3.37} in TM3, H177^{5.36} and C184^{5.43} in TM5, and Y239^{6.51} and R243^{6.55} in TM6, as well as K169 in extracellular loop 2 (Fig. 1B).

Evaluation of the homology model and specificity of MS0015203 for GPR171

To provide an initial assessment of the mode of action of MS0015203 at GPR171, we generated alanine mutants of GPR171 residues predicted to be involved in hydrogen bonding with MS0015203 (Fig. 1B) and stably expressed these mutant receptors tagged with the sequence DYKDDDDK in Chinese hamster ovary (CHO) cells. We confirmed that the abundance of the mutant receptors at the cell surface in the population of stably expressing cells was similar (fig. S4). We individually mutated Y99^{3.37} in TM3, K169 in extracellular loop 2, H177^{5.36} and C184^{5.43} in TM5, and Y239^{6.51} and R243^{6.55} in TM6 to Ala and assessed b-LEN or MS0015203 binding to the mutants by competition assay (Table 1). The increase in the concentration required for 50% displacement of radio-labeled b-LEN by unlabeled b-LEN (IC₅₀) as observed for the Y99A, C184A, Y239A, and R243A mutants indicated a decrease in the affinity of b-LEN for these mutant receptors. However, all of the mutants were activated by b-LEN and b-LEN reduced adenosine 3',5'-monophosphate (cAMP) production in cells expressing the receptors (Table 1).

Unlike b-LEN, which effectively displaced itself at sufficiently high concentrations (10 μ M), MS0015203 binding to each of the mutant receptors except C184A was reduced such that for most of the mutants, MS0015203 displaced less than ~50% of b-LEN (Fig. 1C and Table 1). This reduction in binding correlated with a reduction in MS0015203-induced signaling in the cells expressing these mutants (Table 1).

We also tested the only additional compound (MS0016574) available for purchase and that was in the same cluster with and exhibited the highest chemical similarity to MS0015203 (Tanimoto distance larger than 0.5), yet had different substituents attached to the phenyl ring (table S2). Despite its apparently higher affinity for GPR171, this compound was less effective at displacing b-LEN from GPR171 (only displacing ~40% bound b-LEN, compared with ~90% displacement by MS0015203). These results suggested that substituents to the phenyl ring play an important role in defining the binding characteristics of MS0015203 to GPR171.

To assess the selectivity of MS0015203 for GPR171, we screened the ligand against a panel of 80 different transmembrane and soluble receptors, including ~70 GPCRs (fig. S5). At 10 μ M, MS0015203 failed to displace >20% of a target-specific radiolabeled ligand from all targets except galanin 1 receptor (Gal1R) (fig. S5A). The Gal1R is a GPCR that activates G_{a_i} proteins and plays a role in a number of physiological processes, including food intake and appetite. Because MS0015203 displaced $22 \pm 1\%$ radiolabeled ligand binding to Gal1R, we examined the effect of MS0015203 on signaling in cells expressing tagged mouse Gal1R. Whereas the Gal1R agonist M617 caused a dose-dependent decrease in forskolin-stimulated cAMP (EC_{50} , $\sim 4 \pm 1$ nM), MS0015203 did not. However, MS0015203 at high concentrations attenuated the Gal1R agonist-mediated decrease in cAMP (by ~17%) (fig. S5B). Together, these results suggested that MS0015203 did not activate Gal1R and, at very high doses, may exhibit some antagonistic activity.

We also examined MS0015203-mediated signaling in cells expressing tagged mouse P2Y₁₂. Whereas the P2Y agonist 2MeSADP caused a dose-dependent decrease in cAMP (EC_{50} , $\sim 3 \pm 2$ nM), MS0015203 produced a modest decrease in forskolin-stimulated cAMP at only high concentrations ($EC_{50} > 10$ μ M) and had no effect on the 2MeSADP-mediated decrease in cAMP (fig. S5B). Together, these results suggest that MS0015203 is relatively selective for GPR171 and likely to be useful for the pharmacological characterization of GPR171.

Characterization of MS0015203 as an agonist for GPR171

We assessed the properties of MS0015203 using ligand binding and signaling assays in membranes from rat hypothalamus, as well as mouse Neuro2A neuroblastoma cells in which GPR171 is endogenously present. GPR171 couples to G_{a_i} proteins, and application of b-LEN to Neuro2A cells inhibits adenylyl cyclase activity (6). MS0015203 dose-dependently displaced radiolabeled b-LEN from hypothalamic membranes with an affinity that was lower than that of b-LEN (Fig. 2A). To assess the effect of this putative GPR171 agonist on receptor-associated signaling events, we assayed the effect of MS0015203 on [³⁵S]GTP γ S binding and forskolin-stimulated adenylyl cyclase activity in hypothalamic membranes. MS0015203 dose-dependently increased [³⁵S]GTP γ S binding and inhibited adenylyl cyclase activity in rat hypothalamic membranes, although with a reduced potency and

efficacy compared to b-LEN (Fig. 2, B and C), consistent with MS0015203 functioning as a partial agonist of GPR171.

To examine the specificity of MS0015203 for GPR171, we used Neuro2A cells with reduced GPR171 abundance achieved through lentiviral shRNA-mediated stable knockdown, as described in (6). In wild-type Neuro2A cells, both b-LEN and MS0015203 induced a dose-dependent decrease in cAMP, indicating inhibition of adenylyl cyclase activity, and this response was absent in the Neuro2A cells in which GPR171 was knocked down (Fig. 2D). Neuro2A cells have many family A GPCRs (6, 23–26); however, the results with the GPR171-knockdown cells suggested that MS0015203 is a selective partial agonist of GPR171.

Effects of MS0015203 on neuronal activation in the hypothalamus and on feeding

Previous immunohistochemical analysis showed that GPR171 is present in the PVN of the hypothalamus and colocalized with its ligand b-LEN in a subset of cells (6, 27), consistent with a role for GPR171 in feeding. We monitored the effect of MS0015203 on c-Fos abundance as an indication of increased neuronal activity in the PVN 30 min after intraperitoneal injection of MS0015203 in mice (Fig. 3, A and B). Compared to vehicle-injected animals, MS0015203 caused a significant increase in the total number of c-Fos-positive cells (Fig. 3B), which corresponded to ~30% of total cells (Fig. 3C). In addition, when comparing a subset of GPR171-positive and GPR171-negative cells, we found that c-Fos activation occurred with a higher frequency in GPR171-positive cells after MS0015203 administration (Fig. 3D). These results indicate that MS0015203, or its metabolite, caused an increase in neuronal activity within cells containing GPR171 in the PVN.

To investigate the functional implication of these findings, we examined the effect of MS0015203 on food intake in mice that were fasted overnight and given a single intraperitoneal injection of either vehicle or MS0015203 at the beginning of the light phase of the circadian cycle. We measured food intake for an 8-hour period. MS0015203 administration significantly increased cumulative food intake at 2, 4, and 8 hours after injection compared to that of vehicle-injected control animals (Fig. 4A) and increased food intake monitored hourly as well (fig. S6), consistent with the proposed orexigenic role of the b-LEN/GPR171 system. To assess whether this effect of MS0015203 required GPR171, we administered MS0015203 to mice with shRNA-mediated knockdown of GPR171 in the hypothalamus and evaluated food intake. Whereas a single intraperitoneal administration of MS0015203 significantly increased food intake after 8 hours in mice with control lentiviral shRNA, MS0015203 did not increase food intake in mice with GPR171-targeted lentiviral shRNA knockdown (Fig. 4B). We confirmed that MS0015203 acted within the CNS by showing that a single intracerebroventricular injection of MS0015203 significantly increased food intake at 4 and 8 hours after injection, an effect not observed in mice expressing GPR171-targeted shRNA (Fig. 4C). We confirmed that the GPR171-targeted shRNA mice induced a significant decrease in *GPR171* mRNA in the hypothalamus, as determined by quantitative realtime polymerase chain reaction (PCR) (Fig. 4D) and Western blot analysis (fig. S7). Thus, these data indicated that systemic or central administration of MS0015203 increases feeding through a mechanism involving GPR171.

Analysis of the effect of long-term treatment with MS0015203 on body weight and neuropeptide and neuropeptide mRNA abundance

To examine the effects of long-term activation of GPR171, we administered MS0015203 (2.5 mg/kg) to mice by intraperitoneal injection every third day and fed a high-fat diet to exacerbate the effects of the compound. This diet and drug treatment led to a significantly higher weight gain in the mice receiving MS0015203 compared to their vehicle-treated counterparts (Fig. 5A). Knockdown of GPR171 in the hypothalamus significantly attenuated MS0015203-induced weight gain (Fig. 5B), implying that chronic administration of MS0015203 affects body weight through GPR171.

To determine whether chronic administration of MS0015203 affected the hypothalamic neuropeptides and receptor systems involved in body weight regulation, we quantified mRNA abundances of several neuropeptide-encoding transcripts and relevant receptors by quantitative real-time PCR (qRT-PCR). Administration of MS0015203 for 40 days significantly increased the abundance of the mRNAs encoding proSAAS, NPY, AgRP, or orexin in the ventral hypothalamus compared to their abundances in vehicle-treated controls (Fig. 5C). This treatment also significantly increased the abundance of the mRNAs encoding the NPY receptor Y1R and the orexin receptor HCRT2 and significantly decreased that encoding the orexin receptor HCRT1 in the ventral hypothalamus (Fig. 5D). In the dorsal hypothalamus, this treatment significantly increased the abundance of mRNAs encoding NPY, AgRP, and HCRT2 while decreasing that of HCRT1 (Fig. 5, E and F). Together, these data indicated that manipulation of GPR171 activity with MS0015203 results in adaptive changes in other neuropeptide and receptor systems that regulate body weight.

DISCUSSION

A number of neuropeptides and their receptors have been implicated in the regulation of food intake and body weight. However, studies directly examining the effectiveness of these neuropeptides in the treatment of food-related disorders have been largely unsuccessful because the effects of these peptides are short-lived. Thus, investigators have searched for small drug-like molecule ligands that could be used to investigate the role of neuropeptide-receptor systems in feeding and to examine the therapeutic potential of such ligands. These ligands, both agonists and antagonists, have helped elucidate the physiological role of the targeted neuropeptide receptor. However, to date, compounds targeting the well-characterized neuropeptide receptors involved in feeding, such as the NPY and melanocortin receptors, have proven effective in biochemical assays but ineffective in clinical trials because of their adverse effects including depression-related behaviors (28–30). This has led to a search for other peptide-receptor systems that could modulate food intake and body weight.

Here, we report the identification of a small-molecule partial agonist of GPR171. Previously, we showed the involvement of this receptor and its endogenous ligand in feeding-related behaviors in mice (6). However, intracerebroventricular administration of b-LEN had no effect on food intake, whereas administration of antibodies selective to b-LEN decreased food intake (5). One of the reasons for the lack of effect of b-LEN on feeding behavior could be because the peptide is rapidly degraded after intra-cerebroventricular administration. To

study the role of GPR171 further, we sought to identify small drug-like molecules, agonists or antagonists, that would help elucidate the role of GPR171 in vivo.

We used the crystal structure of P2Y₁₂, a phylogenetically related receptor, to generate a homology model of GPR171, which we then used to virtually screen for possible ligands. With the availability of several high-resolution crystal structures of GPCRs, in silico structure-based methods have proven successful in identifying ligands with novel structures as promising chemical scaffolds for drug discovery (31). The feasibility of these computational strategies with homology models of GPCRs has also been demonstrated (32), as well as their successful application to deorphanize GPCRs (33). Here, using binding and signaling assays, we provide experimental validation that the small-molecule ligand MS0015203 identified through virtual screening at a GPR171 homology model functions as a partial agonist of this receptor.

Studies show that the arcuate nucleus of the hypothalamus plays a key role in the modulation of food intake largely through interactions between two major populations of neurons: Orexigenic neurons increase food intake and are positive for both AgRP and NPY, and anorexigenic neurons decrease food intake and are positive for both pro-opiomelanocortin and cocaine-amphetamine-regulated transcript (7). Thus, the interplay between these two neuronal populations regulates food intake. Activation of AgRP neurons in the arcuate nucleus using a DREADD approach enhances feeding and increases weight gain in mice (10). Whereas ablation of the AgRP/NPY neurons in adult animals (34) leads to starvation, selective genetic ablation of AgRP or NPY does not significantly affect body weight or food intake (35). It is likely that additional orexigenic peptides, such as b-LEN, in these neurons are involved in modulating food intake (5, 6). Consistent with this, the small-molecule ligand identified in this study, which exhibits relative selectivity for GPR171, increased feeding after either acute systemic or central administration. Our data suggested that after systemic administration, MS0015203 or its metabolites may cross the blood-brain barrier and affect GPR171 in the hypothalamus to increase food intake. This is surprising given that MS0015203 has two carboxyl (COOH) groups and such compounds typically poorly penetrate the CNS. However, MS0015203 fulfills other criteria proposed for good CNS penetration (36, 37). In addition, the observation that intraperitoneal administration of MS0015203 also increases food intake and this is not seen in animals in which GPR171 is knocked down in the hypothalamus further supports the notion that MS0015203 can reach the brain from the periphery.

Studies examining the effect of long-term activation of GPR171 on other neuropeptide-receptor systems revealed interesting differences in different regions of the hypothalamus. The increase in NPY- and AgRP-encoding mRNA after 40-day treatment with MS0015203 is consistent with the increased food intake and body weight and may indicate interaction of these peptide-receptor systems, perhaps through upstream control of orexigenic neuropeptide generation by the b-LEN–GPR171 system. These changes could explain why GPR171 manipulation resulted in phenotypic effects despite the redundancy in the neuronal network and the lack of effect of previous manipulation of a single neuropeptide-receptor system. Agonism of GPR171 has an orexigenic effect; thus, this receptor could prove a useful drug target for eating disorders, such as anorexia nervosa or bulimia. Moreover,

because chronic treatment with MS0015203 leads to increases in body weight, this would suggest that antagonism of the receptor could be effective in treating obesity. Together, our results further support the role of the b-LEN/GPR171 system as a regulator of food intake and body weight. The partial agonist MS0015203 may serve as a useful tool to evaluate the physiological role of GPR171 and as a scaffold for the generation of receptor-selective antagonists.

MATERIALS AND METHODS

Materials

Mouse *GPR171* complementary DNA was obtained from Open Biosystems and subcloned into pCMV-myc-N-terminal Epitope Tagging Mammalian Expression Vector (Stratagene) according to the manufacturer's protocol. Neuro2A, a mouse neuroblastoma cell line, and CHO cells were obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were from Corning Cellgro. F12 was from Gibco. Lipofectamine and Fluo-4 NW calcium dye were from Invitrogen. Fetal bovine serum (FBS) was from Biowest. Pierce iodination reagent was from Thermo Scientific. Na¹²⁵I (catalog #NEZ033L001MC) and [³⁵S]GTPγS (catalog #NEG030H250UC) were from PerkinElmer. Rabbit antibodies to GPR171 were from GeneTex (catalog #GTX108131). Rat antibodies to GPR171 were generated using the amino acid sequence MTNSSFFCPVY and tested for specificity using a standard protocol (38) in CHO cells alone or expressing myc-tagged mGPR171, or in Neuro2A cells alone or expressing either myc-tagged mGPR171 or GPR171 shRNA (27). Mouse c-Fos antibody (catalog #C3012) was from Santa Cruz Biotechnology. Rabbit antibody to DYKDDDDK tag (catalog #14793S) was from Cell Signaling Technology. Anti-tubulin antibodies (catalog #T5168), protease inhibitor cocktail (catalog #P2714), ATP, adenosine 5'-O-(3-thiotriphosphate) (ATPγS), 2MeSATP, uridine 5'-triphosphate (UTP), ADP, ADPβS, 2MeSADP, UDP, UDP-galactose, UDP-glucose, α-ketoglutaric acid and succinate were from Sigma-Aldrich. Anti-rabbit IRDye 800 (catalog #926-32211) and anti-mouse IRDye 680 (catalog #926-68070) secondary antibodies were from LI-COR. DYKDDDDK-tagged mouse GPR171 constructs (wild type, Y99A, K169A, H177A, C184A, Y239A, or R234A), DYKDDDDK-tagged mouse Gal1 R, and DYKDDDDK-tagged mouse P2Y₁₂ were from GenScript. MS0015203 and MS0016574 were obtained from ChemBridge.

Peptide synthesis: Tyr-b-LEN, b-LEN (LENPSPQAPARRLLPP), NPSPQAPARRLLPP, SPQAPARRLLPP, QAPARRLLPP, PARRLLPP, RRLPP (R2L2P2), RLLPP (RL2P2), LLPP (L2P2) and LP2 (LPP) were synthesized by solid-phase synthesis (39).

Homology modeling

A homology model of mouse GPR171 was built using as a template the crystal structure of the phylogenetically related P2Y₁₂ receptor (13, 40) corresponding to PDB code 4PXZ (17). The complete amino acid sequences of human P2Y₁₂ (entry Q9H244) and mouse GPR171 (entry Q8BG55) were retrieved in FASTA format from UniProt (www.uniprot.org/) (41) and uploaded to the PROMALS3D multiple sequence and structure alignment server (<http://prodata.swmed.edu/promals3d/promals3d.php>) (42) to generate an initial alignment. This

alignment was then modified according to a recently published alignment of mouse GPR171 and mouse P2Y₁₂ (14), and the N and C termini were removed. The final sequence alignment (fig. S2), which includes residues 15 to 305 of P2Y₁₂ and 6 to 292 of GPR171, was used as the input to the default “model” routine of the MODELLER version 9.13 (43). One hundred models were generated and ranked by means of their DOPE (discrete optimized protein energy) score (44), and the top-ranking model was selected for virtual screening.

In silico screening at GPR171

An in-house chemical library of 10,526 physically available small molecules, obtained by combining three individual libraries from ChemBridge—CNS-Set, DIVERSet Set, and MicroFormats—was used as an initial data set for in silico screening at GPR171. The Schrödinger Suite 2014-2 (Schrödinger LLC) was used to prepare these molecules in a ready-to-dock format using Maestro 9.8 as the graphical interface. Specifically, protonation states at pH 7.0±2 were predicted by Epik version 2.8 (Schrödinger LLC), and 3D structures were built using LigPrep version 3 and the 2005 Optimized Potentials for Liquid Simulations (OPLS) force field (45). Chiralities were determined for these 3D structures, and a total of 4470 tautomers and isomers were predicted by Epik and added to the original library of 10,526 small molecules, resulting in a total of 14,995 entries to be used for docking. Geometry optimization was achieved by Cartesian minimization in the framework of ICM-Pro version 3.8-1 (Molsoft LLC) using the Merck molecular force field (46).

The GPR171 model structure was uploaded to ICM-Pro and prepared for docking. The latter was limited to a rectangular box including a GPR171 region corresponding to the binding pocket of the crystal structure of the P2Y₁₂ receptor (4PXZ) upon superposition. Specifically, this region included a mesh corresponding to the molecular surface of the crystallo-graphic ligand (47) and all receptor residues within a range of 5 Å from this molecular surface.

Virtual screening was carried out using default parameters and the biased probability Monte Carlo (BPMC) stochastic optimizer as implemented in the ICM-Pro program. The GPR171 ligand-binding site was represented by precalculated 0.5 Å spacing potential grid maps, and a distance-dependent dielectric function was used with a dielectric constant set equal to 4.0. The number of BPMC steps to be carried out was calculated by an adaptive algorithm, and the binding energy was assessed with the standard ICM (internal coordinate mechanics) empirical scoring function (21,48,49). Binding solutions for each small molecule were clustered on the basis of their geometry (root mean square deviation between heavy atoms less than 1 Å), the poses within a range of 1 kcal/mol from the lowest energy conformation selected for rescoring, and further identification of the best docking solution. The top-scoring ligands were clustered using the hierarchical clustering method with default settings as implemented in the ICM-Pro program.

Cell culture and transfections

CHO cells were grown in F-12 medium containing glucose (1.802 g/liter) (catalog #11765-054, Gibco Life Sciences), 10% FBS, and 1× penicillin-streptomycin solution at

37°C and 10% CO₂/O₂. CHO cells were transfected with myc-tagged GPR171 along with a chimeric Ga_{15/13} protein using Lipofectamine as per the manufacturer's protocol for experiments measuring intracellular Ca²⁺ signals. CHO cells were transfected with DYKDDDDK-tagged mouse GPR171 constructs (wild type, Y99A, K169A, H177A, C184A, Y239A, or R234A), mGal1R, or P2Y₁₂ using Lipofectamine and stable cell lines generated as described in the manufacturer's protocol. Neuro2A cells were grown in DMEM containing glucose (4.5 g/liter; catalog #10-013-CV, Corning Cellgro), 10% FBS, and 1× penicillin-streptomycin solution (catalog #30-002-CI, Corning) at 37°C and 10% CO₂/O₂. Neuro2A cells were infected with control or GPR171 lentiviral shRNA according to the manufacturer's protocol (Sigma-Aldrich). Neuro2A cells stably expressing GPR171 lentiviral shRNA were generated according to the manufacturer's protocol (Sigma-Aldrich) (6).

Measurement of intracellular Ca²⁺ signals

These assays were carried out as described previously (6,50,51). Briefly, CHO cells expressing myc-tagged mouse GPR171 along with chimeric Ga_{15/13} were plated into black poly-L-lysine-coated, 96-well clear-bottom plates (40,000 cells per well). On the next day, the growth medium was removed and cells were washed twice in Hanks' balanced salt solution (HBSS) containing 20 mM Hepes buffer. Cells were incubated with Fluo-4 NW calcium dye (3 μM in 100 ml of HBSS buffer containing protease inhibitor cocktail) for 1 hour at 37°C and treated with buffer or with 10 μM of the 13 compounds from the screening library, l-LEN, b-LEN, or ATP. Increases in intracellular Ca²⁺ were measured for ~210 s at 494-nm excitation and 516-nm emission. Buffer conditions were set to 100%.

Profile of selectivity of MS0015203

The binding profile of MS0015203 (10 μM) was screened using a panel of 80 transmembrane and soluble receptors, as well as ion channels and monoamine transporters by Eurofins (Lead ProfilingScreen 2, Eurofins Cerep Panlabs; Ref. P3).

Membrane preparation

Membranes from rat hypothalamus were prepared as described previously (6, 52). Briefly, rat hypothalami were homogenized in 25 volumes (1 g wet weight/25 ml) of ice-cold 20 mM tris-Cl buffer (pH 7.4) containing 250 mM sucrose, 2 mM EGTA, and 1 mM MgCl₂, followed by centrifugation at 27,000g for 15 min at 4°C. The pellet was resuspended in 25 ml of the same buffer and the centrifugation step repeated. The resulting membrane pellet was resuspended in 40 volumes (of original wet weight) of 2 mM tris-Cl buffer (pH 7.4) containing 2 mM EGTA and 10% glycerol. The protein content of the homogenates was determined using the Pierce BCA protein assay reagent, after which the homogenates were stored in aliquots at -80°C until use.

Determination of surface receptor abundance

Enzyme-linked immunosorbent assay (ELISA) was carried out to determine cell surface receptor abundance in CHO cells stably expressing DYKDDDDK-tagged mouse GPR171 constructs (wild type, Y99A, K169A, H177A, C184A, Y239A, or R234A), mouse Gal1R, or

mouse P2Y₁₂. Briefly, cells (2×10^5 cells per well) were seeded into 24-well plates. The next day, cells were labeled with rabbit anti-DYKDDDDK antibodies [1:1000 in phosphate-buffered saline (PBS) containing 1% FBS] for 1 hour at 4°C and then washed three times with PBS containing 1% FBS, followed by incubation with anti-rabbit antibody coupled to horseradish peroxidase (1:2000 in PBS containing 1% FBS) for 2 hours at 4°C. Cells were washed three times with 1% FBS in PBS (5 min each wash), and color was developed by addition of the substrate *o*-phenylenediamine [5 mg per 10 ml in 0.15 M citrate buffer (pH 5) containing 15 μ l of H₂O₂]. Absorbance at 490 nm was measured with a Bio-Rad ELISA reader. Nonspecific signal was determined using cells that were not incubated with the primary antibody.

Binding assays

Tyr-b-LEN (200 μ g) was iodinated using [¹²⁵I] and Pierce iodination reagent as described in the manufacturer's protocol (Thermo Scientific). The specific activity of the iodinated peptide was 65.3 Ci/mmol at the time of iodination (the radiolabeled peptide was used within 60 days of iodination). Binding assays were carried out using membranes (100 μ g) from rat hypothalamus or CHO cells (2×10^5 cells) expressing either DYKDDDDK-tagged mouse GPR171 constructs (wild type, Y99A, K169A, H177A, C184A, Y239A, or R234A) using Hepes-buffered HBSS containing protease inhibitor cocktail (Sigma-Aldrich) as described previously (6, 52, 53). Briefly, displacement binding assays were carried out for 1 hour at 37°C using 3 nM of [¹²⁵I]Tyr-b-LEN and different concentrations (0 to 10 μ M) of b-LEN (LENPSPQAPARLLPP), NPSPQAPARLLPP, SPQAPARLLPP, QA-PARLLPP, PARLLPP, RRLPP, RLLPP, LLPP (L2P2), LPP, or MS0015203 or a single concentration (10 μ M) of either b-LEN, ATP, ATP γ S, 2MeSATP, UTP, ADP, ADP β S, 2MeSADP, UDP, UDP-galactose, UDP-glucose, α -ketoglutaric acid, or succinate. At the end of the incubation period, samples were filtered using a Brandel filtration system and GF/B filters. Filters were washed three times with 3 ml of ice-cold 50 mM tris-Cl (pH 7.4), and bound radioactivity was measured using a scintillation counter.

[³⁵S]GTP γ S binding

[³⁵S]GTP γ S binding assays were carried out as described previously (6, 51, 53) using 50 mM Hepes (pH 7.4) containing 5 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin (BSA), and a protease inhibitor cocktail. Briefly, membranes from rat hypothalamus (10 μ g) were incubated for 1 hour at 30°C with b-LEN or MS0015203 (final concentration, 0 to 10 μ M) in the presence of 2 mM guanosine diphosphate (GDP) and 0.5 nM [³⁵S]GTP γ S. Nonspecific binding was determined in the presence of 10 μ M cold [³⁵S]GTP γ S. Basal values represent values obtained in the presence of GDP and the absence of cold ligand. At the end of the incubation period, samples were filtered using a Brandel filtration system and GF/B filters. Filters were washed three times with 3 ml of ice-cold 50 mM tris-Cl (pH 7.4), and bound radioactivity was measured using a scintillation counter.

Determination of cAMP

Rat hypothalamic membranes (10 μ g), CHO cells (10,000 cells per well) expressing either DYKDDDDK-tagged mGPR171 constructs (wild type, Y99^{3,37}A, K169A, H177^{5,36}A,

C184^{5.43}A, Y239^{6.51}A, and R243^{6.55}A) mGal1R or mP2Y₁₂, Neuro2A cells, or Neuro2A cells stably expressing GPR171 lentiviral shRNA (10,000 cells per well) were pretreated for 30 min with 20 μ M forskolin, followed by treatment with either b-LEN or MS0015203 (0 to 10 μ M) in the presence of 1 $\times$ protease inhibitor cocktail for 30 min. cAMP levels were quantified using the HitHunter cAMP detection kit for membranes or cells from DiscoverX according to the manufacturer's protocol.

Animals

Male C57BL/6J mice 10 to 12 weeks of age (The Jackson Laboratory) were used in these studies. Mice were housed individually in a humidity-and temperature-controlled room with a 12-hour light/dark cycle (lights on: 0700 to 1900) and given free access to either RLD-5001 diet (LabDiet) or a high-fat diet (RLD D12451, LabDiet) and water throughout the experiment. All surgical and behavioral procedures were conducted according to ethical guidelines/regulations approved by the Icahn School of Medicine Animal Care and Use Committee.

Acute effects of MS0015203 on food intake

MS0015203 (3 mg/kg, intraperitoneally) was dissolved in 6% DMSO in sterile saline (0.9% NaCl). MS0015203 or vehicle was injected intra-peritoneally to mice that were fasted for 12 hours. Mice were then returned to their individual home cages, and the amount of food consumed at 1, 2, 4, or 8 hours later was measured. These studies were also carried out with mice expressing either control or GPR171 lentiviral shRNA in the ventral hypothalamus using a high-fat diet. For lentiviral-mediated knockdown of GPR171 in the ventral hypothalamus, mice were infused bilaterally [anteroposterior (AP): -1.82 mm, dorsoventral (DV): -6 mm, mediolateral (ML): ± 0.2 mm from bregma) (54) over 5 min with 1 μ l of control or GPR171 lentiviral shRNA per side (2 μ l of total lentivirus). Mice were allowed to recover for 2 weeks before subsequent injections of ligands.

In another set of studies, either 2 μ l of vehicle (2% DMSO in saline) or MS0015203 (2.5 mg/kg body weight) was administered intracerebroventricularly over 4 min (0.5 μ l/min) to wild-type mice or to mice expressing either control or GPR171 lentiviral shRNA in the ventral hypothalamus; these mice were fasted for 12 hours before drug administration. After injection, the obturator was replaced, the mouse was returned to his home cage, and food (normal chow) was provided. The amount of food consumed was measured at 1, 2, 4, or 8 hours after injection. For intracerebro-ventricular drug administration, mice were anesthetized with ketamine and xylazine and were cannulated as described (5). Mice were allowed to heal for 7 to 10 days before injections were performed. Mice were initially injected with 50 ng of angiotensin II in 2 μ l of sterile saline, and drinking response was observed to verify cannulation into the third ventricle. All mice showed a marked stimulation of water consumption after the angiotensin II injection.

Chronic effects of MS0015203 on food intake

MS0015203 (2.5 mg/kg per mouse) was dissolved in 200 μ l of sterile saline (0.9% NaCl) with 0.02% DMSO. Injections of 200 μ l of either DMSO alone or DMSO with MS0015203 were administered intraperitoneally into mice that were fasted for 12 hours (during lights

out). After injection, mice were returned to their home cages and food (high-fat diet) was provided. Injections were carried out every third day. Food intake and bodyweight were measured daily. Two cohorts of mice were treated with MS0015203 chronically in this manner, with the first cohort consisting of five vehicle-treated mice and five MS0015203-treated mice. These mice are the cohort also tested for acute food intake. The second cohort consists of 16 mice, 7 of which were injected with control lentivirus and 9 of which were injected with GPR171-knockdown shRNA virus.

Immunohistochemistry

Mice were deeply anesthetized with ketamine (100 mg/kg, intraperitoneally) and transcardially perfused through the ascending aorta with 4% paraformaldehyde (200 ml). Brains were postfixed for 1 hour and stored in 1× PBS. Immunohistochemistry was performed on free-floating coronal brain slices (50 µm) containing hypothalamus. Sections were incubated in 1% sodium borohydride in PBS for 30 min followed by blocking buffer (5% normal goat serum and 0.3% Triton X-100 in PBS) for 1 hour. Tissue was incubated overnight at 4°C in primary antibodies against b-LEN (rabbit, 1:250), GPR171 (rat, 1:100; rabbit, 1:400), or c-Fos (mouse, 1:100; Santa Cruz Biotechnology) in 1% BSA and 0.1% Triton X-100. Antibodies were visualized with donkey anti-mouse 568, donkey anti-rat 594, goat anti-rabbit 488, or goat anti-rabbit 594 (Invitrogen). After 5 min of incubation with DAPI (100 ng/ml), sections were mounted with ProLong Gold Antifade (Molecular Probes). Images were taken with a Leica DM6000 microscope at the Microscopy CORE at the Icahn School of Medicine at Mount Sinai. For studies examining the effect of MS0015203 on c-Fos activity, mice were injected twice with either vehicle (6% DMSO in 0.9% saline) or MS0015203 (3 mg/kg, intraperitoneally) before perfusion. This protocol was used to detect c-Fos activity in the brain because not much is known about the optimal time and dose of MS0015203 required to induce c-Fos activity. Sections containing PVN were quantified in a $350 \times 250 \mu\text{m}^2$ (87.5 mm^2 total area) region parallel to either side of the third ventricle within 75 µm from the most dorsal point of the ventricle. Quantification of total cells, GPR171-positive cells, and c-Fos-positive cells was performed using the ImageJ particle analysis with auto thresholds. For analysis of the number of c-Fos-positive cells that were also GPR171-positive, merged images of green (GPR171) and DAPI were used to randomly pick 40 cells per section that contained GPR171 or not. The image was then switched to the red (c-Fos) and DAPI image, and colocalization with the selected DAPI cells was determined. All analyses were performed by an experimenter blinded to the treatment conditions.

Quantitative real-time PCR

Intact or bisected hypothalamic tissue was used in these studies. First, mice treated chronically with either vehicle or MS0015203 were sacrificed by cervical dislocation, and brain regions were harvested. The intact hypothalami, dorsal (containing the PVN, the dorsomedial hypothalamus, and the upper part of the lateral hypothalamus) or ventral hypothalami (containing the arcuate nucleus, the ventromedial hypothalamus, and the lower part of the lateral hypothalamus), were individually extracted using phenol-chloroform extraction with TRIzol reagent (Life Technologies) followed by RNeasy Mini RNA preparation kit (Qiagen) according to the respective manufacturers' instructions.

Predesigned primer sets for qRT-PCR from Sigma— AgRP (M_Agrp_1), NPY (M_Npy_1), melanocortin 4 receptor (M_Mc4r_1), orexin (M_Hert_1), hypocretin receptor 1 (M_Hcrt1_2), hypocretin receptor 2 (M_Hcrt2_1), proSAAS (M_Pcsk1n_1), Y1 receptor (M_Npy1r_1), Y5 receptor (M_Npy5r_1), and GPR171 (M_GPR171_1q)—were used. qRT-PCR was carried out as described previously (6).

Western blot analysis

For experiments examining the effect of administration of control or GPR171 lentiviral shRNA to the PVN (AP: −0.9 mm; ML: ±0.2 mm; DV: −4.8 mm) on GPR171 abundance in dorsal and ventral hypothalamus, membranes were solubilized in 2% SDS in 50 mM tris-Cl (pH 6.8) containing protease and phosphatase inhibitor cocktails. Samples (~10 to 15 µg of protein) in 1× Laemmli's sample buffer containing 10% freshly added β-mercaptoethanol and 8 M urea were heated for 30 min at 65°C and subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose membranes at 30 V overnight. Membranes were blocked with 10% nonfat dried milk in tris-buffered saline-Tween 20 (TBS-T) [50 mM tris-Cl (pH 7.4) containing 150 mM NaCl, 1 mM CaCl₂, and 0.1% Tween 20]. After four washes (15 min each) with TBS-T, membranes were probed overnight at 4°C with rabbit anti-GPR171 antibody (1:1000) and mouse anti-tubulin antibodies (1:50,000) diluted in 30% Odyssey blocking buffer (catalog #927–40000, LI-COR) in TBS-T. After four washes (15 min each) with TBS-T, membranes were incubated with anti-rabbit IRDye 800 and anti-mouse IRDye 680 antibodies (1:10,000 in TBS-T containing 30% Odyssey blocking buffer) for 1 hour at 37°C. After four washes (15 min each) with TBS-T, blots were imaged and quantified using the Odyssey Imaging System (LI-COR).

Data and statistical analyses

Results are expressed as means ± SE. Data with one variable were analyzed using one-way ANOVAs, whereas data with two variables were analyzed with two-way ANOVAs followed by Tukey's multiple comparisons or Bonferroni's post hoc comparisons. Data comparing two groups were analyzed using two-tailed unpaired Student's *t* tests. Significance was set at *P* < 0.05. Statistical analyses of data were generated by using Prism software (version 6.0, GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank K. Gagnidze for technical support and members of the Devi Laboratory for active scientific discussions. Computations were run on resources available through the Scientific Computing Facility at Mount Sinai and the Extreme Science and Engineering Discovery Environment under MCB080109N (to M.F.), which is supported by National Science Foundation grant number OCI-1053575.

Funding: This work was supported by NIH awards DA008863 and NS026880 to L.A.D. and DA026434 to M.F.

REFERENCES AND NOTES

1. Fricker LD. Analysis of mouse brain peptides using mass spectrometry-based peptidomics: Implications for novel functions ranging from non-classical neuropeptides to microproteins. *Mol. BioSyst.* 2010; 6:1355–1365. [PubMed: 20428524]
2. Morgan DJ, Mzhavia N, Peng B, Pan H, Devi LA, Pintar JE. Embryonic gene expression and pro-protein processing of proSAAS during rodent development. *J. Neurochem.* 2005; 93:1454–1462. [PubMed: 15935061]
3. Wei S, Feng Y, Che FY, Pan H, Mzhavia N, Devi LA, McKinzie AA, Levin N, Richards WG, Fricker LD. Obesity and diabetes in transgenic mice expressing proSAAS. *J. Endocrinol.* 2004; 180:357–368. [PubMed: 15012590]
4. Morgan DJ, Wei S, Gomes I, Czyzyk T, Mzhavia N, Pan H, Devi LA, Fricker LD, Pintar JE. The propeptide precursor proSAAS is involved in fetal neuropeptide processing and body weight regulation. *J. Neurochem.* 2010; 113:1275–1284. [PubMed: 20367757]
5. Wardman JH, Berezniuk I, Di S, Tasker JG, Fricker LD. ProSAAS-derived peptides are colocalized with neuropeptide Y and function as neuropeptides in the regulation of food intake. *PLOS One.* 2011; 6:e28152. [PubMed: 22164236]
6. Gomes I, Aryal DK, Wardman JH, Gupta A, Gagnidze K, Rodriguiz RM, Kumar S, Wetsel WC, Pintar JE, Fricker LD, Devi LA. GPR171 is a hypothalamic G protein-coupled receptor for BigLEN, a neuropeptide involved in feeding. *Proc. Natl. Acad. Sci. U.S.A.* 2013; 110:16211–16216. [PubMed: 24043826]
7. Sohn J-W. Network of hypothalamic neurons that control appetite. *BMB Rep.* 2015; 48:229–233. [PubMed: 25560696]
8. Betley JN, Cao ZFH, Ritola KD, Sternson SM. Parallel, redundant circuit organization for homeostatic control of feeding behavior. *Cell.* 2013; 155:1337–1350. [PubMed: 24315102]
9. Krashes MJ, Kravitz AV. Optogenetic and chemogenetic insights into the food addiction hypothesis. *Front. Behav. Neurosci.* 2014; 8:57. [PubMed: 24616674]
10. Krashes MJ, Koda S, Ye C, Rogan SC, Adams AC, Cusher DS, Maratos-Flier E, Roth BL, Lowell BB. Rapid, reversible activation of AgRP neurons drives feeding behavior in mice. *J. Clin. Invest.* 2011; 121:1424–1428. [PubMed: 21364278]
11. Krashes MJ, Shah BP, Koda S, Lowell BB. Rapid versus delayed stimulation of feeding by the endogenously released AgRP neuron mediators GABA, NPY, and AgRP. *Cell Metab.* 2013; 18:588–595. [PubMed: 24093681]
12. Aponte Y, Atasoy D, Sternson SM. AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat. Neurosci.* 2011; 14:351–355. [PubMed: 21209617]
13. Kakarala KK, Jamil K. Sequence-structure based phylogeny of GPCR class A rhodopsin receptors. *Mol. Phylogenet. Evol.* 2014; 74:66–96. [PubMed: 24503482]
14. Rossi L, Lemoli RM, Goodell MA. Gpr171, a putative P2Y-like receptor, negatively regulates myeloid differentiation in murine hematopoietic progenitors. *Exp. Hematol.* 2013; 41:102. [PubMed: 23022127]
15. Gomes, I.; Wardman, JH.; Stockton, SDJ.; Devi, LA. *Neuropeptide Receptors*. Devi, L.; Lloyd, DF., editors. Vol. 4. Princeton, NJ: Morgan & Claypool Life Sciences; 2013. p. 1-153.
16. Gonzalez NS, Communi D, Hannedouche S, Boeynaems J-M. The fate of P2Y-related orphan receptors: GPR80/99 and GPR91 are receptors of dicarboxylic acids. *Purinergic Signal.* 2004; 1:17–20. [PubMed: 18404396]
17. Zhang J, Zhang K, Gao Z-G, Paoletta S, Zhang D, Han GW, Li T, Ma L, Zhang W, Müller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q. Agonist-bound structure of the human P2Y₁₂ receptor. *Nature.* 2014; 509:119–122. [PubMed: 24784220]
18. Zhang K, Zhang J, Gao Z-G, Zhang D, Zhu L, Han GW, Moss SM, Paoletta S, Kiselev E, Lu W, Fenalti G, Zhang W, Müller CE, Yang H, Jiang H, Cherezov V, Katritch V, Jacobson KA, Stevens RC, Wu B, Zhao Q. Structure of the human P2Y₁₂ receptor in complex with an antithrombotic drug. *Nature.* 2014; 509:115–118. [PubMed: 24670650]
19. Zhang D, Gao Z-G, Zhang K, Kiselev E, Crane S, Wang J, Paoletta S, Yi C, Ma L, Zhang W, Han GW, Liu H, Cherezov V, Katritch V, Jiang H, Stevens RC, Jacobson KA, Zhao Q, Wu B. Two

- disparate ligand-binding sites in the human P2Y₁ receptor. *Nature*. 2015; 520:317–321. [PubMed: 25822790]
20. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci*. 1995; 25:366–428.
21. Abagyan R, Totrov M. Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. *J. Mol. Biol.* 1994; 235:983–1002. [PubMed: 8289329]
22. Abagyan R, Totrov M, Kuznetsov D. ICM—A new method for protein modeling and design: Applications to docking and structure prediction from the distorted native conformation. *J. Comput. Chem.* 1994; 15:488–506.
23. Hochhaus G, Yu VC, Sadée W. Delta opioid receptors in human neuroblastoma cell lines. *Brain Res.* 1986; 382:327–331. [PubMed: 3019481]
24. Graham ES, Ball N, Scotter EL, Narayan P, Dragunow M, Glass M. Induction of Krox-24 by endogenous cannabinoid type 1 receptors in Neuro2A cells is mediated by the MEK-ERK MAPK pathway and is suppressed by the phosphatidylinositol 3-kinase pathway. *J. Biol. Chem.* 2006; 281:29085–29095. [PubMed: 16864584]
25. Chaki S, Inagami T. Identification and characterization of a new binding site for angiotensin II in mouse neuroblastoma neuro-2A cells. *Biochem. Biophys. Res. Commun.* 1992; 182:388–394. [PubMed: 1731796]
26. Koenig JA, Edwardson JM, Humphrey PPA. Somatostatin receptors in Neuro2A neuroblastoma cells: Ligand internalization. *Br. J. Pharmacol.* 1997; 120:52–59. [PubMed: 9117098]
27. Gomes I, Bobeck EN, Margolis EB, Gupta A, Sierra S, Fakira AK, Fujita W, Müller TD, Müller A, Tschöp MH, Kleinau G, Fricker LD, Devi LA. Identification of GPR83 as the receptor for the abundant neuroendocrine peptide PEN. *Sci. Signal.* 2016; 9:ra43. [PubMed: 27117253]
28. Emmerson PJ, Fisher MJ, Yan LZ, Mayer JP. Melanocortin-4 receptor agonists for the treatment of obesity. *Curr. Top. Med. Chem.* 2007; 7:1121–1130. [PubMed: 17584132]
29. Brothers SP, Wahlestedt C. Therapeutic potential of neuropeptide Y (NPY) receptor ligands. *EMBO Mol. Med.* 2010; 2:429–439. [PubMed: 20972986]
30. Rodgers RJ, Tschöp MH, Wilding JPH. Anti-obesity drugs: Past, present and future. *Dis. Model. Mech.* 2012; 5:621–626. [PubMed: 22915024]
31. Rodriguez D, Ranganathan A, Carlsson J. Discovery of GPCR ligands by molecular docking screening: Novel opportunities provided by crystal structures. *Curr. Top. Med. Chem.* 2015; 15:2484–2503. [PubMed: 26126906]
32. Carlsson J, Coleman RG, Setola V, Irwin JJ, Fan H, Schlessinger A, Sali A, Roth BL, Shoichet BK. Ligand discovery from a dopamine D₃ receptor homology model and crystal structure. *Nat. Chem. Biol.* 2011; 7:769–778. [PubMed: 21926995]
33. Huang X-P, Karpiak J, Kroeze WK, Zhu H, Chen X, Moy SS, Saddoris KA, Nikolova VD, Farrell MS, Wang S, Mangano TJ, Deshpande DA, Jiang A, Penn RB, Jin J, Koller BH, Kenakin T, Shoichet BK, Roth BL. Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65. *Nature*. 2015; 527:477–483. [PubMed: 26550826]
34. Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science*. 2005; 310:683–685. [PubMed: 16254186]
35. Flier JS. AgRP in energy balance: Will the real AgRP please stand up? *Cell Metab.* 2006; 3:83–85. [PubMed: 16459309]
36. Pajouhesh H, Lenz GR. Medicinal chemical properties of successful central nervous system drugs. 2005; 2:541–553.
37. Mikitsh JL, Chacko AM. Pathways for small molecule delivery to the central nervous system across the blood-brain barrier. *Perspect. Medicin. Chem.* 2014; 6:11–24. [PubMed: 24963272]
38. Gupta A, Décaillot FM, Gomes I, Tkalych O, Heimann AS, Ferro ES, Devi LA. Conformation state-sensitive antibodies to G-protein-coupled receptors. *J. Biol. Chem.* 2007; 282:5116–5124. [PubMed: 17148456]
39. Kumar S, Liang F, Lawrence DS, Zhang Z-Y. Small molecule approach to studying protein tyrosine phosphatase. *Methods*. 2005; 35:9–21. [PubMed: 15588981]

40. Gloriam DE, Fredriksson R, Schioth HB. The G protein-coupled receptor subset of the rat genome. *BMC Genomics*. 2007; 8:338. [PubMed: 17892602]
41. UniProt Consortium, Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Res*. 2014; 42:D191–D198. [PubMed: 24253303]
42. Pei J, Kim B-H, Grishin NV. PROMALS3D: A tool for multiple protein sequence and structure alignments. *Nucleic Acids Res*. 2008; 36:2295–2300. [PubMed: 18287115]
43. Šali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol*. 1993; 234:779–815. [PubMed: 8254673]
44. Shen, M-y; Sali, A. Statistical potential for assessment and prediction of protein structures. *Protein Sci*. 2006; 15:2507–2524. [PubMed: 17075131]
45. Kaminski GA, Friesner RA, Tirado-Rives J, Jorgensen WL. Evaluation and reparametrization of the OPLS-AA force field for proteins via comparison with accurate quantum chemical calculations on peptides. *J. Phys. Chem. B*. 2001; 105:6474–6487.
46. Halgren TA, Nachbar RB. Merck molecular force field. IV. Conformational energies and geometries for MMFF94. *J. Comput. Chem*. 1996; 17:587–615.
47. Totrov M, Abagyan R. The contour-buildup algorithm to calculate the analytical molecular surface. *J. Struct. Biol*. 1996; 116:138–143. [PubMed: 8742735]
48. Totrov, MA.; Abagyan, R. *Drug-Receptor Thermodynamics: Introduction and Applications*. Raffa, RB., editor. New York: John Wiley & Sons Ltd; 2001. p. 603-624.
49. Schapira M, Abagyan R, Totrov M. Nuclear hormone receptor targeted virtual screening. *J. Med. Chem*. 2003; 46:3045–3059. [PubMed: 12825943]
50. Gomes I, Grushko JS, Golebiewska U, Hoogendoorn S, Gupta A, Heimann AS, Ferro ES, Scarlata S, Fricker LD, Devi LA. Novel endogenous peptide agonists of cannabinoid receptors. *FASEB J*. 2009; 23:3020–3029. [PubMed: 19380512]
51. Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E, Lim M, Maillet E, Junek M, Cahill CM, Harkany T, Devi LA. Increased abundance of opioid receptor heteromers after chronic morphine administration. *Sci. Signal*. 2010; 3:ra54. [PubMed: 20647592]
52. Gomes I, Filipovska J, Devi LA. Opioid receptor oligomerization. Detection and functional characterization of interacting receptors. *Methods Mol. Med*. 2003; 84:157–183. [PubMed: 12703323]
53. Rozenfeld R, Gupta A, Gagnidze K, Lim MP, Gomes I, Lee-Ramos D, Nieto N, Devi LA. AT1R–CB₁R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II. *EMBO J*. 2011; 30:2350–2363. [PubMed: 21540834]
54. Paxinos, G.; Franklin, KBJ. *The Mouse Brain in Stereotaxic Coordinates*. San Diego, CA: Academic Press; 2001.

Fig. 1. Identification of a GPR171 agonist and its predicted binding mode

(A) The indicated ligands (10 μ M) predicted to bind to GPR171 were tested by measuring stimulation of Ca^{2+} signal in CHO cells expressing GPR171 and $\text{G}_{\alpha 15/\beta 3}$. Ca^{2+} fluorescence was measured with Fluo-4 NW, ATP served as a positive control (activating endogenous purinergic receptors), b-LEN served as a GPR171-selective positive control, and I-LEN (LENSSPQAPA) served as a GPR171-negative control. Left graph shows normalized data (means $\pm$ SE; $n = 6$) with buffer conditions considered 100%. *** $P < 0.001$, one-way analysis of variance (ANOVA), Bonferroni's test; $F_{16,34} = 27.73$. Right graph shows

representative time course data of cells exposed to l-LEN, b-LEN, or MS0015203. Relative fluorescence is normalized to the baseline conditions (100%) before the indicated ligand was added. **(B)** Two-dimensional (2D) structure of MS0015203 and three-dimensional (3D) representation of the MS0015203-GPR171 complex showing amino acid residues involved in direct ligand-receptor interactions in the binding pocket. H-bond interactions are indicated with dotted lines. GPR171 residues are numbered and indicated with Ballesteros-Weinstein numbering (superscript). **(C)** Displacement of [125 I]Tyr-b-LEN binding (3 nM) with MS0015203 (0 to 10 μ M) using CHO cells expressing either mouse GPR171 (wild type) or the indicated mutant. Binding in the absence of MS0015203 was taken as 100%.

Fig. 2. Characterization of molecular pharmacological properties of MS0015203

(A) Ability of MS0015203 (0 to 10 μM) to displace [¹²⁵I]Tyr-b-LEN (3 nM) binding from rat hypothalamic membranes. Binding in the absence of cold ligands was taken as 100%. (B) Effect of MS0015203 (0 to 1 μM) on [³⁵S]GTPγS binding in rat hypothalamic membranes. (C) Effect of MS0015203 (0 to 10 μM) on forskolin-stimulated adenylyl cyclase activity in rat hypothalamic membranes. (D) Effect of MS0015203 (0 to 10 μM) on cAMP concentrations in wild-type Neuro2A cells (N2A) and Neuro2A cells in which GPR171 is knocked down (N2A + KDV). Data in all panels represent means ± SE (*n* = 3 to 6). ***P* < 0.01; ****P* < 0.0001, b-LEN versus MS0015203 (Student's *t* test).

Fig. 3. Effect of systemic administration of MS0015203 on c-Fos activity in PVN neurons (A) Mice were injected twice intraperitoneally at 30 and 60 min with either vehicle [6% dimethyl sulfoxide (DMSO); top panel] or MS0015203 (3 mg/kg, intraperitoneally; bottom panel) before perfusion, and c-Fos activity was measured. Representative images are shown. An area like the one outlined in white (87.5 mm²) was used for analysis and is shown in the enlarged image. Arrows indicate c-Fos activation in GPR171-positive cells. DAPI, 4',6-diamidino-2-phenylindole. (B) Images (×10) were quantified for total cells, GPR171-positive cells, or c-Fos-positive cells using ImageJ particle analysis. Two equal-sized areas from either side of the ventricle were quantified for each image. (C) Percentage of cells quantified in (B) shown as percentage of total number of cells that are positive for GPR171 or c-Fos after vehicle or MS0015203 injection. (D) Cells were randomly selected that contained GPR171 (40 per image) or lacked GPR171 (40 per image). Percent of each of these cells that contain c-Fos was quantified. *n* = 3 to 4 mice per group; two to four sections per mouse. Scale bars, 50 μm. **P* < 0.05; ***P* < 0.01; *****P* < 0.0001, vehicle versus MS0015203, two-way ANOVA with Bonferroni's post hoc test.

Fig. 4. Effect of acute administration of MS0015203 on food intake

(A) Cumulative food intake in fasted mice administered MS0015203 (3 mg/kg, intraperitoneally; $n = 6$) or vehicle (6% DMSO; $n = 5$). $*P < 0.05$; $**P < 0.01$, vehicle versus MS0015203 [two-way ANOVA with Bonferroni's post hoc comparisons; Interaction: $F_{3,27} = 2.261$, $P = 0.1041$; Time: $F_{3,27} = 197.9$, $P < 0.0001$; Drug: $F_{1,9} = 6.946$, $P < 0.0271$; Subjects (matching): $F_{9,27} = 8.511$, $P < 0.0001$]. Food intake on an hour-by-hour basis is shown in fig. S6. (B) Effect of administration of MS0015203 (3 mg/kg, intraperitoneally) or vehicle (6% DMSO) to mice that were administered control virus (CV) or GPR171-knockdown virus (KDV) and that were fed a high-fat diet. $**P < 0.01$, one-way ANOVA (Bonferroni's multiple comparison test). (C) Effect of administration of MS0015203 (2.5 mg/kg, intracerebroventricularly) or vehicle (6% DMSO) to mice that were administered control virus (CV) or GPR171-knockdown virus (KDV) and were fed regular chow. $*P < 0.05$; $**P < 0.01$, vehicle versus MS0015203 (two-way ANOVA with Bonferroni's post hoc comparisons; Interaction: $F_{9,48} = 0.3331$, $P = 0.9595$; Time: $F_{3,48} = 10.07$, $P < 0.0001$; Drug:

$F_{3,48} = 17.52$, $P < 0.0001$). **(D)** Comparison of expression of *GPR171* mRNA in hypothalami of mice administered control virus or GPR171-knockdown virus [$n = 7$ mice (control virus) or 9 mice (GPR171-knockdown virus)]. *** $P < 0.001$, control virus versus GPR171-knockdown virus, Student's t test.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Effect of chronic administration of MS0015203 on body weight

(A) Effect of administration of MS0015203 (3 mg/kg, intraperitoneally; $n = 5$ per group) or vehicle (6% DMSO) every third day on body weight of mice fed a high-fat diet. * $P < 0.05$, vehicle versus MS0015203 (two-way ANOVA with Bonferroni's post hoc comparisons; Interaction: $F_{10,88} = 1.219$, $P = 0.2901$; Time: $F_{10,88} = 14.87$, $P < 0.0001$; Drug: $F_{1,88} = 45.79$, $P < 0.0001$). (B) Effect of administration of MS0015203 (3 mg/kg, intraperitoneally) or vehicle (6% DMSO) every third day to mice that were administered control virus ($n = 7$) or GPR171-knockdown virus ($n = 9$) and that were fed a high-fat diet. ** $P < 0.01$, control

virus versus GPR171-knockdown virus (two-way ANOVA with Bonferroni's post hoc comparisons; Interaction: $F_{7,80X} = 0.1145$, $P = X$; Time: $F_{7,80} = 22.69$, $P < 0.001$; Treatment: $F_{1,80} = 46.15$, $P < 0.0001$). (C and D) Effect of chronic administration of MS0015203 on them RNAs encoding proSAAS, NPY, AgRP, orexin, GPR171, neuropeptide Y1 receptor (Y1R), neuropeptide Y5 receptor (Y5R), melanocortin 4 receptor (MC4R), hypocretin 1 receptor (HCRT1R), and hypocretin 2 receptor (HCRT2R) in the ventral hypothalamus relative to vehicle-injected controls ($n = 5$ per group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, vehicle versus MS0015203, Student's t test. (E and F) Effect of chronic administration of MS0015203 on the mRNAs encoding the indicated neuropeptides or receptors in the dorsal hypothalamus relative to vehicle-injected controls ($n = 5$ per group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, vehicle versus MS0015203, Student's t test.

Table 1
Binding of b-LEN and MS0015203 to different mutants of GPR171

Cells expressing wild-type DYK-tagged mouse GPR171 (wild type) or constructs expressing either Y99A, K169A, H177A, C184A, Y239A, or R243A mutations were incubated with 3 nM [¹²⁵I]b-LEN in the absence or presence of different concentrations of b-LEN or MS0015203 (Binding) (0 to 10 μ M). In a separate set of experiments, cells were incubated with b-LEN or MS0015203 (0 to 10 μ M), and the amount of cAMP formed was measured (Signaling). Values are means $\pm$ SE of two independent experiments in triplicate. IC₅₀, median inhibitory concentration; EC₅₀, median effective concentration; n.d., not determinable.

GPR171	Binding b-LEN		Binding MS0015203		Signaling b-LEN		Signaling MS0015203	
	IC ₅₀ (nM)	% Displaced at 10 μ M	IC ₅₀ (nM)	% Displaced at 10 μ M	EC ₅₀ (nM)	% Inhibition at 10 μ M	EC ₅₀ (nM)	% Inhibition at 10 μ M
Wild type	8 $\pm$ 1	100 $\pm$ 1	81 $\pm$ 1	85 $\pm$ 1	3 $\pm$ 1	49 $\pm$ 3	165 $\pm$ 3	37 $\pm$ 2
Y99A	376 $\pm$ 1	100 $\pm$ 5	445 $\pm$ 3	41 $\pm$ 2	31 $\pm$ 4	38 $\pm$ 2	1934 $\pm$ 5	11 $\pm$ 3
K169A	6 $\pm$ 1	99 $\pm$ 2	18 $\pm$ 2	44 $\pm$ 1	2 $\pm$ 1	44 $\pm$ 5	78 $\pm$ 4	16 $\pm$ 6
H177A	2 $\pm$ 1	99 $\pm$ 8	6 $\pm$ 2	24 $\pm$ 2	12 $\pm$ 5	35 $\pm$ 2	n.d.	7 $\pm$ 2
C184A	35 $\pm$ 1	99 $\pm$ 1	657 $\pm$ 1	79 $\pm$ 2	7 $\pm$ 2	45 $\pm$ 2	353 $\pm$ 1	30 $\pm$ 2
Y239A	42 $\pm$ 2	100 $\pm$ 4	50 $\pm$ 3	27 $\pm$ 1	18 $\pm$ 3	39 $\pm$ 3	1119 $\pm$ 5	11 $\pm$ 1
R243A	92 $\pm$ 2	99 $\pm$ 9	103 $\pm$ 2	20 $\pm$ 2	26 $\pm$ 2	30 $\pm$ 6	n.d.	-7 $\pm$ 2