Differential Activation of “Social” and “Solitary” Variants of the Caenorhabditis elegans G Protein-coupled Receptor NPR-1 by Its Cognate Ligand AF9

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Natural variations of wild Caenorhabditis elegans isolates having either Phe-215 or Val-215 in NPR-1, a putative orphan neuropeptide Y-like G protein-coupled receptor, result in either “social” or “solitary” feeding behaviors (de Bono, M., and Bargmann, C. I. (1998) Cell 94, 679–689). We identified a neuropeptide, GLGPR-PLRF-NH2 (AF9), as a ligand activating the cloned NPR-1 receptor heterologously expressed in mammalian cells. Shifting cell culture temperatures from 37 to 28 °C, implemented 24 h after transfections, was essential for detectable functional expression of NPR-1. AF9 treatments linked both cloned receptor variants to activation of Gi/Go proteins and cAMP inhibition, thus allowing for classification of NPR-1 as an inhibitory G protein-coupled receptor. The Val-215 receptor isoform displayed higher binding and functional activity than its Phe-215 counterpart. This finding parallels the in vivo observation of a more potent repression of social feeding by the npr-1 gene encoding the Val-215 form of the receptor, resulting in dispersing (solitary) animals. Since neuropeptide Y shows no sequence homology to AF9 and was functionally inactive at the cloned NPR-1, we propose to rename NPR-1 and refer to it as an AF9 receptor, AF9-R1.

The finding of de Bono and Bargmann (1) that a single amino acid difference in a G protein-coupled receptor dramatically influences feeding behavior in the nematode Caenorhabditis elegans is remarkable because most behaviors seem to be controlled by multiple genes. The receptor in question is the putative C. elegans neuropeptide GPCR2 (Wormpep designation C39E.6.6) with homology to the vertebrate neuropeptide Y (NPY) receptor family and therefore named NPR-1 (1). Natural variations of wild isolates have either Phe-215 or Val-215 in NPR-1, resulting in either “social” or “solitary” feeders, respectively (1).

Possible roles of NPR-1 are highlighted by the finding that npr-1 knockout worms are strongly clumping, indicating that npr-1 functions to repress social feeding and aggregation. In transgenes that express NPR-1 in the npr-1 null background, both variants can repress social feeding, but Val-215-npr-1 does it more effectively (1). Two recent papers from the de Bono group (2, 3) provided further insight into the complex nature of genetic, molecular, and neural mechanisms involved in nematode social feeding. Evaluation of various mutant strains of C. elegans for mutations affecting social feeding led to the identification of other genes that co-localized or interacted with npr-1 in distinct sets of neurons. These findings point to a network of multiple interactions that control signals from the external environment as well as neuronal activity inside the body cavity of the worm that lead to worm aggregation into feeding groups (2, 3). However, so far none of these studies proposed any link that would connect the two systems or suggest a possible role for the activation of NPR-1 by its cognate ligand in this process.

Since the two strains behave similarly in the absence of food, it has been postulated that food might regulate secretion of a neuropeptide that acts through the NPR-1 receptor to result in different feeding patterns (1). Despite the publication of the C. elegans genome more than four years ago now (4) and prediction of 50 putative peptide GPCRs in this nematode (5), there has been no report to date on any C. elegans neuropeptide GPCR (including NPR-1) successfully matched with its cognate ligand. This is in contrast to numerous orphan vertebrate GPCRs, which have been paired to their natural ligands by reverse pharmacology after heterologous receptor expression, in most cases, using mammalian cells (6).

We describe here the identification of AF9 (GLGPRPLRF-NH2), a nematode FMRFamide-related peptide (FaRP) with no homology to NPY (Fig. 1) as a ligand activating NPR-1. To our knowledge, this is the first successful functional heterologous expression of a C. elegans neuropeptide GPCR and the first account of pairing of any C. elegans FaRP receptor with its cognate ligand.

EXPERIMENTAL PROCEDURES

Materials—Human NPY was from Sigma. Other synthetic peptides were custom made at Auspep Pty. Ltd. (Parkville, Australia). Mutant Chinese hamster ovary cells CHO-10001A (7, 8) (referred to as CHO cells in the text) were a kind gift from Dr. Rita Huff. Cell culture media, peptide Y; CHO, Chinese hamster ovary; RAMP, receptor activity-modifying protein; CRLR, calcitonin receptor-like receptor; h, human.
transfection, and assay reagents were as described previously (9, 10).

Cloning and Plasmid Preparation—Molecular biological techniques followed either manufacturer’s recommendations or general protocols (11). The open reading frame for npr-1 (C39E6.6) was obtained by reverse transcriptase-PCR and cloned directly into the eucaryotic expression vector pCR3.1 (Invitrogen). The only significant modification was the addition of an optimized translational initiation sequence immediately preceding the authentic initiation codon (GCC GGC ATG) (12). A clone was obtained that had the identical sequence to the Phe-215 npr-1 variant (1). Site-directed mutagenesis was used to derive the corresponding Val-215 npr-1 variant. The DNA sequence of all constructs was confirmed before use in transfection studies. Details of molecular biological manipulations are available upon request.

Cell Cultures, Cell Transfection, and Membrane Preparation—CHO-10001A cells were cultured and transfected as described (9, 10). The Phe-215-NPR-1/pCR3.1 or Val-215-NPR-1/pCR3.1 plasmids (5 µg of DNA/10-cm plate) were used for transfections. The cells were harvested 48 h after transfection, and membranes were prepared as described (9).

In cases where the 37 °C shift was implemented, the transfected cells were incubated at 37 °C for 24 h after transfection and then moved to a humidified 28 °C, 3% CO2 incubator for an additional 24 h before harvesting for membrane preparation.

Radioligand Preparation and Receptor Binding Via Scintillation Proximity Assay—[35S]-I-Tyr]AF9 was prepared by radioiodiation of YGLGPRPLRF-NH2 ([35S]AF9) using the chloramine T procedure. The crude products were separated by reversed-phase high performance liquid chromatography on a Vydac C18, 0.45 × 15 cm column using a 0.1 M ammonium acetate, pH 6.5/acetonitrile system. The radioactive fractions with retention time corresponding to that of the cold [3-monoo-iodoY]AF9 standard were collected into vials containing capture buffer (0.1 M NaHPO4, pH 8.0, with 0.5% bovine serum albumin, 1% Triton X-100, and 0.05% Tween 20). The transfected cell membranes were incubated for 30 min at room temperature with occasional shaking with wheat germ agglutinin-coated scintillation proximity assay beads (Amersham Biosciences) in assay buffer (200 mM HEPES, 10 mM MgCl2, pH 7.4) (200 µg of membrane protein/20 µg of beads/ml). After centrifugation at 1200 rpm for 10 min, the beads were resuspended in the assay buffer. In saturation binding experiments, to each well in 96-well scintillation proximity assay plates (Wallac, Turku, Finland), we added 60 µl of the assay buffer, various concentrations of [35S]-I-Tyr]AF9 diluted into the assay buffer (20 µl), and 20 µl of the reconstituted membrane-coated scintillation proximity assay bead suspension (1.5 µg of membrane/150 mg of beads) to result in a total volume of 100 µl/well. Nonspecific binding was determined in the presence of 10 µM AF9 at each radioligand concentration. Plates were counted on a Wallac 1450 Microbeta counter continuously, and the 2-h counts, corrected for nonspecific binding, were used for Kd and Bmax, determinations. Competitive displacement methods were similar to those used for determination of the saturation curve except that a fixed [35S]-I-Tyr]AF9 dose was used (216 and 54 pM with Phe-215- and Val-215-NPR-1 membranes, respectively), added in 10 µl of assay buffer, and followed by the addition of increasing peptide concentrations, also in 10 µl of assay buffer. Total binding was determined in the absence of any peptides and was ~10% of the label added with 80% specific binding at the highest peptide concentrations used (10 µM).

[35S]GTPγS Binding Assay—The GTPγS assay was carried out as described (9, 10). In experiments evaluating hNPR-1 as a potential antagonist, the Phe-215-NPR-1 membranes were incubated with varying concentrations of [35S]GTPγS binding assay buffer (200 µM GTPγS, 10 µM MgCl2, pH 7.4). Each treatment was run in triplicate, values were expressed as means ± S.E. The calculated EC50, Kd, or Ki, values were considered statistically different (p < 0.05) when their 95% confidence limits did not overlap.

RESULTS

Temperature-sensitive Functional Expression of NPR-1 and Receptor-Ligand Matching—The receptor-ligand matching was achieved by functionally expressing NPR-1 in mammalian cells using special cell culturing conditions. The cloned npr-1 genes, encoding the Phe-215 and Val-215 forms of the receptor, were transiently transfected into CHO cells, and a GTPγS assay was utilized as a readout for detection of receptor function based on agonist-driven stimulation of [35S]GTPγS binding to membranes prepared from the transfected cells. A collection of about 200 synthetic peptides representing a variety of C. elegans and other invertebrate FaRPs (based on both the isolated and the predicted peptide sequences) was used as a source of potential matching ligands. Individual peptides were arranged into peptide pools of 5–7 peptides/group (each peptide at 5–6 µM) tested with plasma membranes prepared from the cells transiently transfected with NPR-1. In initial experiments, no stimulation of [35S]GTPγS binding could be detected in response to peptide treatments when standard transfection and cell culture conditions at 37 °C were applied (Fig. 2A). However, when membranes were prepared from the Phe-215-NPR-1/CHO cells, which were moved to 28 °C at 24 h after transfection,
tion, and incubated at this lower temperature for an additional 24 h prior to cell harvesting, a 2.8-fold increase in the \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding was recorded for a single peptide pool (Fig. 2B, pool 7). AF9 was then found as the only active component in this pool, as identified in a subsequent deconvolution experiment run with individual peptides present in pool 7, each tested at 5 mM (Fig. 2C). It is important to stress that the observed AF9 activity was receptor-specific since it was detected only in the NPR-1/CHO membranes, whereas there was no response to AF9 when membranes from the untransfected CHO cells or CHO cells transfected with unrelated \textit{Drosophila} neuropeptide GPCRs (9, 10) were used as controls (data not shown).

In subsequent experiments, the 37 to 28 °C shift consistently resulted in the elevated \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding. Without this cooling step, no stimulation of \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding by AF9 (5 mM) could be seen in most of the experiments, as reflected in the same basal and AF9-evoked activity in the 37 °C membranes, analogous to that depicted in Fig. 2A. Only occasionally, a weak signal was recorded (data not shown). Similar results were obtained with the Val-215-NPR-1 membranes (data not shown). These findings indicate that at 37 °C, the receptor function was compromised, and the observed sporadic activity could be due to different receptor expression levels and efficiency of functional folding in transient transfection experiments.

**Evaluation of NPY for Activation of NPR-1**—Human NPY did not trigger either Phe-215- or Val-215-NPR-1 stimulation when tested at concentrations up to 10 mM in the \[^{35}\text{S}]\text{GTP}^\gamma\text{S} assays carried out with membranes prepared from the transfected cells harvested after the implemented 37 to 28 °C temperature shift (Fig. 3, A and B). Additionally, hNPY did not show any antagonistic activity when tested with the Phe-215-NPR-1 (Fig. 3A) or Val-215-NPR-1 membranes stimulated with 200 nM AF9 (data not shown). In control experiments with the same plasma membranes as tested with hNPY, the AF9-stimulated \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding to the Phe-215- and Val-215-NPR-1 membranes was dose-dependent with calculated EC\textsubscript{50} values of 59.4 and 2.5 nM, respectively (Fig. 3, A and B). These results show that the assay conditions were suitable for detecting NPR-1 activation and confirm that hNPY was not able to functionally activate either variant of the NPR-1 receptor.

**Comparison of Functional Activity for the Phe-215- and Val-215-NPR-1 Variants**—Direct quantitative comparisons of the Phe-215 and Val-215 receptor variants were complicated by the fact that the potency of AF9 varied due to the transient nature of transfections and the time of cell incubation at 28 °C. For example, in the \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding assay, the AF9 potency range (EC\textsubscript{50} values) was from 59.4 to 391 nM in the Phe-215-NPR-1 and from 2.5 to 77 nM in the Val-215-NPR-1 membranes in various independent transfection experiments. Attempts to overcome this problem by creating clonal cell lines stably expressing each receptor were unsuccessful because only a small fraction of the transfected cells survived but then quickly lost the receptor function only after one or two passages. This was even worse when HEK-293 cells were used as a host since the inserted Phe-215- or Val-215-npr-1 genes caused cell death about 16–20 h after transfection.

To minimize the variables of transient transfection, in direct receptor variant comparisons, the same CHO cell pool was used for transfections with the Phe-215- and Val-215-NPR-1 plasmids run in parallel, and identical conditions were utilized for the transfections and the subsequent cooling step before cell harvesting for membrane preparation. Membranes from transfected cells were normalized by their protein content, and the same amounts of membrane proteins were used for each receptor form. As depicted in Fig. 4A, in one of such \[^{35}\text{S}]\text{GTP}^\gamma\text{S} assays, AF9 was ~42-fold more potent at the Val-215 receptor than the Phe-215 counterpart (EC\textsubscript{50} values of 2.5 and 102.2 nM, respectively, p < 0.05). Similar results were obtained in three other independent experiments, performed in an analogous fashion, where the determined differences in the potency of AF9 to activate the Val-215- versus Phe-215-NPR-1 were in the 5–10-fold range (data not shown).

**Receptor Binding**—Before \[^{125}\text{I}]\text{YGLGPRPLRF-NH}_2 (\[^{125}\text{I}]\text{Y-Tyr}^9\text{AF9}) was used as a probe for binding, its cold iodo counterpart was functionally characterized in the \[^{35}\text{S}]\text{GTP}^\gamma\text{S} assay. As shown in Fig. 3A, \[^{125}\text{I}]\text{Y-AF9} acted as a high potency functional agonist to stimulate \[^{35}\text{S}]\text{GTP}^\gamma\text{S} binding in the Phe-215-NPR-1 membranes and was indistinguishable from AF9 (EC\textsubscript{50} values of 42.8 and 59.3 nM, p > 0.05, for \[^{125}\text{I}]\text{Y-AF9} and AF9, respectively). This finding was confirmed in another independent transfection experiment with the determined EC\textsubscript{50} values of 70 and 102.2 nM, respectively, p = 0.05 (Table I). \[^{125}\text{I}]\text{Y-AF9} was also a full agonist at the Val-215 receptor, however, with the potency about 4-fold lower as compared with that of AF9 (EC\textsubscript{50} values of 10.4 and 2.5 nM, respectively, p = 0.05) (Fig. 4B and Table I).

In a saturation binding experiment, \[^{125}\text{I}]\text{Y-Tyr}^9\text{AF9} bound with about 4-fold higher affinity to the Val-215-NPR-1 membranes than to the Phe-215 preparations (K\textsubscript{D} values of 59.5 and 227.2 pM, respectively, p = 0.05). The receptor density in the Val-215-NPR-1 membranes was about 1.7-fold higher than in the Phe-215-NPR-1 (K\textsubscript{D} values of 59.5 and 227.2 pM, respectively, p = 0.05) (Table I). All together, the receptor...
binding/competition and functional (GTP\textsubscript{S}) results indicate that AF9 was significantly more potent at the Val-215-NPR-1 receptor.

In the competition binding assay, carried out with both the Val-215- and the Phe-215-NPR-1 membranes, hNPY weakly competed with the radioactive probe, [\textsuperscript{125}I-Tyr\textsuperscript{\textdegree}]AF9. The calculated $K_i$ values for hNPY at the Val-215 and Phe-215 receptors were 1.6 and 1.2 $\mu$m, respectively (Fig. 4, C and D, and Table I). This makes hNPY 694- and 66-fold less potent than AF9 at the Val-215 and Phe-215 receptor, respectively, which might be not biologically relevant. Despite this weak binding to NPR-1, hNPY was unable to functionally activate this C. elegans GPCR, as determined in the GTP\textsubscript{S} assay (Fig. 3, A and B).

Evaluation of NPR-1 Signaling Pathways—AF9 did not increase intracellular cAMP in cells transfected with either Phe-215- or Val-215-NPR-1, indicating that Gs proteins were not involved in NPR-1 activation (data not shown). However, the AF9-evoked stimulation of [\textsuperscript{35}S]GTP\textsubscript{S} binding to the Phe-215-NPR-1 and Val-215-NPR-1 membranes was completely abolished by pertussis toxin treatments (Fig. 4A). This implicated Gi/Go proteins in both the activation of the receptors and cAMP inhibition as a major signaling pathway. An AF9 dose-dependent inhibition of forskolin-stimulated cAMP release was detected in the CHO cells transiently transfected with either the Phe-215 or the Val-215 receptor variants, both at 37 and 28 °C (Fig. 5, A and B). Qualitatively, the inhibitory effects of AF9 at 37 °C (Fig. 5A) were more potent in the Val-215 than in the Phe-215 cells, and similar results were obtained in two independent experiments. However, quantitatively, the determined IC\textsubscript{50} values for Phe-215- and Val-215-NPR-1 (4.1 and 0.42 nM, respectively) were not statistically different (overlapping 95% confidence limits) (Fig. 5B). The assay noise originated from the fact that the cAMP inhibition was measured in a population of transfected and untransfected cells with the latter contributing to a high background and variability of the forskolin-induced cAMP, which could not be

**Table I**

*Summary of the [\textsuperscript{35}S]GTP\textsubscript{S} and receptor binding data*

| Peptide | [\textsuperscript{35}S]GTP\textsubscript{S} binding EC\textsubscript{50} (nM) | Receptor binding $K_i$ (pM) ($B_{\text{max}}$ (pmol/mg)) | Receptor competition $K_i$ (nM) |
|---------|-------------------------------|---------------------------------|-----------------------------|
|         | Val-215 | Phe-215 | Val-215 | Phe-215 | Val-215 | Phe-215 | Val-215 | Phe-215 |
| AF9     | 2.5$^a$ | 102.2$^b$ | NA | NA | 2.3$^f$ | 17.8$^g$ |
| [\textsuperscript{1}Y]AF9 | 10.4$^c$ | 70.0$^d$ | 59.5$^e$ (1.5)$^h$ | 227.2$^i$ (0.9)$^j$ | 2.6$^d$ | 21.5$^e$ |
| hNPY    | Inactive | Inactive | NA | NA | 1574$^k$ | 1179$^l$ |

$^{a}$ Saturation binding with the radiolabel, [\textsuperscript{125}I-Tyr\textsuperscript{\textdegree}]AF9. NA, not applicable.

$^{b,c,f}$ EC\textsubscript{50} values with different superscripts were significantly different, $p = 0.05$ (non-overlapping 95% confidence limits).

$^{c,d,h}$ $K_i$ values with different superscripts were significantly different, $p = 0.05$ (non-overlapping 95% confidence limits).

$^{c,e,i}$ $B_{\text{max}}$ values with different superscripts were significantly different, $p = 0.05$ (non-overlapping 95% confidence limits).

$^{d,e,k}$ IC\textsubscript{50} values with different superscripts were significantly different, $p = 0.05$ (non-overlapping 95% confidence limits).
inhibited by AF9. Additionally, it is also possible that the cooling step could have helped with proper folding of the expressed nematode receptors, but at the same time, could have been detrimental to the activity of the mammalian adenyl cyclase in the CHO cells incubated at 28 °C. For this reason, the cAMP inhibition, as a further downstream effect, might not have paralleled the differences observed at the receptor and G protein levels. Attempts to overcome problems related to transient transfections by creating stable CHO cell lines expressing each receptor were unsuccessful, as mentioned earlier.

It has been suggested (1) that the two *npr-1* alleles could activate different G proteins and different downstream signaling pathways since position 215 in NPR-1/AF9-R1 is predicted to be in the third intracellular loop, the region, which was shown to be important for coupling to G proteins in other GPCRs. This hypothesis was based on the report that an aromatic amino acid residue in an equivalent position in a vertebrate counterpart has its structural counterparts but use a completely different set of peptides to activate them. One such example includes an orphan *Drosophila* GPCR homologous to mammalian opioid/somatostatin receptors that has been paired with allatostatin-C, a *Drosophila* peptide unrelated to any known mammalian peptides (16). Another *Drosophila* orphan GPCR, predicted to be a vasopressin-like receptor, was found to be activated by two peptides with no sequence homology to vasopressin (17). These findings stress the importance of the experimental identification of true cognate ligands for the accurate functional annotation of the newly deorphaned receptors.

AF9 was originally isolated from a parasitic nematode *Ascaris suum* (18), but recently, its sequence was also found to be encoded on a putative *C. elegans* precursor gene *flp21* (19). In *A. suum*, AF9 was reported to produce strong electrophysiological effects on motor neurons and dramatic behavioral effects after AF9 injection into whole worms (decrease in locomotion and reduced general movement) (20). The effects and role of AF9 in *C. elegans* have not yet been explored.

We would like to stress that the main factor that contributed to the successful functional expression of AF9-R1 in mammalian cells was the 37 to 28 °C cooling step implemented 24 h after transfection. The idea for the temperature shift came from the realization that the optimal temperature for free living *C. elegans* is ~15–19 °C, whereas mammalian cell cultures are maintained at 37 °C. We hypothesized that this temperature difference could contribute to misfolding and aggregation of heterologously expressed *C. elegans* GPCRs and thus prevented their functional activity. Support for this hypothesis comes from a report by Zhang et al. (21) providing evidence for the cytosolic localization of a misfolded odorant *C. elegans* GPCR, ODR-10, heterologously expressed in human embryonic kidney cells, HEK-293 cultured at 37 °C. Only 0.3–1.0% of the transfected cells displayed ODR-10 in the plasma membrane, whereas in the majority of cells, the receptor protein remained mostly cytoplasmic (21). We thought that perhaps cell cooling could help with better GPCR folding, as was reported for temperature-sensitive expression of other transmembrane protein, *e.g.*, *Torpedo* fish (22, 23) or *Drosophila* (24) nicotinic acetylcholine receptors. These channel proteins are expressed in mammalian cells at both 37 and 26–28 °C but properly assemble into functional channels only at temperatures lower than 37 °C (22–24). Misfolding and a lack of function was also reported for mutant and native mammalian GPCRs (25, 26) and channel proteins (27, 28) heterologously expressed in mammalian cells, which points to amino acid sequence-dependent rather than species-dependent problems. This misfolding and defective function could also be overcome by growing transfected mammalian cells at lower temperatures (25–28). Our success with functional expression of AF9-R1 at lower temperatures in mammalian cells, as described here, presents the
potential for utilizing similar approaches to annotate other numerous putative orphan C. elegans neuropeptide GPCRs whose cognate ligands are yet to be discovered.

The mammalian Gao proteins appear to be the most logical partners for coupling to AF9-R1 in CHO cells because they are ~80–87% identical to the C. elegans GOA-1 (Gao homologue) (29, 30). Besides GOA-1, the C. elegans genome contains one clear homologue for each of the other three vertebrate classes of Gα genes, Gαs (gao-1), Gαq (egl-30), and Go12 (gpa-12). Additionally, there are 16 new Gα genes, most likely involved in perception (29, 30). They are unique to C. elegans with no sequence homology to any of the known Gα subunits (29, 30). At present, the identity of a G protein(s) in the worm that couples to NPR-1/AF9-R1 is not known.

Although NPR-1/AF9-R1 is predominantly expressed in the head (nervous system), it was also found in the pharyngeal neurons, in the ventral nerve cord, the sensory neurons in the tail, as well as in a muscle in the terminal bulb of the pharynx and in the excretory duct cell and excretory canal (3). Such a wide distribution of NPR-1/AF9-R1 might imply highly complex and specialized functions of the receptor depending on its localization and the presence of other interacting proteins, which either co-localize with NPR-1/AF9-R1 or connect to NPR-1/AF9-R1 via a network of downstream signaling pathways. It is possible that one class of G protein(s) could be responsible for the activation of NPR-1/AF9-R1 present in the C. elegans neurons exposed to the body fluid. This receptor system appears to suppress social feeding by antagonizing signaling through a cGMP-gated ion channel (3). It will be interesting to find out whether the same or a different G protein(s) couple to the NPR-1/AF9-R1 expressed in the neurons, which localize to the anterior of the animal and have been postulated to transmit external aversive stimuli coming from bacterial food to a circuit promoting social feeding (aggregation) via activated NPR-1 (2).

It is tempting to speculate that the complexity of signaling via the NPR-1/AF9-R1 receptor could be even more complicated by the possibility that this receptor might be activated not only by AF9 but potentially also by a different peptide ligand (yet to be identified), depending on where the receptor is expressed and the repertoire of accessory proteins present in the same cell. This idea comes to mind by analogy with the recent discovery of a group of receptor activity-modifying proteins (RAMPs), which cannot only influence functional expression of GPCRs but can also define receptor pharmacology, as was shown for the calcitonin receptor-like receptor (CRLR) (31, 32). CRLR can function either as a calcitonin gene-related peptide (CGRP) receptor or as an adrenomedullin receptor, depending on which member of the RAMP family co-expresses with CRLR (31, 32). Such a possibility could now be tested by designing proper experiments aimed at finding C. elegans RAMPs, possibly co-expressing with NPR-1/AF9-R1.

Our finding that AF9 is more potent to activate the cloned Val-215 than the Phe-215 form of the NPR-1/AF9-R1 receptor parallels the in vivo observations that although both natural variants of the npr-1 gene can repress social feeding, the Val-215 receptor does it more potently (1). The observed lower activity of the cloned Phe-215-AF9-R1 is also in line with the prediction that the naturally existing Phe-215 social nematode strains do not lack npr-1 function but might rather have the receptor that functions at a lower level (33). This was suggested because the npr-1 null mutation causes a stronger clumping phenotype than the Phe-215 allele and also because overexpression of the 215F allele was able to convert social npr-1 null mutant into solitary feeders (1, 33). The knowledge of the cognate ligand for NPR-1/AF9-R1 will now allow the testing of the hypothesis (1) that food is likely to regulate secretion of a neuropeptide (AF9) that acts through the NPR-1 receptor(s) to result in different feeding patterns of the Phe-215- and Val-215-NPR-1-expressing C. elegans strains. In addition, reverse genetics may now be used to assess direct involvement of fbp21, the gene encoding AF9, in C. elegans feeding and behavior.

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REFERENCES
1. de Bono, M., and Bargmann, C. I. (1998) Cell 94, 679–689
2. Coates, J. C., and de Bono, M. (2002) Nature 418, 925–929
3. de Bono, M., Tobin, D. M., Davis, M. W., Avery, L., and Bargmann, C. I. (2002) Nature 419, 899–903
4. The C. elegans Sequencing Consortium (1998) Science 282, 2012–2018
5. Bargmann, C. I. (1998) Science 282, 2028–2033
6. Cevelli, O., Nethercough, H.-P., Saito, Y., Wang, Z., Lin, S. H. S., and Reinscheid, R. K. (2001) Trends Neurosci. 24, 230–237
7. Gottesman, M. M., LeComam, A., Buchkowski, M., and Pastan, I. (1988) Somatic Cell Genet. 6, 45–61
8. Lajiness, M. E., Chio, C. L., and Huff, R. M. (1993) J. Pharmacol. Exp. Ther. 267, 1573–1581
9. Larsen, M. J., Burton, K. J., Zambello, M. R., Smith, V. G., Lowery, D. E, and Kubiak, T. M. (2001) Biochem. Biophys. Res. Commun. 286, 895–901
10. Kubiak, T. M., Larsen, M. J., Burton, K. J., Bannow, C. A., Martin, R. A., Zambello, M. R., and Lowery, D. E. (2002) Biochem. Biophys. Res. Commun. 291, 313–320
11. Aasuble, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidam, J. G., Smith, J. A., Struhl, K., Albright, L. M., Coen, D. M., Yarki, A., Chanda, V. B. (eds) (1991) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York, NY
12. Konzak, M. (1987) Nucleic Acids Res. 15, 8125–8148
13. Chiulli, A. C., Trompeter, K., and Palmer, M. (2000) J. Biomol. Screen. 5, 229–247
14. Blum, K., Mutschler, E., and Wess, J. (1994) J. Biol. Chem. 269, 402–405
15. Burstein, E. S., Spalding, T. A., and Brann, M. R. (1996) J. Biol. Chem. 271, 3862–3865
16. Iversen, L. L., Cazzaipalli, G., Williamson, M., Hauser, F., and Grimmelikhuijzen, S. J. P. (1988) Biochem. Biophys. Res. Commun. 168, 1705–1717
17. Park, Y., Kim, Y.-J., and Adams, M. E. (2002) Peptides 23, 1688–1694
18. Jaquette, J., and Segaloff, D. L. (1997) Science 276, 8942–8950
19. Jansen, G., Thijssen, K. L., Werner, P., van der Horst, M., Hazendonk, E., and Solari, R., Lee, M. G., and Foord, S. M. (1998) Nat. Genet. 21, 1688–1694
20. Davis, R. E., and Stretton, A. O. (2001) J. Cell Biol. 150, 705–717
21. Claudio, T., Green, W. N., Hartman, D. S., Hayden, D., Paulson, H. L., Sigworth, F. J., Sine, S. M., and Swedlund, A. (1997) Science 238, 1688–1694
22. Lansdell, S. J., Schmitt, B., Betz, H., Sattelle, D., Millar, N. S. (1997) J. Neurochem. 68, 1812–1819
23. Jaquette, J., and Segaloff, D. L. (1997) Endocrinology 138, 85–91
24. Jeyaraj, S. C., Chotani, M. A., Mitra, S., Gregg, H. E., Flavahan, N. A., and Morrison, K. J. (2001) Mol. Pharmacol. 60, 1195–1200
25. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) Nature 358, 761–764
26. Sharma, M., Benharouga, M., Hu, W., and Lukacs, G. L. (2001) J. Biol. Chem. 276, 8842–8850
27. Lombard, M. A., Mendel, J. E., Sternberg, P. W., and Simon, M. I. (1991) Cell Regul. 2, 135–154
28. Jansen, G., Thijsen, K. L., Werner, P., van der Horst, M., Hazendonk, E., and Plasterk, R. H. (1999) Nat. Genet. 21, 414–419
29. McLaughlin, I. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Froud, S. M. (1998) Nature 393, 333–339
30. Kamitani, S., Anakawa, M., Shimakake, Y., Kozakoso, K., Nakahara, K., and Sakata, T. (1999) FEBS Lett. 448, 111–114
31. Thomas, J. H. (1998) Cell 94, 549–550