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Soluble Guanylate Cyclases Act in Neurons Exposed to the Body Fluid to Promote *C. elegans* Aggregation Behavior

Benny H.H. Cheung, Fausto Arellano-Carbajal, Irene Rybicki, and Mario de Bono*
The Medical Research Council Laboratory of Molecular Biology
Hills Road
Cambridge CB2 2QH
United Kingdom

Summary

The genome of the nematode *Caenorhabditis elegans* encodes seven soluble guanylate cyclases (sGCs) [1]. In mammals, sGCs function as α/β heterodimers activated by gaseous ligands binding to a haem prosthetic group [2, 3]. The principal activator is nitric oxide, which acts through sGCs to regulate diverse cellular events. In *C. elegans* the function of sGCs is mysterious: the worm genome does not appear to encode nitric oxide synthase, and all *C. elegans* sGC subunits are more closely related to mammalian β than α subunits [1]. Here, we show that two of the seven *C. elegans* sGCs, GCY-35 and GCY-36, promote aggregation behavior. *gcy-35* and *gcy-36* are expressed in a small number of neurons. These include the body cavity neurons AQR, PQR, and URX, which are directly exposed to the blood equivalent of *C. elegans* and regulate aggregation behavior [4]. We show that GCY-35 and GCY-36 act as α-like and β-like sGC subunits and that their function in the URX sensory neurons is sufficient for strong nematode aggregation. Neither GCY-35 nor GCY-36 is absolutely required for *C. elegans* to aggregate. Instead, these molecules may transduce one of several pathways that induce *C. elegans* to aggregate or may modulate aggregation by responding to cues in *C. elegans* body fluid.

Results and Discussion

Mutations in the Soluble Guanylate Cyclases GCY-35 and GCY-36 Disrupt Social Feeding

Wild races of *C. elegans* feed on bacteria either alone or in groups [5]. The behavioral variation between "solitary" and "social" feeding races is associated with two natural isoforms of the G protein-coupled neuropeptide receptor NPR-1 [5], which have different ligand responses [6]. Null mutants of *npr-1* aggregate strongly on bacterial food, and NPR-1 acts partly in neurons exposed to the body fluid of the animal to inhibit social feeding [4].

To unravel the molecular and neural networks that regulate *C. elegans* aggregation, we mutagenized social *npr-1* animals and screened for mutants that failed to aggregate into groups. One complementation group we identified, represented by six mutations, was disrupted in *gcy-36* (guanylate cyclase 36). A second complementation group represented by two alleles was disrupted in *gcy-35*. *gcy-35* and *gcy-36* are two of seven genes, *gcy-31* through *gcy-37*, that encode *C. elegans* homologs of mammalian soluble guanylate cyclases (sGC) [1]. We identified the lesions associated with all eight alleles of *gcy-35* and *gcy-36*. Four of the *gcy-36* alleles and both *gcy-35* mutations altered the guanylate cyclase active site (see Figure S1), suggesting that GCY-35 and GCY-36 function as guanylate cyclases. A further allele of *gcy-35, gcy-35(ok769)*, was recently isolated by the International *C. elegans* Gene Knockout Consortium. This allele deletes sequences encoding the guanylate cyclase active site. For our further studies we focused on the *gcy-35(ok769)* and *gcy-36(db66)* alleles, which are both predicted to be null (see Experimental Procedures and Supplemental Data for a molecular description of *gcy* alleles). Both *gcy-35(ok769)* and *gcy-36(db66)* mutations strongly disrupted aggregation of *npr-1* animals (Figure 1A). The mutations also suppressed the preference of *npr-1* animals for the edge of the *E. coli* food lawn, where bacteria grow thickest; we call this phenotype "bordering" (Figures 1B and S2). Thus these soluble guanylate cyclases regulate both aggregation and bordering behaviors in *C. elegans*.

npr-1 mutants and wild aggregating races of *C. elegans* show an additional difference from solitary races in their locomotory response to food. Individuals from solitary races respond to food by strongly reducing movement, whereas animals from social strains continue to move rapidly upon encountering food [5]. This behavioral polymorphism is highly reminiscent of rover and sitter foraging variants of wild *Drosophila* that are associated with different alleles of the cGMP-dependent protein kinase *forager* [7]. The high locomotory rate on food of *C. elegans* aggregating strains requires the activity of a cGMP-gated ion channel [4] and might therefore also require GCY-35 and GCY-36 function. However, *gcy-35; npr-1* and *npr-1 gcy-36* animals did not strongly reduce their locomotion in response to food and, in this regard, behaved more like social animals than individuals from solitary wild strains such as the standard N2 strain [5, 8] (Figure 1C). Thus the GCY-35 and GCY-36 guanylate cyclases promote the aggregation and bordering behavior of *npr-1* animals but are not required for their high locomotory activity on food.

gcy-35 and *gcy-36* mutants displayed robust responses in other behavioral paradigms: they showed good olfactory behavior, appropriately avoided regions of high osmotic tension, and mated well (see Figure S3 and data not shown). Finally, *gcy-35; npr-1* and *npr-1 gcy-36* animals behaved similarly to each other and to *gcy-35; npr-1 gcy-36* triple mutants in all of our assays, suggesting that *gcy-35* and *gcy-36* may act in the same pathway to promote *C. elegans* aggregation and accumulation on thick bacterial food.

GCY-35 and GCY-36 Are Expressed in the Body Cavity Neurons

To examine where *gcy-35* and *gcy-36* function to regulate aggregation and bordering behaviors, we fused the

*Correspondence: debono@mrc-lmb.cam.ac.uk

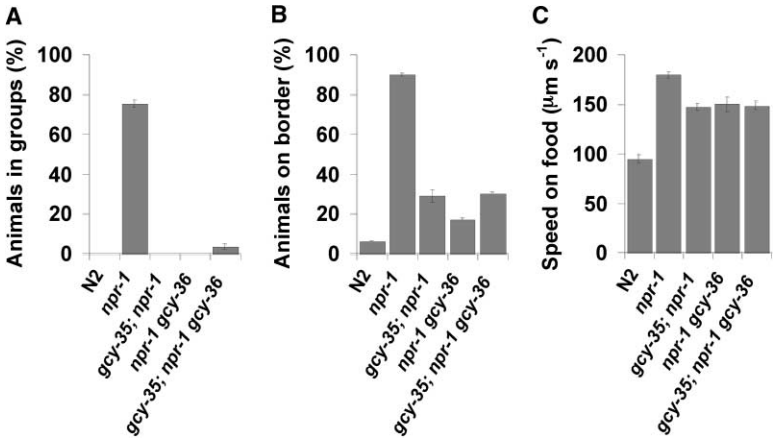

Figure 1. Mutations in *gcy-35* or *gcy-36* Disrupt Aggregation and Bordering Behaviors in *C. elegans*

(A and B) Mutations in *gcy-35* or *gcy-36* disrupt aggregation of *npr-1* animals on food (A) and accumulation of *npr-1* animals at the border of the bacterial lawn (B). In both (A) and (B) $p < 0.001$ for comparisons between *npr-1* and all other strains.

(C) *npr-1* mutants show high locomotory activity on food compared to N2 animals, even in the absence of *gcy-35* and *gcy-36* activity. $p < 0.001$ for comparisons between N2 and all other strains.

upstream promoter regions of these genes to DNA encoding green fluorescent protein (GFP) and used these expression constructs to make transgenic animals. Both fusion constructs were expressed in a small set of neurons: *gcy-36* was expressed in the body cavity neurons AQR, PQR, and URX, whereas *gcy-35* was expressed in AQR, PQR, and URX, in the interneurons SDQL/R, the mechanosensory neuron AVM, the putative sensory neurons ALN and PLN, and occasionally in one other unidentified cell (Figures 2A–2C). For *gcy-36*, we next created a polycistronic construct in which the *gcy-36* promoter was used to express an operon consisting of *gcy-36* cDNA and *gfp*. The *pgcy-36::gcy-36::gfp* operon restored aggregation and bordering behaviors to *npr-1 gcy-36* animals (data not shown) and recapitulated the expression pattern in AQR, PQR, and URX observed from the *gcy-36* promoter construct, suggesting that GCY-36 acts in these neurons to promote these behaviors.

GCY-35 and GCY-36 Function in the URX Body Cavity Neurons to Promote Aggregation and Bordering Behaviors

Previous work has suggested that a cGMP-gated ion channel encoded by the *tax-2* and *tax-4* genes is required in one or more of the AQR, PQR, and URX body cavity neurons for social feeding [4]. To examine if GCY-35 and GCY-36 both act in these neurons to mediate aggregation behavior, we made constructs in which we expressed *gcy-35::gfp* and *gcy-36::gfp* operons under the control of the *gcy-32* and the *flp-8* promoters. The *gcy-32* promoter is expressed exclusively in the AQR, PQR, and URX neurons [9], whereas the *flp-8* promoter is expressed in the URX, AUA, and PVM neurons [4, 30]. Both the *pgcy-32::gcy-35::gfp* and the *pflp-8::gcy-35::gfp* constructs restored strong aggregation and bordering behaviors to *gcy-35; npr-1* animals (Figure 2D). Similarly, expression of *pgcy-32::gcy-36::gfp* or *pflp-8::gcy-36::gfp* constructs restored aggregation and bordering to *gcy-36; npr-1* mutants (Figure 2E). The transgenes all showed GFP expression in the expected neurons (data not shown). These results suggest that GCY-35 and GCY-36 function in the body cavity neurons AQR, PQR, and URX to promote these behaviors. Moreover, the results with the *flp-8* promoter constructs suggest that GCY-35 and GCY-36 activity in the URX neurons alone is sufficient for strong aggregation and bordering, although we cannot exclude the possibility that AQR and PQR function redundantly with URX.

A Predicted C-Terminal Isoprenylation Site Is Important for GCY-36 Function

Like the rat β2 sGC that they most closely resemble [1], GCY-35 and GCY-36 have a CAAX sequence (cysteine, aliphatic, aliphatic, any amino acid) at their carboxy terminus, suggesting that the cysteine residue may be isoprenylated and the proteins targeted to membranes. To investigate this possibility, we engineered a fusion protein in which GFP was added to the N terminus of GCY-36. Expression of this fusion under the control of the *gcy-36* promoter restored social feeding to *npr-1 gcy-36* animals, suggesting it was functionally active (Figure 2E). Interestingly, the GFP fusion protein was enriched in the sensory cilia of AQR and PQR neurons (URX neurons are not ciliated), although it was expressed at some level throughout the neuron (Figures 2F and 2G). Previous data have suggested that TAX-2/TAX-4 cGMP-gated ion channels are also enriched at sensory cilia [10, 11]. Disrupting the CAAX box by mutating the cysteine residue to a serine disrupted the ability of the *gfp:gcy-36* transgene to restore social feeding to *npr-1 gcy-36* mutant animals (Figure 2E) and prevented enrichment at the cilia (Figures 2H and 2I). These data suggest that isoprenylation and appropriate subcellular localization may be important for GCY-36 function. Recent data suggest that the mammalian α1/β1 and α2/β1 sGCs are both partly localized to the membrane [12, 13]. For the α1/β1 sGC, membrane localization appears to enhance the sensitivity of the cyclase to nitric oxide stimulation [13]. Membrane localization may also be important for activation of GCY-35 and GCY-36 cyclases.

GCY-35 and GCY-36 Act as α and β Soluble Guanylate Cyclase Subunits, Respectively

Mammalian sGC function as α/β heterodimers [14]. However, the seven *C. elegans* soluble guanylate cyclases all appear more closely related to mammalian β than α subunits [1], raising the possibilities that they act

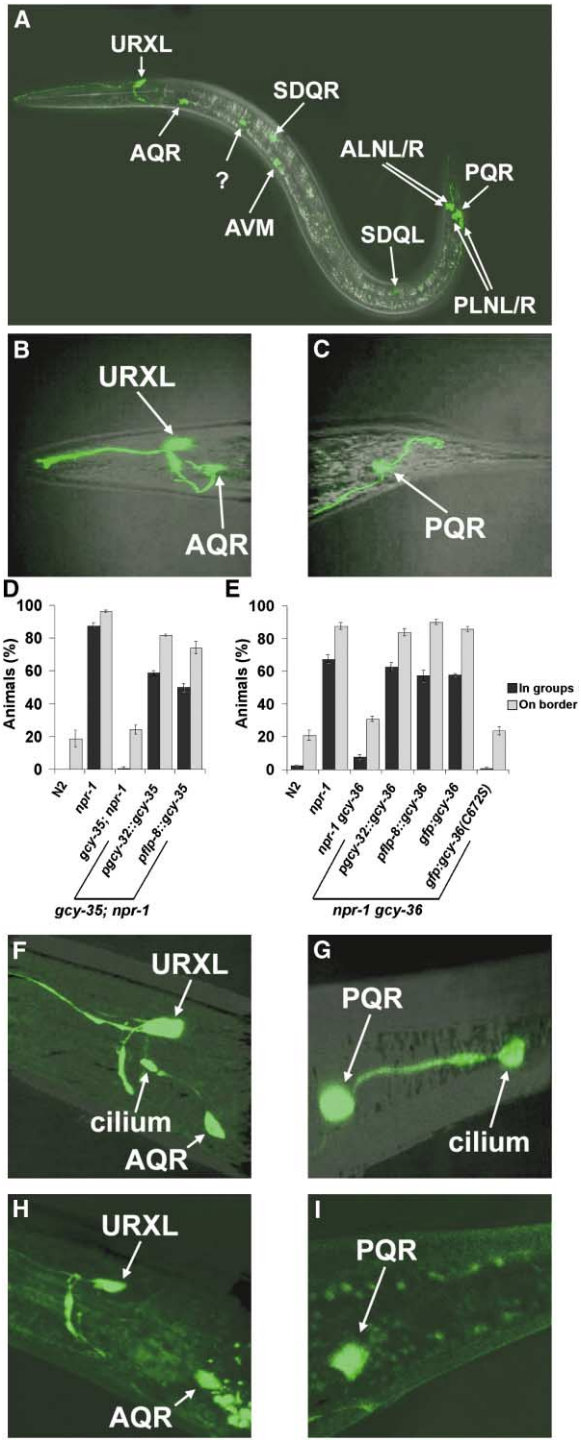

Figure 2. GCY-35 and GCY-36 Activity in the URX Body Cavity Neurons Is Sufficient to Promote *C. elegans* Social Feeding

(A–C) Both *gcy-35* (A) and *gcy-36* (B and C) reporters are expressed in the neurons AQR, PQR, and URX. These neurons are directly exposed to the body fluid of *C. elegans*. *gcy-35* expression is also seen in SDQL/R, ALN, PLN, AVM, and occasionally in another unidentified cell.

(D and E) Targeted expression of *gcy-35* in AQR, PQR, and URX by using the *gcy-32* promoter or in URX by using the *flp-8* promoter is sufficient to restore social feeding behavior to *gcy-35; npr-1* animals (D). Similarly, targeted expression of *gcy-36* from the *gcy-32* or *flp-8* promoter restores social feeding to *gcy-36; npr-1* animals.

either as homodimers or as β-β′ heterodimers [2]. Since GCY-35 and GCY-36 appeared to function in the same neurons for aggregation and bordering behaviors and were required nonredundantly, we speculated that these two proteins might act in heterodimeric complexes that are functionally similar to human α1/β1 sGC heterodimers. Human α1/β1 sGC heterodimers are thought to form a single active site to which each subunit contributes different residues (Figure 3A) [15]. The α subunit contributes an aspartate residue that is thought to act as a catalytic base, whereas the β subunit contributes an asparagine residue proposed to stabilize an oxyanion-like nucleophile (Figure 3A) [15]. To test the hypothesis that GCY-35 and GCY-36 function in α/β-like heterodimer complexes, we mutated amino acids corresponding to these active site residues in *gcy-35* and *gcy-36* and assayed the mutant proteins for their ability to restore social feeding in the absence of endogenous wild-type protein. Substituting the α subunit catalytic base aspartate residue with alanine did not disrupt the rescuing activity of GCY-36 (D499A) but abolished GCY-35 rescuing activity (D473A) (Figures 3B and 3C). Conversely, substituting the β subunit oxyanion-stabilizing asparagine residue with alanine strongly reduced rescuing activity in GCY-36 (N572A), but not in GCY-35 (N546A) (Figures 3B and 3C). These data suggest that GCY-36 functions as a β-like subunit, whereas GCY-35 acts as an α-like subunit in heterodimeric complexes that upregulate aggregation behavior.

Interestingly, none of the active site mutants of GCY-35 or GCY-36 displayed a dominant-negative phenotype: *npr-1* mutants carrying these transgenes retained strong aggregation and bordering phenotypes (data not shown), perhaps because expression levels were not high enough to outcompete endogenous wild-type protein.

GCY-35 and GCY-36 could function together as an α/β heterodimer. Alternatively they could form complexes with one or more of the other five *C. elegans* soluble guanylate cyclases; at least one of these, GCY-32, is also expressed in AQR, PQR, and URX[9]. To test this possibility, we built double mutant strains between *npr-1* and deletion mutations recently isolated by the International *C. elegans* Gene Knockout Consortium for four of these genes (*gcy-31*, *gcy-32*, *gcy-33* and *gcy-34*) (we are grateful to the consortium for these mutants; see Supplemental Data for details of deletions). To our knowledge no knockout strain is available for *gcy-37*. None of these *gcy* mutations disrupted aggregation or

Expressing N terminally GFP-tagged GCY-36 from the *gcy-36* promoter also restores aggregation and bordering, but this rescue is disrupted if the CAAX box is mutated (E). In this and subsequent figures, the genotype to which transgenes were added is indicated under the bracket. In (D) and (E), with the exception of the *gfp:gcy-36(C672S)* transgene, $p < 0.001$ for comparisons between *gcy; npr-1* transgenic animals and nontransgenic controls for both aggregation and bordering behaviors.

(F–I) GFP-tagged GCY-36 is enriched in sensory cilia of AQR (F) and PQR (G). URX neurons are not ciliated. This enrichment is dependent on an intact C-terminal isoprenylation site: mutating this site disrupts GCY-36 rescuing activity (E) and prevents GCY-36 accumulation in cilia (H and I).

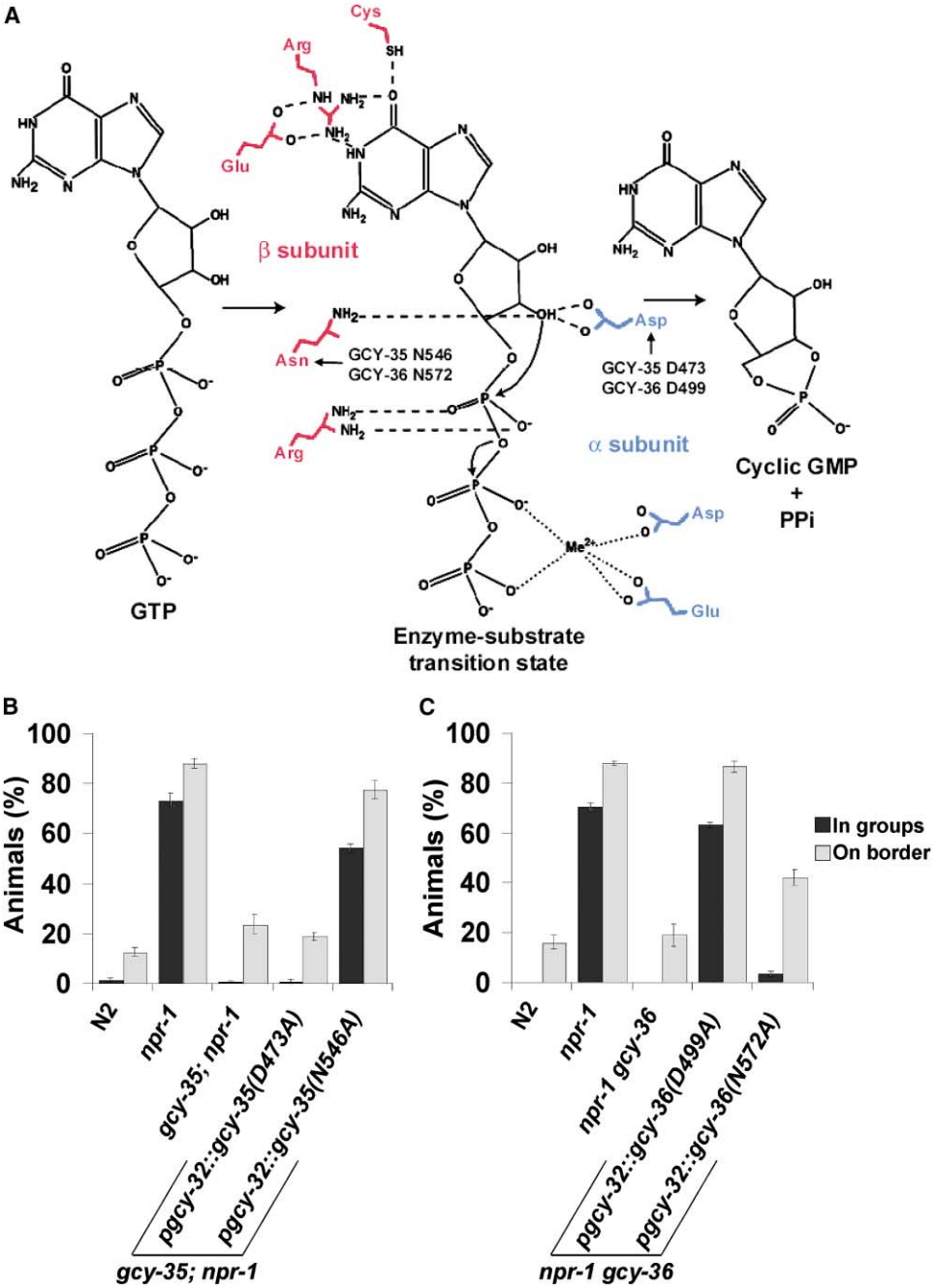

Figure 3. GCY-35 Functions as an α-like Subunit and GCY-36 as a β-like Subunit in Heterodimeric Complexes

(A) Proposed catalytic mechanism for guanylate cyclases [3, 15] highlighting corresponding active site residues in GCY-35 and GCY-36.

(B and C) Site-specific mutation of the aspartate residue contributed by the α subunit and proposed to act as a catalytic base disrupts GCY-35, but not GCY-36, rescuing activity. Conversely, site-specific mutation of the asparagine residue contributed by the β subunit and proposed to stabilize an oxyanion-like nucleophile disrupts GCY-36, but not GCY-35, function. For location of active site residues in GCY-35 and GCY-36, see Figure S1. In (B), for both aggregation and bordering, $p > 0.05$ for a comparison of *gcy-35; npr-1* with *gcy-35; npr-1; Ex[pgcy-32::gcy-35(D473A)]* and $p < 0.001$ for a comparison of *gcy-35; npr-1* with *gcy-35; npr-1; Ex[pgcy-32::gcy-35(N546A)]*. In (C), for both aggregation and bordering, $p < 0.001$ for a comparison of *gcy-36; npr-1* with *gcy-36; npr-1; Ex[pgcy-32::gcy-36(D499A)]*. For a comparison of aggregation behavior, $p > 0.05$ between *gcy-36; npr-1* and *gcy-36; npr-1; Ex[pgcy-32::gcy-36(N572A)]*. However, $p < 0.05$ for a similar comparison for bordering, suggesting that the *pgcy-32::gcy-36(N572A)* transgene can weakly rescue bordering behavior.

bordering behavior of *npr-1* animals (data not shown). These results suggest that of the six sGC tested, only GCY-35 and GCY-36 are required for aggregation of *npr-1* animals under our assay conditions, consistent with GCY-35 and GCY-36 functioning together as a heterodimer. However, we cannot exclude the possibility

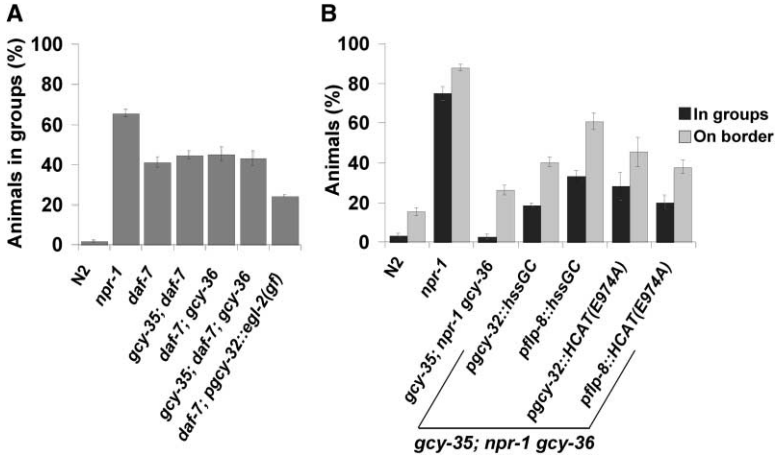

Figure 4. GCY-35 and GCY-36 Are Not Absolutely Required for Aggregation Behavior, and Expression of a Constitutively Active Guanylate Cyclase in Body Cavity Neurons Can Partially Restore Aggregation to *gcy-35; npr-1 gcy-36* Mutant Animals

(A) Aggregation of *daf-7* animals on a lawn of *E. coli* is not disrupted by *gcy-35* or *gcy-36* mutations ($p > 0.05$) and is only partially suppressed by expression of a constitutively active K^+ channel, EGL-2(gf), in AQR, PQR, and URX.

(B) Coexpression of human α1 and β1 sGC subunits (*hssGC*) or of a constitutively active fragment of the A type natriuretic peptide receptor guanylate cyclase; HCAT(E974A) in AQR, PQR, and URX; or URX can partially restore aggregation behavior to *gcy-35; npr-1 gcy-36* mutant animals. HCAT(E974A) corresponds to a dominantly active soluble fragment of the A type natriuretic peptide (ANP) receptor. It includes the hinge and catalytic regions of the receptor with an E974A mutation that makes the fragment constitutively active [19, 28]. In (B), for both bordering and aggregation, $p < 0.01$ between *gcy-35; npr-1 gcy-36* in the presence and absence of the *hssGC* transgenes. For the *HCAT(E974A)* transgenes, $p < 0.05$ for aggregation when transgenic animals are compared to nontransgenic controls; however, bordering behavior is not significantly restored by HCAT(E974A).

that two or more of the other *gcy* genes are redundantly required for aggregation behavior and form heterodimers with GCY-35 and GCY-36.

Neither GCY-35 Nor GCY-36 Are Required for Aggregation of *daf-7 TGF-*β Mutant Animals

We next examined whether aggregation behavior was always dependent on *gcy-35* and *gcy-36* function by looking at *daf-7* mutants. DAF-7 TGF-β acts in a parallel pathway to NPR-1 to suppress social feeding: *daf-7* mutants aggregate to feed in groups [16], although less strongly than *npr-1* animals, and *daf-7; npr-1* double mutants aggregate more strongly than either mutant alone [17]. We found that *daf-7* mutants lacking *gcy-35*, *gcy-36*, or both aggregated indistinguishably from *daf-7* animals in response to bacterial food (Figure 4A). Thus GCY-35 and GCY-36 are not always required for aggregation.

To investigate further whether activity of the body cavity neurons was required for aggregation of *daf-7* animals, we inhibited the activity of these neurons by expressing an activated form of the *ether-a-go-go* (*eag*) K^+ channel EGL-2(gf) [18] from the *gcy-32* promoter. Expression of EGL-2(gf) in body cavity neurons strongly suppresses social feeding of *npr-1* mutant animals [4]. In contrast, aggregation of *daf-7* animals was only weakly suppressed by the same transgene (Figure 4A), suggesting that aggregation behavior can be largely independent of the activity of the AQR, PQR, and URX body cavity neurons in some circumstances.

Constitutively Active Guanylate Cyclase Activity Partially Restores Aggregation Behavior to *gcy-35; npr-1 gcy-36* Animals

Our results with *daf-7* mutants indicated that GCY-35 and GCY-36 are not absolutely required for animals to aggregate together. To investigate the roles of these genes further, we asked whether constitutive guanylate cyclase activity in the body cavity neurons might be sufficient to restore social feeding to *gcy-35; npr-1 gcy-36* mutant animals. First, we coexpressed human α1 and β1 soluble guanylate cyclase subunits in AQR, PQR, and URX or URX from the *gcy-32* and *flp-8* promoters. The human α1/β1 sGC is usually activated by nitric oxide [2, 14], but since *C. elegans* does not appear to encode nitric oxide synthase, these subunits would be expected to have only a basal level of activity. For both promoter constructs, transgene expression restored significant aggregation behavior to *gcy-35; npr-1 gcy-36* triple mutant animals (Figure 4B). We next used the same promoters to express a constitutively active fragment of the A type natriuretic peptide (ANP) receptor guanylate cyclase, HCAT(E974A) [19], in *gcy-35; npr-1 gcy-36* mutant animals (see Experimental Procedures). The ANP receptor belongs to a separate family of guanylate cyclases from soluble guanylate cyclases, the transmembrane guanylate cyclases. It does not bind haem and is activated by a peptide rather than a gaseous ligand. The ANP receptor fragment we used, HCAT, includes the hinge and catalytic regions of the protein and is soluble. Both *pgcy-32::HCAT(E974A)::gfp* and *pflp-8::HCAT(E974A)::gfp* transgenes restored significant aggregation behavior to *gcy-35; npr-1 gcy-36* animals (Figure 4B).

In a further set of experiments, we tested whether overexpression of the human soluble guanylate cyclase transgenes or of the HCAT(E974A) guanylate cyclase in the body cavity neurons of *npr-1* mutant animals, using the *gcy-32* or *flp-8* promoters, would disrupt aggregation behavior. Neither transgene reduced the ability of *npr-1* mutants to aggregate (data not shown).

Together, these data suggest that regulated cGMP production from GCY-35 and GCY-36 is not absolutely required for *C. elegans* to aggregate. Constitutively active guanylate cyclase activity would otherwise not be expected to restore aggregation behavior to *gcy-35; npr-1 gcy-36* animals. One possibility is that several neural pathways mediate *C. elegans* aggregation; GCY-

35 and GCY-36 are required for one pathway, but their absence can be compensated for in some circumstances. Another possibility is that GCY-35 and GCY-36 do not mediate aggegation per se but transduce a contextual signal; for example, a signal in the body fluid that promotes aggregation.

In mammals, sGCs are activated when a gaseous ligand, usually nitric oxide (NO), binds to the iron of the haem prosthetic group. This binding breaks a bond between the haem and a conserved histidine residue, activating the enzyme [20]. All seven *C. elegans* sGCs conserve the haem binding histidine residue. However, since *C. elegans* appears to lack NO synthase and NO fails to stimulate cGMP production in homogenates of *C. elegans* [1], GCY-35 and GCY-36 are likely to be activated by a ligand other than NO [1]. Potential other ligands are carbon monoxide and oxygen, both of which bind haem proteins [21].

Our data indicate that GCY-35 and GCY-36 activity in the URX neurons is sufficient to promote aggregation and bordering behaviors. One of the main synaptic outputs of URX is to the AUA and RIA interneurons, via exclusively dyadic synapses [22]. Previous results indicate that the NPR-1 neuropeptide receptor is expressed in both the URX and the AUA neurons and that targeted expression of NPR-1 in AUA, AQR, PQR, and URX is more potent at suppressing aggregation of *npr-1* mutant animals than NPR-1 expression in AQR, PQR, and URX alone [4]. Thus the URX, AUA, and RIA neurons may form a circuit that modulates aggregation behavior. We speculate that activation of the GCY-35/GCY-36 sGC in URX leads to depolarization of this neuron by opening the TAX-2/TAX-4 cGMP-gated ion channel. It will be interesting to investigate the effect of this depolarization on the activity of the postsynaptic AUA and RIA neurons and to explore how these neurons impinge on the downstream circuitry coordinating aggregation behavior.

Experimental Procedures

Strains

Nematodes were grown at 20°C under standard conditions [23]. The *gcy-35* (*db113* and *db117*) and *gcy-36* (*db39*, *db42*, *db65, db66, db67*,and *db110*) alleles were generated from a mutagenesis screen for mutations that disrupt aggregation behavior. Details of the screen will be described elsewhere (B.H.H.C., N. Tremain, and M.d.B., unpublished data). *gcy-35* and *gcy-36* mutations were mapped by using single nucleotide polymorphisms between the N2 (Bristol) and the CB4856 (Hawaii) strains following established protocols [24]. Once the mutations had been mapped to small genomic intervals, candidate genes were sequenced and lesions identified in all alleles of the complementation group (see Figure S1 for details). The *gcy-35(ok769)* allele was obtained from the International *C. elegans* Gene Knockout Consortium. It deletes 668 bp, corresponding to sequences from 31962–32629 in cosmid T04D3. This removes the GCY-35 active site and is predicted to be a null allele (see Figure S1). The *ok769* deletion junction sequence is TTTATCTTCGTT/AACGTGGCGAAC.

Double mutant strains were constructed by using balancer chromosomes in an N2 background. Strains used or generated in this study are listed in the Supplemental Data. Germline transformation was carried out as described [25] by using *lin-15* (pJMZ, 30 ng μl^{-1}) [26] as a coinjection marker, except for the *gcy-35* transgenic strains in Figure 2D in which *punc-122::gfp* [29] was used as a coinjection marker. Strains injected were AX206 *lin-15(n765ts) X*, AX1265 *gcy-35(ok769) I; npr-1(ad609) lin-15(n765) X*, AX48 *npr-1(ad609) gcy-36(db66) lin-15(n765) X*, and AX1266 *gcy-35(ok769) I; npr-1(ad609) gcy-36(db66) lin-15(n765) X*. Test DNA was injected at 50 ng μl^{-1}. At least two transgenic lines were assayed for each construct; the figures show data from one representative line. Strains carrying *promoter::gene* transgenes were examined by Nomarski and fluorescence confocal microscopy (Biorad Radiance confocal head on a Nikon E800 microscope) to confirm GFP expression in the appropriate cells.

Behavioral Assays

Aggregation and bordering behaviors on food were quantified as described previously [5]. Speed of locomotion was quantified from 5 min video recordings of behaving animals with the DIAS program, as previously described [5]. All behavioral data represent means of at least four and usually six or more assays carried out over several days. Statistical significance was determined using the two-tailed t test. In all figures, error bars represent the standard error of the mean.

Molecular Biology

General molecular manipulations followed standard protocols [27]. The sequences of the *gcy-35* and *gcy-36* genomic regions were established by the *C. elegans* Sequencing Consortium. Reporter transgene constructs (*promoter::gfp*) for *gcy-35* and *gcy-36* were made by inserting PCR-amplified promoter fragments corresponding to 3.2 kb upstream of the *gcy-35* ATG start site and 2 kb upstream of the *gcy-36* ATG start site into pPD95.75 and pDest 49.26, respectively. *gcy-35* and *gcy-36* cDNA were obtained by RT-PCR.

gcy-35, *gcy-36*, *egl-2(gf)*, *hs sGC*α*1/*β*1*, and *HCAT(E974A)* expression constructs were generated with the Gateway system (Invitrogen) [4]. A library of destination vectors was created to target expression of genes of interest to specific *C. elegans* cells. The promoters used (with neuronal expression in parentheses) were *pgcy-32* (AQR, PQR, and URX) and *pflp-8* (AUA, URX, and PVM). Entry vectors were designed to place the gene of interest in an artificial operon with DNA encoding GFP[4]. To construct the *gfp*-tagged *gcy-36* construct (Figures 2F–2I), *gfp* sequences in a pEntr *gcy-36::gfp* vector was first excised, and a *gfp* fragment amplified by PCR cloned into this pEntr *gcy-36* vector 5′ of *gcy-36*. This resulted in the in-frame insertion of GFP upstream of the ATG start codon of *gcy-36*. Site directed mutageneses (Figures 2E, 3B, and 3C) were carried out by using Quikchange™ (Stratagene). Further details of plasmid construction are available on request.

Supplemental Data

Supplemental Data including Experimental Procedures and three figures are available at http://www.current-biology.com/cgi/content/full/14/12/1105/DC1/.

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