

Neurotransmitter signaling through heterotrimeric G proteins: insights from studies in *C. elegans*

Michael R. Koelle[§]

Department of Molecular Biophysics & Biochemistry, Yale University, New Haven CT
06520 USA

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[§]To whom correspondence should be addressed. E-mail: michael.koelle@yale.edu

Abstract

Neurotransmitters signal via G protein coupled receptors (GPCRs) to modulate activity of neurons and muscles. *C. elegans* has ~150 G protein coupled neuropeptide receptor homologs and 28 additional GPCRs for small-molecule neurotransmitters. Genetic studies in *C. elegans* demonstrate that neurotransmitters diffuse far from their release sites to activate GPCRs on distant cells. Individual receptor types are expressed on limited numbers of cells and thus can provide very specific regulation of an individual neural circuit and behavior. G protein coupled neurotransmitter receptors signal principally via the three types of heterotrimeric G proteins defined by the G alpha subunits $\text{G}\alpha_o$, $\text{G}\alpha_q$, and $\text{G}\alpha_s$. Each of these G alpha proteins is found in all neurons plus some muscles. $\text{G}\alpha_o$ and $\text{G}\alpha_q$ signaling inhibit and activate neurotransmitter release, respectively. $\text{G}\alpha_s$ signaling, like $\text{G}\alpha_q$ signaling, promotes neurotransmitter release. Many details of the signaling mechanisms downstream of $\text{G}\alpha_q$ and $\text{G}\alpha_s$ have been delineated and are consistent with those of their mammalian orthologs. The details of the signaling mechanism downstream of $\text{G}\alpha_o$ remain a mystery. Forward genetic screens in *C. elegans* have identified new molecular components of neural G protein signaling mechanisms, including Regulators of G protein Signaling (RGS proteins) that inhibit signaling, a new $\text{G}\alpha_q$ effector (the Trio RhoGEF domain), and the RIC-8 protein that is required for neuronal $\text{G}\alpha$ signaling. A model is presented in which G proteins sum up the variety of neuromodulator signals that impinge on a neuron to calculate its appropriate output level.

1. Introduction

The nervous system functions through the use of neurotransmitters that act as chemical signals between cells. Small-molecule neurotransmitters such as acetylcholine, glutamate, and GABA (gamma aminobutyric acid) are released from vesicles clustered at synapses. Neuropeptides (secreted proteins of <50 amino acids) are released at synaptic and non-synaptic sites from a different class of vesicles, known as dense-core vesicles for their appearance in the electron microscope (Merighi et al., 2011: PMID 21385606). Certain small-molecule neurotransmitters such as serotonin can be released from either class of vesicle. All these types of neurotransmitters act on neurons and muscles to generate dynamic patterns of activity that constitute thoughts and behaviors. A principal objective in neuroscience is to understand the molecular mechanisms by which neurons and muscles respond to neurotransmitters. This objective is important to advance our basic science understanding of the brain, but also is of great medical significance since many pharmaceuticals used to treat psychiatric disorders act by mimicking, antagonizing, or altering the levels of naturally-occurring neurotransmitters (Conn and Roth, 2008: PMID 18216778), and drugs of abuse also act by altering neurotransmitter signaling (Joffe et al., 2014: PMID 24999377).

1.1. Neurotransmitters signal by gating ion channels and by activating G protein coupled receptors

Neurotransmitters signal via two distinct classes of receptors, known within the neuroscience field as ionotropic and metabotropic receptors. Ionotropic receptors are neurotransmitter-gated ion channels, and most small-molecule neurotransmitters each have a number of such receptors (Lemoine et al., 2012: PMID 22988962). Binding of neurotransmitter to an ionotropic receptor favors channel opening, and communication between neurons using ionotropic receptors can occur in less than a millisecond (Sabatini and Regehr, 1996: PMID 8906792). Metabotropic receptors are known outside the neuroscience field as G protein coupled receptors (GPCRs) because they activate intracellular signaling proteins called heterotrimeric G proteins. All small-molecule neurotransmitters have G protein coupled receptors, as do most neuropeptides (Hall, 2004, PMID: 15125891; Beaulieu and Gainetdinov, 2011: PMID 21303898; Pytliaik et al., 2011: PMID 22530579; Hoyer and Bartfai, 2012: PMID 23161624; Vaidya et al., 2013: PMID 22530579; Kruse et al., 2014: PMID 24903776). An individual small-molecule neurotransmitter might have up to a dozen different GPCRs. There are over 100 neuropeptide genes in both humans and *C. elegans*, and each organism also has about an equal number of GPCRs that are likely to be neuropeptide receptors, as they are similar to the few G protein coupled neuropeptide receptors that have been characterized so far (Li and Kim, 2008: PMID 18819171; Li and Kim, 2010: PMID 21189676; Frooninckx et al., 2012: PMID: 23267347; Hoyer and Bartfai, 2012: PMID 23161624). Binding of a neurotransmitter to a GPCR, as opposed to an ionotropic receptor, leads to slower and longer lasting effects. For example, GPCR signaling can involve biochemical amplification of a signal (e.g. production of pool of second messenger) that is much slower than the rapid voltage changes induced by opening ion channels, and GPCR signaling can result in changes to the transcriptional program and structure of a neuron that last days or longer (Kandel, 2004: PMID 16134023). Whereas ionotropic receptors mediate signaling underlying such prosaic neural functions as the knee-jerk response, GPCRs mediate signaling underlying more poetical functions of the brain, such as feelings of pleasure and love that result from dopamine and oxytocin (Love, 2014: PMID 23850525), the psychedelic effects of hallucinogens (Fantegrossi et al., 2008: PMID 17977517), and the regulation of mood by serotonin (Donaldson et al., 2013: PMID 23385115).

1.2. *C. elegans* as a model system for studies of neural signaling

This review focuses on insights into the molecular mechanisms and biological functions of neurotransmitter signaling through GPCRs that arise from studies in the model organism *C. elegans*. But why study neurotransmitter signaling in the worm? Signaling through ionotropic receptors has already been studied in a sophisticated manner in other species using electrophysiological techniques to analyze ion channel activity. Such electrophysiological studies have often used model organisms such as slug, squid, leech, or crab that have large neurons easily accessible to electrodes.

GPCRs activate intracellular signaling pathways that can have indirect effects on ion channel activity, and electrophysiological studies in the same model organisms have given important insights into how GPCR signaling modulates the function of neural circuits (Bailey and Kandel, 2008: PMID 18394474; Marder, 2012: PMID 23040802). However, methods for studying intracellular signaling pathways have been most highly developed outside of the neuroscience field, with the greatest successes coming from applying a combination of biochemistry and genetics, techniques not easily applied in the model organisms suited to electrophysiology. A remarkable body of biochemical studies of signaling by heterotrimeric G proteins, mostly in non-neuronal mammalian cells, has been in progress for decades and has resulted in many Nobel prizes (Cori and Cori, 1947; Sutherland, 1971; Krebs, 1992; Fischer 1992; Gilman 1994; Rodbell 1994; Kobilka, 2013: PMID 23650120; Lefkowitz 2013: PMID 23650015). Prior to the studies in *C. elegans* described in this review, neural G protein signaling had not been seriously studied using genetic approaches. Thus, despite the excellence of the body of biochemical work on G protein signaling, there remained significant gaps in understanding the molecular mechanisms of this type of signaling in neurons, and also in achieving a big-picture understanding of how and why such signaling is used to control the activity of neural circuits.

As described in this review, the molecular mechanisms of neural G protein signaling are strongly conserved between humans and *C. elegans*, and *C. elegans* provides two advantages that complement the past electrophysiological and biochemical work on neural G protein signaling. First, the power of forward genetics in *C. elegans* has allowed new neural signaling proteins to be discovered. Second, the simplicity of the *C. elegans* nervous system, combined with the use of genetics, has allowed biological functions to be assigned to signaling by specific neurotransmitters acting through specific GPCRs on individual identified neurons. Enough such biological functions of neural G protein signaling have now been described that the long-elusive big-picture understanding of the whole purpose of this mode of neurotransmission is beginning to emerge.

1.3. The heterotrimeric G protein activity cycle

Here I outline the activity cycle for a generic heterotrimeric G protein as a prelude to diving more deeply into how specific GPCRs, G proteins, and their downstream signaling pathways regulate neural function in *C. elegans*. The G protein activity cycle proceeds through five states labeled in Figure 1. Most steps in the cycle were discovered and characterized through biochemical studies of mammalian proteins, but the RGS and RIC-8 proteins that catalyze the transitions between states 4 and 5 were discovered through studies in *C. elegans* and subsequently characterized in mammals.

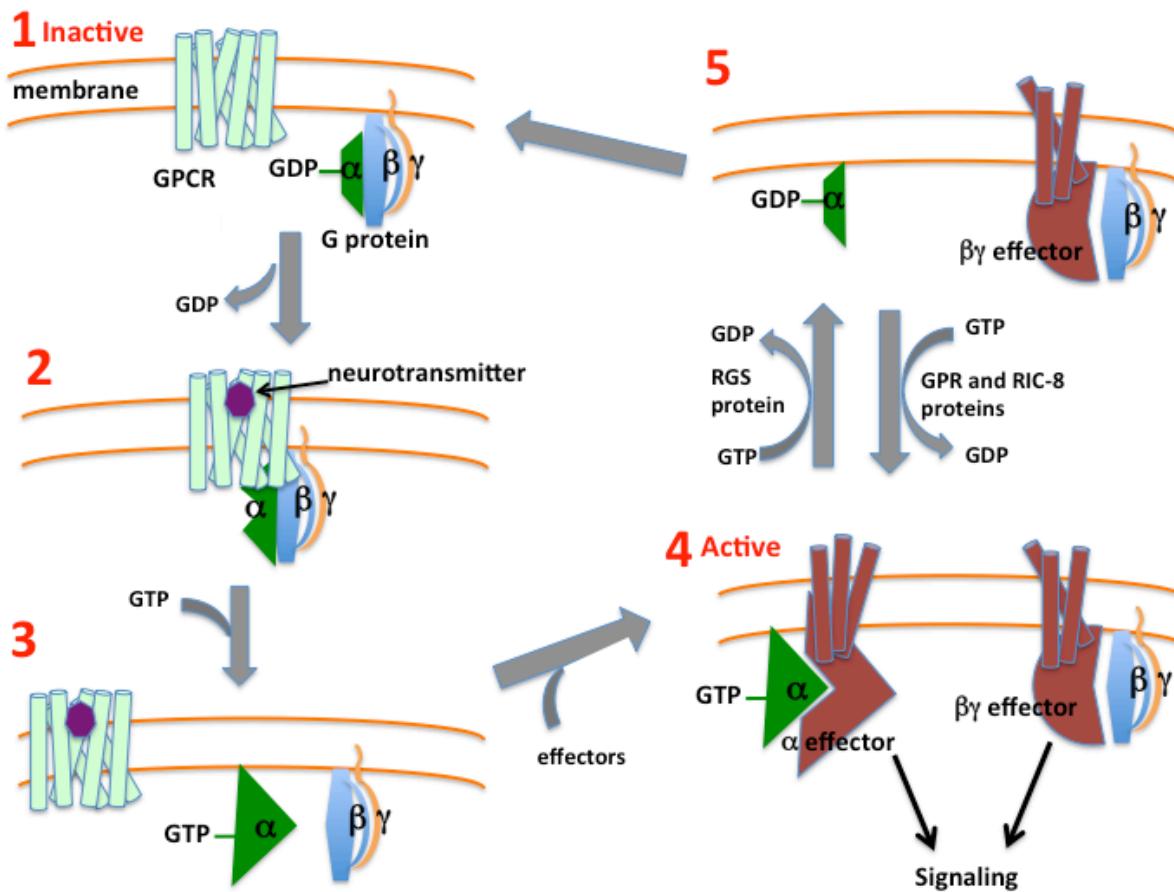


Figure 1. Activity cycle for a generic heterotrimeric G protein. In the inactive state (1), the G protein is an $\alpha\beta\gamma$ heterotrimer bound to GDP. When neurotransmitter binds the receptor (2), it assumes an active conformation that can bind the G protein heterotrimer and induce a conformational change in $\text{G}\alpha$ that releases GDP. GTP can then bind the open nucleotide site (3), inducing another conformational change in $\text{G}\alpha$ that releases both the receptor and $\text{G}\beta\gamma$. The separate $\text{G}\alpha\text{-GTP}$ and $\text{G}\beta\gamma$ complexes can then associate with their respective effector proteins (4), which are typically transmembrane protein complexes, activating those effectors for downstream signaling. $\text{G}\alpha$ signaling is terminated when a Regulator of G protein Signaling (RGS) protein induces $\text{G}\alpha$ to hydrolyze GTP (5), and $\text{G}\beta\gamma$ signaling is terminated when $\text{G}\beta\gamma$ re-associates with $\text{G}\alpha\text{-GDP}$ (1). The RIC-8 non-receptor nucleotide-exchange protein, working with GPR domain proteins, can prolong signaling by converting $\text{G}\alpha\text{-GDP}$ (5) back to $\text{G}\alpha\text{-GTP}$ (4). RIC-8 is also a chaperone required for $\text{G}\alpha$ folding and stability, which may be its principal function.

1.3.1. State 1, the inactive state

GPCRs are integral membrane proteins with seven transmembrane helices. Heterotrimeric G proteins have α , β , and γ subunits and are peripheral membrane proteins tethered to the inner leaflet of the plasma membrane by lipid modifications (Hepler and Gilman, 1992: PMID 1455506; Wedegaertner et al., 1995: PMID 7822269). The $\text{G}\alpha$ subunit changes conformation depending on whether it is bound to GDP, GTP, or no guanine nucleotide, and these conformational changes allow $\text{G}\alpha$ to alter its

association with other proteins (Noel et al., 1993: PMID 8259210; Coleman et al., 1994: PMID 8073283; Lambright et al., 1994: PMID 8208289; Wall et al., 1995: PMID 8521505). In its GDP-bound inactive state, $G\alpha$ is typically bound to $G\beta\gamma$ to form an inactive G protein heterotrimer.

1.3.2. State 2, activated GPCR promotes release of GDP by the $G\alpha$

Neurotransmitter binding induces conformational shifts in GPCRs that have recently been delineated by X-ray crystallographic studies (Katritch et al., 2013: PMID 23140243). GPCRs and G proteins are able to diffuse laterally due to the fluid nature of biological membranes, and a neurotransmitter-bound, activated receptor can thus collide with an inactive G protein heterotrimer. This interaction induces a large conformational change in $G\alpha$ that allows the release of bound GDP (Fung et al., 1981: PMID 6264430); Brandt and Ross, 1986: PMID 2868003; Rasmussen et al., 2011: PMID 21772288). The active GPCR and nucleotide-free G protein heterotrimer form a stable complex that dissociates in the next step of the G protein cycle.

1.3.3. State 3, GTP binding induces dissociation of GPCR, $G\alpha$, and $G\beta\gamma$

The open nucleotide-binding site on $G\alpha$ can bind GTP which causes $G\alpha$ to undergo another conformational change that causes $G\alpha$ -GTP to dissociate from both the receptor and $G\beta\gamma$ (Noel et al., 1993: PMID 8259210; Coleman et al., 1994: PMID 8073283; Lambright et al., 1994: PMID 8208289; Wall et al., 1995: PMID 8521505). The release of GDP and subsequent GTP binding by $G\alpha$ is referred to as “nucleotide exchange”. The receptor, which remains bound to neurotransmitter, can diffuse laterally in the membrane and proceed to promote nucleotide exchange on additional G proteins. Thus the active receptor can function as an enzyme to catalytically promote conversion of inactive $G\alpha\beta\gamma$ -GDP to the active signaling species $G\alpha$ -GTP and $G\beta\gamma$ (Ross, 2014: PMID 25279250).

1.3.4. State 4, $G\alpha$ -GTP and $G\beta\gamma$ signal by forming stable complexes with effectors

The separated $G\alpha$ -GTP and $G\beta\gamma$ complexes are able to stably bind to and regulate activity of other proteins to promote responses in the cell, and these other proteins are collectively termed G protein “effectors”. Effectors have been identified for $G\beta\gamma$ (Reuveny et al., 1994: PMID 8022483; Herlitze et al., 1996: PMID 8637576) and for the $G\alpha$ isoforms $G\alpha_s$ (Sunahara et al., 1996: PMID 8725398), $G\alpha_q$ (Kadmur and Ross, 2013: PMID 23140367I; Rohas et al., 2007: PMID 17606614; Williams et al., 2007: PMID 17942708), and $G\alpha_{12}$ (Siehler, 2009: PMID 19226283), and $G\alpha_i$ (Taussig et al., 1994: 8119955). Most G protein effectors are transmembrane protein complexes. Some G protein effectors are enzymes that catalyze the production of second messengers, small molecules that can diffuse in the cell and evoke responses. $G\alpha$ proteins bind their effectors via the same “switch” regions they use to bind $G\beta\gamma$. $G\alpha$ must be in its GTP-bound conformation to bind and activate effectors, but must be in its GDP-bound

conformation to bind G $\beta\gamma$. Thus G α serves as a molecular switch that alternates between an active GTP-bound state and an inactive GDP-bound state. G $\beta\gamma$ can only activate its effectors once it has dissociated from G α .

1.3.5. State 5, inactivation of G α via GTP hydrolysis, and reactivation via receptor-independent nucleotide exchange

G α subunits of heterotrimeric G proteins have a slow but significant intrinsic GTPase activity, such that purified G α protein can hydrolyze bound GTP to GDP with a half time on the order of a few minutes. A key contribution of *C. elegans* and yeast genetics was the discovery of a class of “regulator of G protein signaling” (RGS) proteins (Koelle and Horvitz, 1996; PMID 8548815; Dohlman et al., 1996; PMID 8756677) that serve as GTPase activators for G α proteins, speeding up the hydrolysis reaction by orders of magnitude (Berman et al., 1996; PMID 8910288; Tesmer et al., 1997; PMID 9108480). Physiologically, G α signaling is typically inactivated with the help of an RGS protein (Figure 1).

A second key contribution of *C. elegans* genetics was the discovery of RIC-8, a soluble protein that promotes G protein signaling (Miller et al., 2000; PMID 11102364), sometimes with the help of other proteins containing the G protein regulatory (GPR) motif that binds G α proteins (Colombo et al., 2003; PMID 12750478; Gotta et al., 2003; PMID 12814548; Srinivasan et al., 2003; PMID 12730122; Hofler and Koelle, 2011; PMID 218321860). Genetic studies in *C. elegans* show that GPR proteins and RIC-8 promote G protein activity, and the biochemical activities of these proteins *in vitro* suggest that they may do so by reactivating G α -GDP as illustrated in Figure 1. However, studies in mammalian cells show that RIC-8, in addition to catalyzing nucleotide exchange *in vitro*, also acts in living cells as a chaperone to promote folding and stability of G α proteins (Gabay et al., 2011; PMID 22114146; Chan et al., 2013; PMID 23431197). The *C. elegans* genetic data are consistent with the alternative model that GPR and RIC-8 proteins are simply required for G α folding and stability.

The G protein activity cycle is completed when G α -GDP re-associates with G $\beta\gamma$ to re-form the inactive G $\alpha\beta\gamma$ heterotrimer. G $\beta\gamma$ is sequestered in the heterotrimer so that it can no longer associate with its effectors.

2. Neurotransmitters and receptors that signal through heterotrimeric G proteins

In this section, I describe analysis of the surprisingly large set of neurotransmitters and neural G protein coupled receptors present in *C. elegans*. Each neurotransmitter and each receptor is expressed in a very specific and limited set of neurons, and as a result each affects a very specific and limited set of behaviors. An important result from *C. elegans* is that a neurotransmitter can signal via a GPCR expressed on cells that are not postsynaptic to the neurons that release that neurotransmitter. Thus neurotransmitters travel through tissue to signal at sites distant

from their site of release. The pattern of neurotransmitter signaling via GPCRs is thus not determined by the synaptic wiring of the nervous system, but rather by the specific expression patterns of the neurotransmitters and their GPCR receptors. The widespread nature of extrasynaptic neurotransmitter signaling forces us to expand our notion of a neural circuit to a unit consisting of neurons that function together but that may lack direct anatomical connections.

2.1. GPCRs for small-molecule neurotransmitters

Most major small-molecule neurotransmitters used in humans are also found in *C. elegans*, (exceptions are norepinephrine, histamine, and possibly glycine and ATP). Tables I and II summarize studies of a set of 28 *C. elegans* GPCRs for small-molecule neurotransmitters. This includes four serotonin receptors, four dopamine receptors, three octopamine receptors, three tyramine receptor, three acetylcholine receptors, two GABA receptors that function together as an obligate dimer, three apparent glutamate receptors, and six additional predicted receptors with sequence similarity to small molecule neurotransmitter GPCRs that have yet to have ligands assigned to them or to be studied genetically. Many of these 28 GPCRs are produced in multiple isoforms via alternative splicing of their RNA transcripts

2.1.1. Identification of ligands for individual GPCRs

Putative small-molecule neurotransmitter GPCRs in *C. elegans* were first identified as homologs of mammalian neurotransmitter receptors (e.g. Komuniecki et al., 2004: PMID 15279946). Such analyses can make predictions for the ligand that might activate a GPCR homolog, but these are weak predictions. For example, the *C. elegans* receptors SER-2, SER-3, and SER-6 were originally assigned their names due to similarity with serotonin receptors, but later proved experimentally to be receptors for other biogenic amine neurotransmitters (Table 1). Thus, assigning a ligand to a GPCR requires experimental evidence. Three lines of such experimental evidence are described below, and Table 1 lists which of these lines of evidence are available for each of 22 *C. elegans* receptors.

The first line of experimental evidence is listed in Table 1 as “binding studies”. Here, a GPCR is expressed in heterologous cells inducing a binding activity in their membranes for a radiolabeled ligand. Since characterizing binding by many different radioligands is inconvenient, typically a single radioligand with binding activity is identified (e.g. ^3H -LSD, which binds with high affinity to most biogenic amine receptors), and then the ability of many unlabeled neurotransmitters and pharmaceuticals to compete off the radioligand is measured as a “ K_i ”. Such studies measure the relative binding affinity of different ligands, but do not determine if an individual ligand is an agonist (activator) or antagonist (inhibitor) for the receptor. Based on such binding data, a GPCR is considered likely to be a receptor for the neurotransmitter that appears to bind it with highest affinity.

The second line of experimental evidence listed in Table 1 is “heterologous cell signaling”. Here, a GPCR is expressed in heterologous cultured cells, typically mammalian cells or *Xenopus* oocytes, potential ligands are applied in the medium, and activation of downstream signaling is measured (e.g. through use of fluorescent Ca²⁺ indicators, electrophysiological recording of G protein regulated ion channels, etc.). This method allows the concentration of a ligand that gives half-maximal response (EC₅₀) to be measured, and can determine if a ligand is an agonist or antagonist. Further, it provides evidence for the type of G α protein activated by the receptor (see section 2.2.2. below). Based on such heterologous cell signaling studies, a GPCR is considered likely to be a receptor for the neurotransmitter that activates it with the lowest EC₅₀.

The third line of experimental evidence listed in Table I is genetic studies in *C. elegans*. In many cases, a mutant for a GPCR renders worms insensitive to the effects of a specific neurotransmitter, either applied exogenously or released endogenously via optogenetic stimulation. Another type of genetic study demonstrates that a GPCR mutant shows behavioral defects similar to those of a mutant lacking a particular neurotransmitter. Such genetic studies provide evidence that GPCR functions *in vivo* as a receptor for a specific neurotransmitter.

Table 1 shows that for the 22 GPCRs listed as assigned a ligand, the number of experimental lines of evidence for that assignment varies from zero to three, and the quality of each piece of evidence can also vary widely. Clearly, the more experimental evidence that is available, the more confident a ligand assignment will be.

Both *C. elegans* and mammals have multiple GPCRs for individual neurotransmitters. A large number of pharmaceuticals have been developed that activate or antagonize specific mammalian receptor isoforms. It is tempting to try to use *C. elegans* as a model system to investigate the functions of specific neurotransmitter receptor isoforms to better understand the actions of these important drugs. However, when the sequences of *C. elegans* neurotransmitter receptors are lined up with those of mammalian receptors, individual *C. elegans* receptors cannot be unambiguously assigned as orthologs of specific mammalian receptors. For example, the *C. elegans* serotonin receptors are more similar to mammalian serotonin receptors than they are to mammalian receptors for other neurotransmitters, but an additional *C. elegans* receptor (named SER-2) is also most similar to mammalian serotonin receptors in sequence and turned out to be a tyramine receptor (Rex et al., 2004: PMID 15569254). By sequence analysis, the *bona fide* *C. elegans* serotonin receptors do not unambiguously match up with individual mammalian serotonin receptor isoforms. Furthermore, when *C. elegans* serotonin receptors are expressed in cultured cells or *Xenopus* oocytes, their profiles of binding and activation by drugs do not match up to the pharmacology of specific mammalian serotonin receptor isoforms (Komuniecki et al., 2004: PMID 15279946). Thus, while *C. elegans* generally provides an excellent model for studying small-molecule neurotransmitter signaling through G proteins, there are limits to the level of conservation of receptors between humans and worms.

An important issue in assigning ligands to GPCRs is the potential that a single GPCR might physiologically mediate signaling by more than one type of neurotransmitter. The heterologous cell signaling studies cited in Table 1 show that

certain receptors have significant affinities for more than one biogenic amine neurotransmitter. For example, the dopamine receptor DOP-3 is activated in heterologous cell signaling studies by dopamine with an EC₅₀ of 27 nm, but also by tyramine with an EC₅₀ of 500 nm (Sugiura et al., 2005: PMID 16001968). Binding studies show significant affinities for more than one biogenic amine by the receptors DOP-1, DOP-2 (Suo et al., 2002: PMID 11814642), OCTR-1 (Wragg et al., 2007: PMID 18057198), SER-2 (Rex and Komuniecki, 2002: PMID 12354282), TYRA-2 (Rex et al., 2005: PMID 15953361), and TYRA-3 (Wragg et al., 2007: PMID 18057198). Since the synaptic concentrations of released neurotransmitters are thought to go above one millimolar (Barberis et al., 2011: PMID 21734864), the high nanomolar or micromolar binding affinities of certain receptors for a secondary neurotransmitter could be biologically meaningful. Studies in *C. elegans* could resolve this issue. Given the complete wiring diagram in *C. elegans*, along with the known identities of neurons that release specific neurotransmitters and that express specific GPCRs, there is the potential to identify synapses at which a GPCR is exposed to a high concentration of one of its lower-affinity ligands. Genetic analysis could then determine if this results in physiologically significant signaling.

2.1.2. Identification of G proteins activated by individual GPCRs

Sequence comparison of *C. elegans* GPCRs to homologous mammalian GPCRs can be used to predict which G α protein a particular *C. elegans* GPCR might couple to (e.g. Wragg et al., 2007: PMID 18057198). However, a more definitive assignment requires experimental evidence. Two lines of such experimental evidence are described below, and Table 1 lists the lines of evidence for G protein coupling available for each of 22 *C. elegans* receptors.

The first line of experimental evidence is listed in Table 1 as “heterologous cell” studies. Here, a GPCR is expressed in heterologous cultured cells for ligand activation studies, as described above in section 2.1.1. In addition to identifying an activating ligand, these studies can determine what type of G α protein is activated by the GPCR in heterologous cells. For example, activation of Ca²⁺ release generally indicates G α_q signaling, activation of cAMP production generally indicates G α_s activation, and inhibition of cAMP production and/or sensitivity to pertussis toxin generally indicates G $\alpha_{i/o}$ activation.

The second line of experimental evidence listed in Table 1 comes from genetic studies in *C. elegans*. Here, a *C. elegans* GPCR mutant can be used to demonstrate that a receptor acts in a particular cell type to support a particular behavior, and genetic studies can similarly show that a particular G α protein is also required in the same cell type for the same behavior.

Table 1 shows that 20 of 22 GPCRs assigned to a ligand are also assigned coupling to a specific G α protein, with the number of experimental lines of evidence for that G α assignment varying from zero to two. G α assignments from heterologous cell studies generally agree with assignments from *C. elegans* genetics in cases where both lines of evidence are available, bolstering confidence in assignments from the many

cases with only one line of evidence. An interesting case is that of the dopamine receptor DOP-2, which was assigned to $G\alpha_{i/o}$ in heterologous cell studies, and for which *C. elegans* genetics suggests that three different $G\alpha_o$ -related $G\alpha$ proteins may mediate DOP-2 signaling in worms (Suo et al., 2003: PMID 12887685; Suo et al., 2009: PMID 19609300; Correa et al., 2012: PMID 23166505; Pandey and Harbinder, 2012: PMID 22280843; Mersha et al. 2013: PMID 23607404).

2.2. Cataloging GPCRs for neuropeptides

At present at least 119 *C. elegans* genes encoding over 250 neuropeptides have been cataloged (Li and Kim, 2008: PMID 18819171; Li and Kim, 2010: PMID 21189676). Identifying the receptors for all these neuropeptides is a major challenge. Of the >1,000 putative GPCRs encoded in the *C. elegans* genome, most are chemosensory receptors, and a much smaller small subset are likely to be neuropeptide receptors. For tables itemizing these receptors, I refer readers to several recent reviews that have cataloged the likely *C. elegans* neuropeptide receptors by looking for GPCRs most similar to known neuropeptide receptors (Altun, 2011; Frooninckx et al., 2012: PMID: 23267347; Hobert, 2013: PMID 24081909). These cataloging efforts generally predict about 150 genes encoding neuropeptide receptors, with individual genes often producing a number of differentially spliced isoforms.

Only a relatively small number of putative neuropeptide receptors have been assigned as receptors for specific neuropeptide(s) – one recent count put the number of such “deorphanized” neuropeptide receptors at 23 (Frooninckx et al., 2012: PMID: 23267347). The quality of the data varies for each receptor, but a definitive assignment of a peptide to a receptor should include data showing the peptide binds the receptor with high affinity and specificity, as well as *C. elegans* genetic data demonstrating that the peptide functions via the receptor to regulate a specific behavior. An example of such a definitive assignment is work showing that the sensory BAG neurons release two different peptides encoded by the *flp-17* gene which then act via the EGL-6 receptor on the HSN motor neuron to inhibit egg-laying behavior (Ringstad and Horvitz, 2008: PMID 18806786).

Can *C. elegans* neuropeptides or neuropeptide receptors be matched up with mammalian orthologs? In the case of the neuropeptides, the sequences are so short that there just is not enough information content in them to make such an analysis possible. In the case of the receptors, their information-rich sequences do allow such an analysis. Mammalian neuropeptide receptors can be broken down into several subfamilies based on sequence relationships, and many worm receptors can similarly be fitted into the same families (Altun, 2011; Frooninckx et al., 2012: PMID: 23267347; Hobert, 2013: PMID 24081909). Most *C. elegans* putative neuropeptide receptors cannot be definitively assigned as orthologs of specific mammalian receptors simply based on sequence analysis. However, based on sequence similarity as well as additional functional data, some worm receptors have been described as models for specific mammalian receptors. Thus *C. elegans* NPR-1 is similar to the mammalian neuropeptide Y receptor (de Bono and Bargmann, 1998: PMID 9741632), *C. elegans*

PDFR-1 is similar to the *Drosophila* pigment dispersing factor receptor and mammalian vasoactive peptide and calcitonin receptors (Janssen et al., 2008: PMID 18390545), and *C. elegans* NPR-17 is similar to mammalian opioid receptors (Cheong et al., 2015: PMID 25898004).

2.3. Neural GPCRs are each expressed in very restricted sets of cells

An important result from analysis of G protein coupled neurotransmitter receptors in *C. elegans* is that each receptor tends to be expressed on a small number of specific neurons, allowing that receptor to mediate very specific effects on behavior. *C. elegans* hermaphrodites have just 302 neurons, which due to symmetries and repeated structures in the anatomy can be classified into 118 types. Each neuron can be identified by its unique position and morphology within the animal. Further, all the synaptic connections of each neuron with other neurons and muscles have been mapped, making *C. elegans* the only animal for which a complete neural wiring diagram is available (White et al., 1986: PMID 22462104). Because of these features, identifying all the specific neurons that express a neurotransmitter and its receptor is uniquely possible in *C. elegans*, and this information, once obtained, can be interpreted using the wiring diagram to obtain unique insights into neural signaling. In this section I begin a discussion of this area of investigation.

The expression patterns of a significant subset of *C. elegans* neural GPCRs have been studied. These experiments generally involve creating transgenic worms in which the promoter and other regulatory sequences for a GPCR gene are used to drive expression of the green fluorescent protein (GFP), and specific cells expressing GFP are identified by fluorescence microscopy. Data for the expression patterns of 22 GPCR receptors for small-molecule neurotransmitters are summarized in Table 2, and expression patterns for 27 of the ~150 putative neuropeptide receptors are also available (Hobert, 2013: PMID 24081909). The quality and completeness of the work varies greatly for each receptor. In some cases, only relatively short promoter regions were used to construct the GFP reporter transgenes, raising questions as to whether the results obtained fully represent the expression patterns of the endogenous GPCR genes. In other cases, only a cursory analysis of the fluorescently labeled cells is presented, with little or no attempt to identify the GFP-expressing cells.

Despite these caveats, an important generalization can be made about the expression patterns of neural GPCRs: an individual neurotransmitter receptor type tends to be expressed in a limited set of specific cells. This can be illustrated with examples from receptors for which relatively high-quality expression data are available and which typify the results obtained with other receptors. A GFP reporter for the EGL-6 neuropeptide receptor was expressed in just three types of neurons (HSN, SDQ, DVA) and one type of glial cell (GLR) (Ringstad and Horvitz, 2008: PMID 18806786). A GFP reporter for the NPR-1 neuropeptide receptor was expressed in approximately 20 neuron cell types (Coates and de Bono, 2002: PMID 12410311). A reporter for the NPR-4 neuropeptide receptor was expressed in five neuron types, the intestine, and the rectal gland cell, while a reporter for the NPR-5 neuropeptide receptor was expressed

13 neuron types and in all body wall muscles (Cohen et al., 2009: PMID 19356718). Expression patterns of G protein coupled receptors for small-molecule neurotransmitters (Table 1) show similar characteristics to those seen in the above examples of neuropeptide receptors – each receptor is typically expressed in a limited number of neuron types plus sometimes additional non-neuronal cell types. For example, a reporter for the TYRA-2 tyramine receptor is expressed in about 14 neuron types (Rex et al., 2005; PMID 15953361). Reporters for the SER-2 tyramine receptor are expressed in a total of 24 neuron types, plus body wall muscles, pharyngeal muscles, and the excretory gland (Tsalik et al., 2003: PMID 14568548).

2.4. Neurotransmitters signal extrasynaptically through GPCRs

The predominant model of neural signaling has been that synapses, specialized physical connections between neurons, are the sites of neurotransmitter signaling between neurons. A presynaptic neuron releases neurotransmitter from vesicles clustered at a presynaptic terminus, flooding the narrow synaptic cleft with neurotransmitter, which binds to neurotransmitter receptors clustered on the postsynaptic membrane. The idea that synapses are central to understanding neural function inspired the landmark achievement of mapping all the synaptic connections in the *C. elegans* nervous system (White et al., 1986: PMID 22462104), and is behind current efforts, for example within the BRAIN initiative, to map synaptic connections within more complex nervous systems (Jorgenson et al., 2015: PMID 25823863).

While ionotropic neurotransmitter signaling may typically be restricted to synapses, studies in *C. elegans* provide ample evidence that neurotransmitter signaling through G protein coupled receptors may be predominantly extrasynaptic. The concept of extrasynaptic neurotransmission (also called volume transmission) originated in 1986 based on studies of mammalian brain (Agnati et al., 1986: PMID 3022556). Since then, many studies in mammalian brain demonstrated the release of neurotransmitters from extrasynaptic sites, the ability of neurotransmitters to diffuse through the extracellular space, and the localization of G protein coupled neurotransmitter receptors to extrasynaptic sites (Agnati et al., 2010: PMID 20347870). Indeed, given the submicromolar affinities of many GPCRs for their neurotransmitter ligands (see Table 1 for examples), it would make little sense for such receptors to function at synapses where neurotransmitter concentrations can rise to the millimolar level (Barberis et al., 2011: PMID 21734864) and may never be reduced to low enough concentrations to allow high-affinity receptors to become unliganded. Despite this evidence, it has been difficult to provide genetic evidence demonstrating the functional significance of extrasynaptic signaling in mammalian brain. In contrast, *C. elegans* is ideally suited to such work. In this system, it is possible to use rigorous genetic experiments to define the specific neurons that release a neurotransmitter to induce a particular behavioral response, and to also define the specific neurons that express the G protein coupled receptor that functionally receives the neurotransmitter signal to execute the response. Remarkably, results of such analyses show over and over again that the neurotransmitter releasing neuron and the receptor-expressing receiving neuron are not

synaptically connected. Ironically, the complete synaptic wiring diagram of the *C. elegans* nervous system has thus been the key tool for demonstrating that synapses are often not needed to mediate neurotransmitter signaling via G protein coupled receptors. Here I summarize the evidence for extrasynaptic neurotransmitter signaling in *C. elegans*.

2.4.1. Neurotransmitters are released from extrasynaptic sites

Neuropeptides and some small-molecule neurotransmitters are released from dense-core vesicles, which are distinct from the small-clear vesicles clustered at presynaptic termini. In *C. elegans* neurons that make synapses, dense-core vesicles are excluded from the synaptic active zones where synaptic vesicles are released, although many are localized nearby (Hammarlund et al., 2008: PMID 18250196). Furthermore, *C. elegans*, like the human brain, has specialized neuroendocrine cells that synthesize and release neurotransmitters but that do not form synaptic connections with any other cells. The uv1 neuroendocrine cells release tyramine and neuropeptides to inhibit egg-laying behavior (Jose and Koelle, 2007: PMID 17057248), and the NSM neuroendocrine cells release serotonin to regulate locomotion (Sawin et al., 2000: PMID 10896158; Gürel et al., 2012: PMID 23023001). The extrasynaptic neurotransmitter release sites of NSM have been studied in some detail (Nelson and Colón-Ramos, 2012: PMID 23345213). The documented functions of neurotransmitter release from uv1 and NSM provide one set of evidence that neurotransmitters do signal extrasynaptically. Some additional cells in *C. elegans* do not form any synapses, yet express neuropeptide genes, so any functions of peptides secreted from these cells must also be extrasynaptic. Cells in this category include the CAN cell, intestinal cells, muscle cells, and hypodermal cells (White et al., 1986: PMID 22462104; Li and Kim, 2008: PMID 18819171; Li and Kim, 2010: PMID 21189676).

2.4.2. Neurotransmitters can signal through GPCR receptors on cells not postsynaptic to their release sites

The specific *C. elegans* neurons that synthesize and release individual small molecule neurotransmitters or neuropeptides have been mapped in considerable detail (Jorgensen, 2005; Rand, 2007; Chase and Koelle, 2007; Li and Kim, 2008: PMID 18819171; Li and Kim, 2010: PMID 21189676; Jafari et al., 2011: PMID 21677178; Serrano-Saiz et al., 2013: PMID 24243022; Periera et al., 2015: PMID 26705699). Thus when the expression pattern of a G protein coupled receptor for a specific neurotransmitter is determined, we can use the synaptic wiring diagram of *C. elegans* to see if the cells that produce that neurotransmitter are presynaptic to the cells that express its receptor. In many cases, the answer is no.

G protein coupled receptors for small-molecule neurotransmitters are expressed on cells not postsynaptic to neurons that release the corresponding neurotransmitter. This was first noted for the dopamine receptors DOP-1 and DOP-3, which are expressed, among other places, on ventral cord motor neurons (Chase et al., 2004:

PMID 15378064). Dopamine is released from exactly three types of neurons in *C. elegans*, and none of these neurons make synapses onto the ventral cord motor neurons. Genetic studies show that dopamine regulates locomotion via the DOP-1 and DOP-3 receptors, and the locomotion defects of *dop-1* and *dop-3* mutants can be rescued by re-expressing the receptors specifically in ventral cord motor neurons (Chase et al., 2004: PMID 15378064). These experiments rigorously demonstrate that dopamine receptors are not only found on cells distant from dopamine release sites, but that they also function to mediate dopamine signaling in these distant cells.

Since those initial studies of dopamine signaling, similar experiments have demonstrated extrasynaptic signaling by other small-molecule neurotransmitters. One striking case is that of the neurotransmitter tyramine, which is released from only the neuroendocrine uv1 cells (which makes no synapses) and from the RIM interneuron, which makes synapses only onto four other types of neurons and onto neck muscles (White et al., 1986: PMID 22462104). When *C. elegans* is touched on the head, tyramine released from RIM mediates a complex escape response. One aspect of this response is mediated by tyramine signaling at synapses from RIM onto neck muscles using an ionotropic receptor (Pirri et al., 2009: PMID 19477154). However, another aspect of the escape response is mediated by tyramine signaling from RIM through a G protein coupled receptor. This GPCR, SER-2 is expressed on GABAergic motor neurons, which are not post-synaptic to RIM, and defects in the escape response seen in *ser-2* mutants are rescued by cell-specific re-expression of *ser-2* in GABAergic motor neurons, demonstrating that SER-2 acts in these neurons to mediate extrasynaptic signaling by tyramine that originates from RIM (Donnelly et al., 2013: PMID 23565061).

There have now been similar genetic demonstrations that neuropeptides signal onto cells not postsynaptic to the neurons that release them. For example, FLP-17 neuropeptides are released from BAG sensory neurons and signal via the EGL-6 neuropeptide receptor on the HSN motor neurons, which are not postsynaptic to BAG, to inhibit egg laying (Ringstad and Horvitz, 2008: PMID 18806786). The *C. elegans* defecation motor program is coordinated by NLP-40 peptides released from intestinal cells, which, being non-neuronal, make no synapses, and these NLP-40 peptides signal onto GABAergic motor neurons via the AEX-2 peptide receptor (Wang et al., 2013: PMID 23583549). Beyond these rigorous genetic demonstrations that specific peptides signal extrasynaptically via particular neuropeptide receptors, there are additional data showing that some neuropeptide receptors are expressed on cells that do not receive any synapses, so that any signaling they mediate on these cells must be extrasynaptic. These receptor-expressing cells include intestinal, hypodermal, and glial cells (Hobert, 2013: PMID 24081909).

2.5. The genetics of G protein coupled neurotransmitter receptors

Null mutants or RNAi knockdowns for many *C. elegans* G protein coupled neurotransmitter receptors have been analyzed, and the main finding is that phenotypic defects are very hard to detect in them. Most of the time no defects are observed, and the few mutant phenotypes that have been described for neural GPCRs are typically not

gross behavioral defects obvious upon inspection of individual animals, but rather narrow behavioral defects detectable only when populations of worms are put through specific phenotypic assays.

An initial effort at identifying neural GPCR phenotypes used RNAi to knock down expression of 60 G protein coupled neurotransmitter receptors (Keating et al., 2003: PMID 14521838) and simply looked for uncoordinated movement or defects in reproduction. Knockdown of seven of the receptors had detectable effects on the frequency and/or amplitude of body bends, while knockdown of five receptors had some effect on the production of progeny. Later large-scale RNAi screens for genes that affect acetylcholine release/signaling identified hits in five GPCR genes, of which three were validated using genetic knockout mutations (Sieburth et al., 2005: PMID 16049479; Vashlishan et al., 2008: PMID 18466746). It is difficult to interpret some of these RNAi results: not all the defects seen with RNAi were reproduced with genetic mutations, the RNAi results did not always reproduce in different labs, and the phenotypes that were observed have not been characterized in much detail. Negative results from RNAi for GPCRs are also difficult to interpret since gene expression in *C. elegans* neurons is not always efficiently knocked down by RNAi, even in mutant backgrounds that enhance neuronal RNAi (Poole et al., 2011: PMID 21698137).

Mutations in genes encoding GPCRs have almost never been generated in forward genetic screens, despite the large number of GPCR genes in the worm genome. Since Brenner's first screen for uncoordinated mutants (Brenner, 1974: PMID 4366476), *C. elegans* mutants for thousands of genes have been generated in genetic screens, including a large number with defects in neural structure and/or function. Included in this trove are many mutants for heterotrimeric G proteins and other signaling proteins that act downstream of neural GPCRs (Perez-Mansilla and Nurrish, 2009: PMID 19615533), making the absence of neural GPCR mutants even more conspicuous.

Considering the few neural GPCR mutants that have arisen in forward genetic screens is instructive. The EGL-6 neuropeptide receptor was identified by a rare gain-of-function mutation that inhibits egg laying (by increasing EGL-6 signaling), yet knockout mutations of EGL-6 do not produce detectable egg-laying defects (Ringstad and Horvitz, 2008: PMID 18806786). Loss-of-function mutations in G protein coupled serotonin and dopamine receptors have arisen in forward genetic screens, but in these cases the phenotype screened for was resistance to the paralysis induced by applying these neurotransmitters to worms at high concentrations (Chase et al., 2004: PMID 15378064; Gürel et al., 2012: PMID 23023001). These mutants do not have obvious behavioral defects when animals are not bathed in a neurotransmitter, although defects have been detected when populations of mutant animals are put through very specific behavioral assays known to depend on serotonin or dopamine. There is perhaps only a single example of a neural GPCR for which loss-of-function mutants have been recovered from a forward genetic screen and for which the mutant phenotype is easily observable in single animals: mutants lacking AEX-2, the receptor for NLP-40 neuropeptides, are defective in the expulsion step of defecation (Mahoney et al., 2008: PMID 18852466; Wang et al., 2013: PMID 23583549).

C. elegans mutants for many G protein coupled neurotransmitter receptors are available as a result of large-scale efforts to produce gene knockouts for all *C. elegans* genes (*C. elegans* deletion mutant consortium, 2012: PMID 23173093). However, phenotypic defects have been described for only a small subset of these GPCR mutants. The cases in which phenotypic defects have been detected have generally come when either the neurotransmitter that activates the GPCR was known so that specialized assays for behaviors dependent on that neurotransmitter could be assayed for defects, or in cases in which the expression pattern of the GPCR was known so that specialized assays for behaviors dependent on neuron(s) that express the receptor could be tested for defects. An example that illustrates both strategies comes from the work of Harris et al. (2009: PMID 19193891), which analyzed how *C. elegans* backs away from the odor of dilute octanol, a response known to depend on a both on the neurotransmitter serotonin and on a neural circuit containing specific sensory neurons and interneurons. This information was used to focus in on two G protein coupled serotonin receptors, one expressed in the interneurons, another in the sensory neurons, and further experiments showed that knockouts and knock downs of these receptors resulted in specific behavioral defects in response to octanol. The two serotonin receptors studied by Harris et al. (2009) are part of the set of 22 receptors for small molecule neurotransmitters listed in Table 2, which are the most intensively studied GPCRs in *C. elegans*. As shown in the Table, at this point mutant defects have been described for 20 of these receptors. The situation is less encouraging in the case of neuropeptide receptors, with mutant phenotypic defect described for only a few out of this large set of ~150 receptors.

Considering the totality of available genetic studies of *C. elegans* G protein coupled neurotransmitter receptors, it appears that the knockout phenotypes for these proteins are generally so narrow that they will not be easily detected. Because individual receptors are expressed in only a few types of neurons, it is reasonable to expect that just the very specific behaviors affected by those neurons will be affected, and that specialized behavioral assays will be required to detect these defects. Another possible reason that GPCR mutant phenotypic defects are rarely detected could be that the large family of GPCRs contains many functionally redundant receptors, such that knocking out one receptor will not give obvious defects unless its redundant partners are also knocked out simultaneously. Several examples support the idea that neurotransmitter receptors can function redundantly. Two serotonin receptors appear to be co-expressed on the vulval muscles and both appear to promote activity of these muscles (Hapiak et al., 2009: PMID 19001289). Serotonin released from the NSM neurosecretory cells inhibits locomotion, and two different serotonin receptors expressed on largely non-overlapping sets of neurons redundantly mediate this effect (Gürel et al., 2012: PMID 23023001). Neuropeptides encoded by FLP-18 modulate locomotion, and three different receptors, NPR-1, NPR-4, and NPR-5, expressed in different sets of cells, redundantly mediate these effects of FLP-18 (Stawicki et al., 2013: PMID 23658528). FLP-18 signaling is quite complex, because other behavioral effects of FLP-18 peptides are mediated non-redundantly by NPR-1, and for these effects FLP-18 and FLP-21 neuropeptides both seem to activate NPR-1 (Choi et al., 2013: PMID 23764289). If such

complex functional relationships between neurotransmitters and receptors are widespread, this will make assigning functions to receptors using knockout mutations very challenging.

A tool that may prove generally useful in genetically characterizing redundant or subtle GPCR functions is transgenic overexpression of GPCRs. One example comes from studies of the EGL-6 neuropeptide receptor. Loss-of-function mutants for EGL-6 do not result in obvious defects under standard lab growth conditions. However, using a transgene carrying many copies of *egl-6* genomic DNA to presumably overexpress the receptor does result in an obvious egg-laying defective phenotype, apparently due to increased activation of the overexpressed EGL-6 receptor by the FLP-10 and FLP-17 neuropeptides (Ringstad and Horvitz, 2008: PMID 18806786). Another example comes from studies of the NPR-17 neuropeptide receptor. NPR-17 mediates an effect of NLP-3 peptides to modulate how *C. elegans* backs away from aversive stimuli, and a transgene carrying many copies of the *npr-17* gene causes a gain-of-function phenotype opposite that of an *nlp-3* or *npr-17* knockout, and that depends on the presence of a wild-type NLP-3, suggesting that the overexpressed NPR-17 receptor causes increased NPR-17 signaling (Harris et al., 2010: PMID 20534837). If transgenically overexpressing a GPCR is generally able to induce gain-of-function phenotypes, this could be a valuable approach for identifying subtle or redundant functions of GPCRs.

2.6. Heterodimerization of G protein coupled neurotransmitter receptors

A major area of current research on mammalian GPCRs concerns the homo- and hetero-oligomerization of these receptors (González-Maeso, 2011: PMID 21619615). It appears that in many cases, two different types of GPCRs can exist and function *in vivo* as a heterodimeric complex, and that a heteromeric receptor can have very different signaling properties than homomers of its subunits, including the ability to bind to different ligands. While GPCR heteromerization has so far been studied mainly using biochemical and biophysical methods, genetic analysis has much to contribute to understanding the physiological significance of this phenomenon. Heteromerization also potentially vastly complicates the challenge of understanding GPCR function: if *C. elegans* has ~150 G protein coupled neurotransmitter receptor genes, how many types of heteromeric GPCRs might actually be the functional units *in vivo*?

There have so far been only a few efforts to analyze GPCR heteromer function in *C. elegans*. One example is that of the G protein coupled receptor for the neurotransmitter GABA. In mammals this GABA_B receptor is an obligate heteromer between the GABA_{B1} and GABA_{B2} subunits (Kaupman et al., 1998: PMID 9872317). *C. elegans* has orthologs of each subunit, known as GBB-1 and GBB-2, respectively. Knockouts of either GBB-1 or GBB-2 can block all G protein coupled responses to GABA in worms (Schultheis et al., 2011: PMID 21613582), and GBB-1 and GBB-2 act together to alter responses to the drug aldicarb (Dittman and Kaplan 2008: PMID 18614679; Vashlishan et al., 2008: PMID 18466746), consistent with the idea that the GABA_B receptor is an obligatory heterodimer in worms just as it is in mammals. A

second example comes from studies of signaling onto sensory neurons, not by a neurotransmitter, but by the mixture of small molecules known as ascarosides that collectively make up the worm dauer pheromone. The functional receptor for the specific ascaroside isoform ascr#2 appears to be a heteromer of the GPCRs DAF-37 and DAF-38 (Park et al., 2012: PMID 22665789). ascr#2 binds directly to DAF-37, and DAF-37 is essential for response to ascr#2 but is not involved in the response to other ascaroside isoforms. Genetic studies show that DAF-38 assists but is not essential for response to ascr#2, and similarly assists response to other ascaroside isoforms that do not signal through DAF-37, suggesting DAF-38 may heteromize with and assist signaling by a several different GPCRs that bind directly to different ascaroside isoforms. These studies demonstrate the potential of *C. elegans* genetic studies to sort out the intricacies of how GPCR subunits function together as heteromeric receptors *in vivo*.

2.7. Deorphanizing neural G protein coupled neurotransmitter receptors

One of the principal issues in studying neural GPCRs is identifying the specific neurotransmitters that activate them. At this point, 22 of 28 GPCRs encoded in the worm genome that seem likely to be receptors for small-molecule neurotransmitters have been matched with their activating ligands (Table 1), but about 85% of the ~150 putative neuropeptide receptors remain “orphans”, that is receptors with unknown activators (Frooninckx et al., 2012: PMID: 23267347).

The general strategy to deorphanize neuropeptide receptors is to express them in heterologous cells, to apply synthetic versions of each neuropeptide encoded in the genome, and to test which specific peptides can activate receptor signaling. There are a number of cell types and signaling assays that have been used successfully for this purpose (Mertens et al., 2004: PMID 15335287). This strategy has been applied to a number of *C. elegans* neuropeptide receptors, for example EGL-6 (Ringstad and Horvitz, 2008: PMID 18806786) and NPR-1 (Rogers et al., 2003: PMID 14555955 Kubiak et al., 2003: PMID 12821653). The reverse strategy has also been used, screening through a set of *C. elegans* neuropeptide receptors to identify those that can be activated by a particular neuropeptide of interest (Cohen et al., 2009: PMID 19356718). The results of receptor deorphanization experiments are complex: often a single receptor can be activated by multiple different peptides, the same peptides can activate multiple different receptors, the results can vary depending on the cell type and assay system used, and the EC₅₀ values measuring the potencies with which peptides activate receptors vary from the nanomolar to micromolar ranges (Peyman et al., 2014: PMID 24982652).

C. elegans provides the opportunity to analyze mutants for the receptors and peptides putatively matched by such deorphanizing experiments to determine if they actually function together *in vivo*. Genetic approaches can also be used to match peptides and their receptors, for example screening RNAi knockdowns of many neuropeptide genes to find those that phenocopy a particular orphan receptor mutant (Cohen et al., 2009: PMID 19356718). Such genetic studies have generated results that

are similar in complexity to those from using signaling assays in cultured cells for receptor deorphanization. Thus it appears that *in vivo*, individual receptors can be activated by multiple peptides and individual peptides can act through multiple receptors (Choi et al., 2013: PMID 23764289; Stawicki et al., 2013: PMID 23658528).

The gold standard in matching neuropeptides with receptors is to combine studies of receptor activation by purified peptides in cultured cells with genetic studies in *C. elegans*. For the most part, it has been possible to achieve consistent results between the two approaches, generating strong confidence in the results, although there have been occasional puzzling exceptions (Cheong et al., 2015: PMID 25898004). Overall, the data matching neuropeptides to their cognate receptors remains sparse and we have a long way to go to fully match up these signaling molecules.

2.8. Studies of receptor desensitization in *C. elegans*

In order for signaling by an activated GPCR to terminate, the receptor must eventually be inactivated. One mechanism for terminating receptor activity is to clear neurotransmitter from the extracellular space so that it no longer remains bound to the receptor, and there are enzymes that degrade neurotransmitters and transporters that take them back up into cells that function for this purpose (Zimmerman and Soreq, 2006: PMID 16802134; Kanner and Zomot, 2008: PMID 18393466). Another mechanism for terminating signaling involves G protein coupled receptor kinases (GRKs) that specifically recognize and phosphorylate active GPCRs, and arrestin proteins that bind phosphorylated receptors. Phosphorylation and arrestin binding can block the ability of receptors to activate G proteins, and also cause cells to internalize receptors from the cell surface, down-regulating signaling (Kohout and Lefkowitz, 2003: PMID 12488531). These mechanisms, referred to as receptor desensitization, have been extensively studied in mammalian cells. Studies in *C. elegans* of GRK and arrestin homologs have identified specific functions of these proteins in sensory neurons (Fukoto et al., 2004: PMID 15157420; Palmitessa et al., 2005: PMID 15878875, Pereira and van der Kooy, 2012: PMID 22745502; Singh and Aballay, 2012: PMID 22875856), but have so far failed to show any striking effects of GRK or arrestin on neurotransmitter signaling in the rest of the nervous system.

2.9. The overall landscape of neurotransmitter signaling through GPCRs

It is interesting to extrapolate from the existing data on *C. elegans* G protein coupled neurotransmitter receptors to consider what the overall landscape of neural signaling through GPCRs may look like. If we imagine that the ~175 neural GPCRs in *C. elegans* are each expressed on average in 10 of the 118 neural cell types, then a typical neuron would express about 15 GPCRs, about two of which might be small-molecule neurotransmitter receptors, with the remainder being neuropeptide receptors. This typical neuron would then face the task of simultaneously sensing the levels of ~15 neurotransmitters in its extracellular space and executing appropriate responses to the

dynamic mix of these signals it receives over time. All the GPCRs in this typical neuron may signal through about three types of G proteins, which do not act separately but rather collaborate to modulate neurotransmitter release from the neuron (see section 3 below). Thinking about neurotransmitter signaling through GPCRs this way, it does not make sense to consider the action of a single neurotransmitter at a time, which is the way that we currently investigate neural signaling. Rather, it may be more appropriate to investigate how a single neuron computes appropriate responses to the entire mix of neurotransmitters in its environment. A prerequisite to such an investigation would be to know all the GPCRs that are present on that neuron, and we currently do not have that information for even a single *C. elegans* neuron.

Perhaps the most striking result from analysis of *C. elegans* neural GPCRs is that neurotransmitters signal extrasynaptically through these receptors. Thus neurons that have no physical connections can signal each other and work together to control specific behaviors. The predominant model for understanding nervous system function has been that the functional ensembles of neurons that control thoughts and behaviors are circuits defined by the synaptic connections between the neurons in the ensemble. We have had the synaptic wiring diagram for the *C. elegans* nervous system for almost 30 years (White et al., 1986: PMID 22462104) and it has proven insufficient to allow us to understand the neural control of behaviors in this organism. Now that we understand the widespread nature of extrasynaptic neurotransmitter signaling, we need to expand our understanding of a neural circuit to be a functional ensemble of neurons that signal each other but that may lack direct anatomical connections. The patterns of signaling via GPCRs are not determined by the synaptic wiring of the nervous system, but rather by the specific expression patterns of neurotransmitters and their GPCR receptors. Thus to help understand neural circuit function, we need to supplement the existing synaptic wiring diagram with an additional diagram in which the specific cells that express each neurotransmitter and its cognate receptor(s) are defined. This goal, while ambitious, is potentially achievable in the *C. elegans* system, perhaps aided by new cell-specific RNAseq technologies (Spencer et al., 2014: PMID 25372608).

3. The mechanism of signaling by neural heterotrimeric G proteins

In this section, I focus on genetic studies in *C. elegans* of signaling by the neural G proteins $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ that mediate signaling by G protein coupled neurotransmitter receptors. The G proteins themselves and their downstream signaling pathways are strongly conserved comparing mammalian brain and *C. elegans*. Genetic studies in *C. elegans* show that all three types of $G\alpha$ protein signal to regulate neurotransmitter release. *C. elegans* genetic screens have been used to discover new signaling molecules that regulate neural G protein signaling, including Regulators of G protein Signaling (RGS proteins) that help terminate signaling, the RIC-8 protein that is required for signaling, and a new $G\alpha_q$ effector (Trio's RhoGEF domain). The *in vivo* studies of neural signaling in *C. elegans* suggest a model in which the heterotrimeric G

proteins present in a neuron act to integrate signaling by the multiple GPCRs present on the neuron and produce its appropriate output level.

3.1. Heterotrimeric G proteins that mediate neurotransmitter signaling

I will focus here on the G protein subunits that mediate neurotransmitter signaling, and refer readers to an earlier review for a table listing all $\text{G}\alpha$, $\text{G}\beta$, and $\text{G}\gamma$ subunits in *C. elegans*, and for discussion of the large family of specialized *C. elegans* $\text{G}\alpha$ proteins found in sensory neurons that mediate chemosensation (Bastiani and Mendel 2006: PMID 18050432).

In *C. elegans*, as in mammals, there are multiple G protein α , β , and γ subunits that can potentially combine to form a larger number of heterotrimeric combinations. In mammals there are few functional differences between the various β and γ subunits (Khan et al., 2013: PMID 23406670). In *C. elegans* only one β subunit homolog is actually used in G protein heterotrimers (Zwaal et al., 1996: PMID 8752216; Chase et al., 2001: PMID 11250150; Robatzek et al., 2001: PMID 11250160; van der Linden et al., 2001: PMID 11333232), and only one γ subunit is expressed outside the chemosensory neurons (Jansen et al. 2002: PMID 11867526). Thus in *C. elegans*, as in mammals, functional diversity among G protein heterotrimers rests primarily on which α subunit is used. Mammals have multiple members of each of four families of α subunits, while *C. elegans* has just one member for each of these four α families (Jansen et al., 1999: PMID 10192394). The four conserved *C. elegans* α subunits, with their corresponding mammalian orthologs and percent sequence identities to them, are: GOA-1 ($\text{G}\alpha_o$, >80%), EGL-30 ($\text{G}\alpha_q$, >80%), GSA-1 ($\text{G}\alpha_s$, 66%), and GPA-12 ($\text{G}\alpha_{12}$, 52%). These worm $\text{G}\alpha$ proteins are each more similar to their mammalian orthologs than they are to each other. Below I will use the mammalian names to refer to the worm $\text{G}\alpha$ proteins, for example using $\text{G}\alpha_o$ to refer to its *C. elegans* ortholog GOA-1. The worm $\text{G}\alpha_o$, $\text{G}\alpha_q$, and $\text{G}\alpha_s$ proteins are each widely expressed in most or all neurons, plus some muscle and other cells (Mendel et al., 1995: PMID 7886455; Ségalat et al., 1995: PMID 7886454; Park et al., 1997: PMID 9272860; Korswagen et al., 1997: PMID 9203577; Lackner et al., 1999: PMID 10571228; Bastiani et al., 2003: PMID 14704167). $\text{G}\alpha_{12}$, in contrast, is expressed in only a small subset of neurons plus some muscle and hypodermal cells (van der Linden et al., 2003: PMID 12646136; Yau et al., 2003: PMID 14657363).

I note as an aside that while GOA-1 appears to be the $\text{G}\alpha_o$ ortholog in *C. elegans*, there are also two more distantly-related $\text{G}\alpha_o$ homologs that have some functional redundancy with GOA-1. GPA-16 is co-expressed with GOA-1 in some neurons (Jansen et al., 1999: PMID 10192394) and in early embryonic cells, where these two $\text{G}\alpha$ proteins function redundantly to control mitotic spindle positioning during asymmetric cell divisions (reviewed by Rose and Gönczy, 2014: PMID: 25548889). GPA-7 is another $\text{G}\alpha_o$ -related protein that shows expression in many neurons (Jansen et al., 1999: PMID 10192394). The functions of GPA-7 have not been carefully investigated, but in one study GPA-7 and GOA-1 were shown to function redundantly to

mediate dopamine signaling in *C. elegans* male copulatory neurons and muscles (Correa et al., 2012; PMID 23166505).

Studies of mammalian GPCRs demonstrate that any one receptor may activate $G\alpha_o$, $G\alpha_q$, $G\alpha_s$, or $G\alpha_{12}$, but will generally not couple to more than one of these $G\alpha$ types (Moreira, 2014: PMID 24016604). Currently it is not possible to predict from the sequence of a GPCR which $G\alpha$ protein it will activate, so this must be determined experimentally (see section 2.1.2).

C. elegans $G\alpha_{12}$, like mammalian $G\alpha_{12}$, can activate its effector protein RGS-RhoGEF protein (RHGF-1 in *C. elegans*) to in turn activate the small GTPase Rho, and genetic work in *C. elegans* shows this can regulate a pathway involving diacylglycerol and protein kinase C to increase neurotransmitter release (van der Linden et al., 2003: PMID 12646136; Yau et al., 2003: PMID 14657363; Hiley et al., 2006: PMID 17139250). However, these results all arise from studies of worms expressing constitutively active mutants of $G\alpha_{12}$, and no defects have yet been observed in worms carrying loss of function mutations in $G\alpha_{12}$ or its effector RHGF-1. Thus the normal physiological functions of the $G\alpha_{12}$ pathway in *C. elegans* remain to be elucidated. I will not further consider $G\alpha_{12}$ signaling here, but see Perez-Mansilla and Nurrish (2009: PMID 19615533) for a detailed review of *C. elegans* $G\alpha_{12}$ signaling.

3.2. Introduction to the genetics of $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$

Before delving into the details of the signaling pathways for the three major neural G proteins, I begin with an overview of what these pathways do and a description of the genetic approaches in *C. elegans* that have been used to study them.

3.2.1. A simplified overview: $G\alpha_o$, $G\alpha_q$, $G\alpha_s$ signaling regulate neurotransmitter release

The simplified take-home message from genetic analysis of $G\alpha_o$, $G\alpha_q$, $G\alpha_s$ signaling in *C. elegans* neurons is that $G\alpha_o$ signaling inhibits neurotransmitter release, while $G\alpha_q$ and $G\alpha_s$ signaling activate neurotransmitter release or promote synaptic activity (Figure 2). $G\alpha_o$ and $G\alpha_q$ signaling appear to affect the localization of specific molecules at presynaptic release sites that regulate the neurotransmitter release machinery itself (Lackner et al., 1999: PMID 10571228; Nurrish et al., 1999: PMID 10677040; Chan et al., 2012: PMID 22588719), while it remains less clear how $G\alpha_s$ signaling promotes neurotransmitter release (Reynolds et al., 2005: PMID 15489511). A single neuron expresses many GPCRs (see section 2 above), so at any one time all three pathways may be active simultaneously, and indeed there may be receptors for different neurotransmitters activating a single type of $G\alpha$ protein simultaneously. Thus $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ sum up signaling by the several GPCRs active on a neuron, and their three downstream signaling pathways function together to compute an appropriate efficiency for the neurotransmitter release machinery in the neuron.

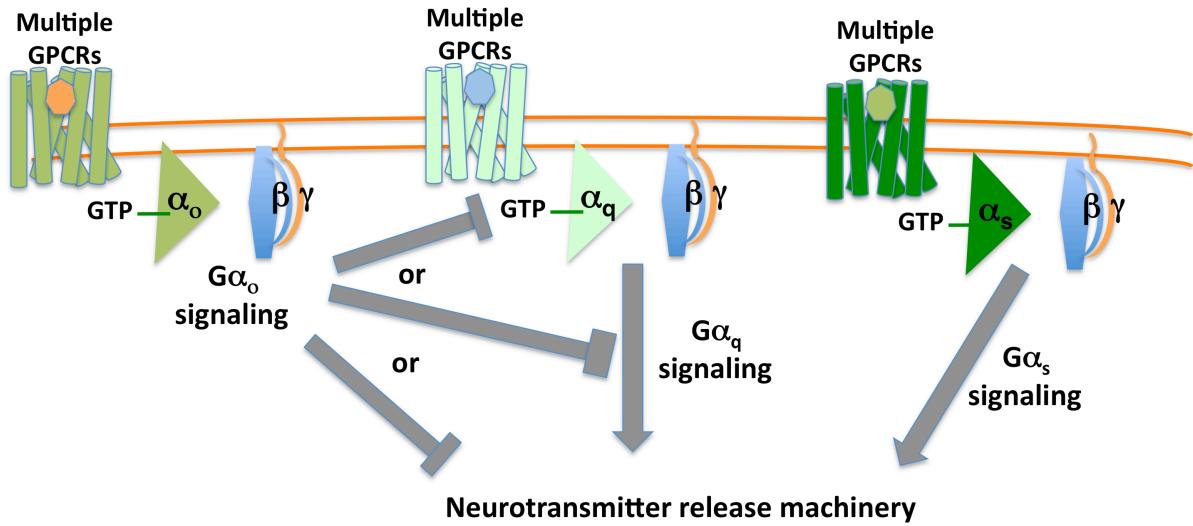


Figure 2. Schematic diagram illustrating how $\text{G}\alpha_o$, $\text{G}\alpha_q$, and $\text{G}\alpha_s$ signaling act single neuron to together regulate neurotransmitter release. A schematic summary of the effects of signaling by the three $\text{G}\alpha$ proteins on neurotransmitter release, as predicted from genetic studies in *C. elegans*. In such diagrams, an arrow indicates promotion or activation of a target, while a bar denotes an inhibitory effect. The bars extending from $\text{G}\alpha_o$ indicate that genetic experiments show $\text{G}\alpha_o$ signaling inhibits $\text{G}\alpha_q$ signaling, but do not determine whether this inhibition occurs upstream of $\text{G}\alpha_q$, at the level of $\text{G}\alpha_q$, or at some level downstream of $\text{G}\alpha_o$.

Figure 2 is a gross simplification. The neurotransmitter release machinery is complex, with distinct pools of small-clear vesicles present at presynaptic termini, as well as dense-core vesicles at non-synaptic sites, and release can occur tonically or after being evoked by depolarization. $\text{G}\alpha$ signaling may differentially affect the various types of vesicle release (Hu et al., 2015: PMID 25609620). Further, $\text{G}\alpha_o$, $\text{G}\alpha_q$, and $\text{G}\alpha_s$ signaling are known from electrophysiological studies to affect activity of specific ion channels, and from studies in other experimental systems to affect gene expression and synaptic structure. These G proteins are expressed not only in neurons but also in other cell types (e.g. muscles). So, clearly signaling by $\text{G}\alpha_o$, $\text{G}\alpha_q$, and $\text{G}\alpha_s$ must have effects other than on the neurotransmitter release machinery. Despite this, studies in *C. elegans* have focused on the effects of G protein signaling on synaptic neurotransmitter release because genetic experiments demonstrate that they actually affect behavior.

There are multiple lines of evidence that $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ signaling affect neurotransmitter release in *C. elegans*, with the most extensive evidence coming from studies of acetylcholine release by ventral chord motor neurons that control locomotion behavior. The four major lines of evidence are: 1) mutations in the G proteins and/or their signaling pathways alter locomotion behavior; 2) mutations in the G proteins and/or their signaling pathways alter response to aldicarb, an inhibitor of the acetylcholinesterase enzyme that clears released acetylcholine from synapses; 3) mutations in the G proteins and/or their signaling pathways alter the localization of GFP-tagged presynaptic proteins at cholinergic synapses; and 4) mutations in the G proteins and/or their signaling pathways cause changes in acetylcholine release that can be measured with electrophysiological methods. The specific studies detailing these lines of evidence for $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ are described and cited below in the remainder of section 3. As these details will show, all four lines of experimental evidence have established the effects of $G\alpha_o$ and $G\alpha_q$ signaling on acetylcholine release in *C. elegans*, while the effects of $G\alpha_s$ signaling on acetylcholine release from *C. elegans* ventral cord motor neurons rest on just the first two lines of evidence. However additional lines of evidence for the effects of $G\alpha_s$ signaling on neurotransmitter release come from studies of other cell types and species. For example studies in *C. elegans* ALA neurons show that the $G\alpha_s$ signaling pathway affects dense-core vesicle release (Zhou et al., 2007: PMID 18031683), and electrophysiological studies in mammalian and *Drosophila* neurons have also established effects of $G\alpha_s$ signaling on release of small clear neurotransmitter vesicles (Trudeau et al., 1996: PMID 8893035; Chen and Regehr, 1997: PMID 9348337; Kuromi and Kidokoro, 2000: PMID 10939337).

3.2.2. Isolation of $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ signaling mutants

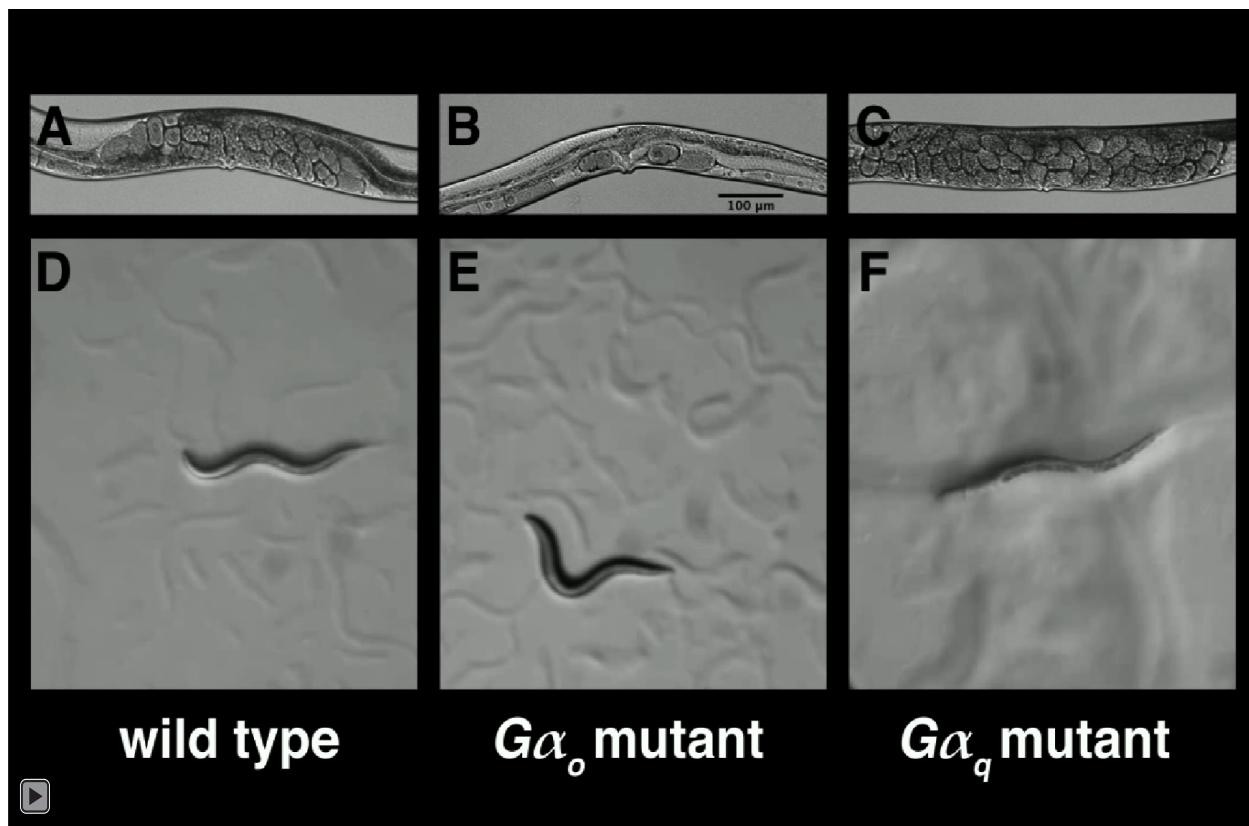
Studies of *C. elegans* neural G protein signaling have been carried out in parallel through both forward and reverse genetics. In the reverse genetic approach, *C. elegans* homologs of signaling proteins mammals are knocked out by gene-targeting technologies. Thus, for example, the entire sets of G protein subunit genes and RGS genes have been knocked out and analyzed (Jansen et al., 1999: PMID 10192394; Hess et al., 2004: PMID 154796380). The special power of the *C. elegans* system, however, is its capacity for large-scale forward genetic screens in which the genome is randomly mutagenized and vast numbers of animals are screened for specific phenotypes associated with neural G protein signaling defects. The initial screens for mutants with general neural G protein signaling defects took advantage of the fact that heterotrimeric G proteins regulate neurotransmitter release from the egg-laying motor neurons and from the cholinergic motor neurons that control locomotion. Thus these screens looked for mutants that fail to lay eggs (Trent et al., 1983: PMID 11813735; Desai and Horvitz, 1989: PMID 2721931), that lay eggs too frequently (Bany et al., 2003: PMID 12954868), that alter acetylcholine release (Miller et al., 1996: PMID 8901627; Miller et al., 1999: PMID 10571227; Miller et al., 2000: PMID 10985349; Sieburth et al., 2005: PMID 16049479; Vashlishan et al., 2008: PMID 18466746), that carry suppressors of previously-isolated G protein signaling mutations (Miller et al.,

1999; PMID 10571227; Schade et al., 2005: PMID 15489510; Charlie et al., 2006b: PMID 16624912; Williams et al., 2007: PMID 17942708), or that have hyperactive locomotion (Schade et al., 2005: PMID 15489510). Additional screens were for mutants that fail to respond to specific neurotransmitters that signal through GPCRs, including dopamine (Chase et al., 2004: PMID 15378064) and serotonin (Gürel et al., 2012: PMID 23023001).

3.2.3. Phenotypes of $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ signaling mutants

All genetic studies of neural G protein signaling in *C. elegans* involve assaying mutant phenotypes that arise from G protein signaling defects. Here I describe some of the mutant phenotypes used for this work.

$G\alpha_o$ null and partial loss-of-function mutants have a “hyperactive” phenotype (Mendel et al., 1995: PMID 7886455; Ségalat et al., 1995: PMID 7886454), which, as described below, appears to arise from increased neurotransmitter release from neurons throughout the animal. This phenotype can easily be recognized by observing in animals growing on a standard laboratory petri dish (Movie 1) and includes body bends that are deeper and more frequent than in the wild type. This defect arises at least in part from increased release of acetylcholine from the ventral cord motor neurons that control locomotion (Vashlishan et al., 2008: PMID 18466746). The hyperactive phenotype also includes an increased frequency of egg-laying behavior, such that animals lay almost all their eggs as soon as they are produced, so that whereas a wild-type animal might carry ~12 unlaid eggs on average, a hyperactive mutant might carry only one or two (Movie 1). This defect arises at least in part from increased neurotransmitter release from the HSN motor neurons that stimulate egg laying (Tanis et al., 2008: PMID 18202365). Specific serotonin and dopamine receptors signal through $G\alpha_o$ to inhibit locomotion, such that treating worms with sufficiently high concentrations of dopamine or serotonin paralyzes wild-type worms, but hyperactive $G\alpha_o$ mutants are resistant to paralysis by serotonin or dopamine (Ségalat et al., 1995: PMID 7886454; Chase et al., 2004: PMID 15378064; Gürel et al., 2012: PMID 23023001). As might be expected, since $G\alpha_o$ is expressed many neurons and non-neuronal cells, $G\alpha_o$ mutants have a number of additional phenotypic defects, including defects in mitotic spindle movements in early embryonic cells (Miller and Rand, 2000: PMID 11102364; Gotta and Ahringer, 2001: PMID 11231580) and defects in meiotic maturation of germ cells (Govindan et al., 2006: PMID 16824915).



Movie 1. The opposite effects of $G\alpha_o$ and $G\alpha_q$ loss-of-function mutations on behavior. A-C, still images of adult mid-body regions of wild-type, *goa-1(n1134)* $G\alpha_o$ partial loss-of-function mutant, and *egl-30(n686)* $G\alpha_q$ partial loss-of-function mutant animals, respectively. Unlaid eggs are visible as oval objects inside the body. While wild-type animals average ~12-16 unlaid eggs, $G\alpha_o$ mutants lay almost all their eggs and thus accumulate only 2-3 unlaid eggs, while $G\alpha_q$ mutants fail to lay eggs and accumulate >40 unlaid eggs. D-F, videos of locomotion by larvae of the same genotypes. The locomotion defects seen are most obvious in larvae but adults show qualitatively similar defects. $G\alpha_o$ mutants show hyperactive locomotion, with more frequent and deeper body bends, and more frequent reversals. $G\alpha_q$ mutants show sluggish locomotion, with infrequent, shallow body bends. Magnification is the same in all images, so the scale bar in B applies to all panels.

$G\alpha_q$ null mutants undergo developmental arrest as young larvae (Reynolds et al., 2005: PMID 15489511), but partial loss-of-function mutants develop to adulthood and show a “sluggish” phenotype that appears to arise from decreased neurotransmitter release (Lackner et al., 1999: PMID 10571228; Miller et al., 1999: PMID 10571227) from neurons throughout the animal (Bastiani et al., 2003: PMID 14704167). Sluggish animals have less frequent and shallower body bends than in the wild type, and in the case of strong loss-of-function or null $G\alpha_q$ mutants are virtually paralyzed (Movie 1) (Reynolds et al., 2005: PMID 15489511; Williams et al., 2007: PMID 17942708). This defect results at least in part from decreased acetylcholine release from ventral cord motor neurons (Lackner et al., 1999: PMID 10571228; Hu et al., 2015: PMID 25609620). Sluggish animals rarely engage in egg-laying behavior, such that adult

animals can become bloated with up to ~50 unlaid eggs (Movie 1), and this defect arises at least in part from decreased neurotransmitter release from the HSN motor neurons (Tanis et al., 2008: PMID 18202365).

The $G\alpha_o$ and $G\alpha_q$ loss-of-function phenotypes are in many respects precisely the opposite of each other. This interpretation is reinforced by the fact that mutations and transgenes that increase $G\alpha_o$ signaling cause a sluggish phenotype essentially indistinguishable from that seen in $G\alpha_q$ loss-of-function mutants (Mendel et al., 1995: PMID 7886455; Ségalat et al., 1995: PMID 7886454; Koelle and Horvitz, 1996: PMID 8548815), while transgenes and mutations that increase $G\alpha_q$ signaling cause a hyperactive phenotype virtually indistinguishable from that seen in $G\alpha_o$ loss-of-function mutants (Bastiani et al., 2003: PMID 14704167; Hajdu-Cronin et al., 1999: PMID 10421631; Schade et al., 2005: PMID 15489510; Reynolds et al., 2005: PMID 15489511; Charlie et al., 2006a: PMID 16272411; Williams et al., 2007: PMID 17942708; Matsuki et al., 2006: PMID 16418272).

$G\alpha_s$ null mutations are lethal (Korngreen et al., 1997: PMID 9203577), but mutations that decrease $G\alpha_s$ signaling cause a sluggish, near-paralyzed locomotion phenotype similar to that of $G\alpha_q$ loss-of-function mutants (Moorman and Plasterk, 2002: PMID 12019229; Reynolds et al., 2005: PMID 15489511). Mutations and transgenes that increase $G\alpha_s$ signaling induce a smoothly sinusoidal hyperactive phenotype, distinctly different from the deep body bends seen in $G\alpha_o$ loss-of-function or $G\alpha_q$ gain-of-function mutants (Reynolds et al., 2005: PMID 15489511; Schade et al., 2005: PMID 15489510; Charlie et al., 2006a: PMID 16272411).

3.2.4. The neuromuscular junction and the egg-laying synapse are often used to study neural G protein signaling in *C. elegans*

While $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ signaling affect many *C. elegans* behaviors, locomotion and egg-laying behaviors are two readouts of neural G protein signaling that have been frequently used to carry out genetic studies of the mechanism of neural G protein signaling due to unique advantages of each.

Egg-laying behavior depends on release of serotonin and other neurotransmitters from the HSN motor neuron. $G\alpha_o$ and $G\alpha_q$ have opposing effects on HSN neurotransmitter release (Tanis et al., 2008: PMID 18202365), and mutations in their signaling pathways result in easily scored and quantitated defects in egg laying (Chase and Koelle, 2004: PMID 15313573). Thus it has been possible to carry out genetic screens for mutants that are defective or hyperactive for egg-laying behavior to isolate mutants with defects in $G\alpha_o$ and $G\alpha_q$ signaling (Trent et al., 1983: PMID 11813735; Desai and Horvitz, 1989: PMID 2721931; Bany et al., 2003: PMID 12954868). Such mutants were used to originally discover and subsequently characterize the Regulators of G protein Signaling (RGS proteins) that inhibit most neural G protein signaling in *C. elegans* and in mammals (Koelle and Horvitz, 1996: PMID 8548815; Hajdu-Cronin et al., 1999: PMID 10421631). The neural circuit that controls egg laying is particularly simple and well-characterized, and tools to express transgenes in any cell of the circuit, to monitor activity of the circuit in freely-behaving animals with the fluorescent Ca^{2+}

indicator GCaMP, and to optogenetically manipulate the circuit have all been developed (Schafer, 2006: PMID 17094742; Emage et al., 2012: PMID 23152612; Collins and Koelle, 2013: PMID 23303953).

C. elegans locomotion behavior involves release of acetylcholine from ventral cord motor neurons onto body wall muscles, and $G\alpha_o$, $G\alpha_q$, and $G\alpha_s$ all affect acetylcholine release at this neuromuscular junction. The drug aldicarb paralyzes worms by preventing acetylcholine released at this neuromuscular junction from being degraded, and a powerful genetic screen for mutants resistant to aldicarb paralysis has been used to isolate mutants with decreased acetylcholine release that lie in the $G\alpha_o$ and $G\alpha_q$ signaling pathways (Miller et al., 1996: PMID 8901627; Miller et al., 1999: PMID 10571227; Miller et al., 2000: PMID 10985349). Screens for suppressors of aldicarb-resistant mutants and screens for mutants with hyperactive locomotion were used to isolate mutants with increased signaling in the $G\alpha_s$ and $G\alpha_q$ pathways (Miller et al., 1999: PMID 10571227; Schade et al., 2005: PMID 15489510; Charlie et al., 2006b: PMID 16624912). Mutations that affect acetylcholine release were used to discover the RIC-8 protein that promotes signaling by heterotrimeric G proteins in *C. elegans* and in mammals (Miller et al., 2000: PMID 11102364). The cholinergic neuromuscular junctions in ventral cord have been used for important experiments visualizing the effects of G protein signaling mutations on the localization of synaptic vesicle release proteins (Lackner et al., 1999: PMID 10571228; Nurrish et al., 1999: PMID 10677040; Chan et al., 2012: PMID 22588719). These neuromuscular junctions are also the most accessible synapses for electrophysiological studies in *C. elegans*, and have allowed studies of the fine details of G protein signaling mutations on neurotransmitter release (Hu et al., 2015: PMID 25609620).

While mutations in the $G\alpha_o$, $G\alpha_q$ and $G\alpha_s$ signaling pathways have powerful effects on egg-laying behavior by affecting neurotransmitter release from the HSN neurons and on locomotion by affecting acetylcholine release from ventral cord motor neurons, it has been hard to identify the GPCRs that activate the G proteins in these neurons. This difficulty is in line with the understanding, described in section 2 above, that an individual neuron appears to express many different GPCRs so that mutations in a single GPCR are expected to have very weak effects compared to mutations in a G protein. In the HSN neuron, the EGL-6 neuropeptide receptor signals through $G\alpha_o$ to inhibit egg laying, but this effect is only obvious in gain-of-function EGL-6 mutants, whereas EGL-6 null mutants do not have detectable defects. In the ventral cord motor neurons, the neuropeptide receptor CKR-2 and the G protein coupled acetylcholine receptor GAR-3 both appear to signal through $G\alpha_q$ to increase acetylcholine release (Hu et al., 2011: PMID 21745640; Chan et al., 2013: PMID 3986249), while a different G protein coupled acetylcholine receptor GAR-2, the heterodimeric G protein coupled GABA_B receptor GBB-1/GBB-2, and the dopamine receptor DOP-3 all appear to signal through $G\alpha_o$ to decrease acetylcholine release (Chase et al., 2004: PMID 15378064; Dittman and Kaplan, 2008: PMID 18614679). Mutations in these ventral cord motor neuron GPCRs have detectable effects on locomotion (Chase et al., 2004: PMID 15378064; Dittman and Kaplan, 2008: PMID 18614679; Hu et al., 2011: PMID 21745640; Chan et al., 2013: PMID 3986249), but knocking out a single one of these

GPCRs, as expected, has much weaker effects than does knocking out its downstream G protein (Mendel et al., 1995: PMID 7886455; Ségalat et al., 1995: PMID 7886454; Brundage et al., 1996: PMID 8630258).

3.3. The mechanism of $G\alpha_q$ signaling

In mammalian cells, active $G\alpha_q$ -GTP is classically known to signal by directly binding to and activating its effector, the transmembrane enzyme phospholipase C β (PLC β), which hydrolyzes the membrane lipid phosphatidylinositol 4,5 bisphosphate (PIP₂) to generate the soluble molecule inositol 1,4,5 trisphosphate (IP₃) and the membrane lipid diacylglycerol (DAG). IP₃ can go on to increase intracellular Ca²⁺, which along with DAG can bind and activate protein kinase C (Figure 3; reviewed by Sánchez-Fernández et al., 2014: PMID 24440667). Prior to studies of $G\alpha_q$ signaling in *C. elegans*, the only confirmed direct $G\alpha_q$ effector was PLC β . Genetic analysis of $G\alpha_q$ signaling in *C. elegans* (previously reviewed by Perez-Mansilla and Nurrish, 2009: PMID 19615533) supported the idea that PLC β (EGL-8 in *C. elegans*) is a physiologically important effector (Lackner et al., 1999, Miller et al., 1999; Bastiani et al., 2003: PMID 14704167). However, these same studies also suggested that there had to be additional $G\alpha_q$ effector(s) since the phenotypes of PLC β null mutants were much less severe than those of $G\alpha_q$ null mutants.

The missing $G\alpha_q$ effector was identified as the Trio RhoGEF through genetic screens for suppressors of the slow growth and hyperactive locomotion phenotypes caused by excessive $G\alpha_q$ pathway activity (Williams et al., 2007: PMID 17942708). Analysis using loss- and gain-of-function mutants in various double-mutant combinations showed that for control of growth, locomotion, and egg laying, $G\alpha_q$ signals through both PLC β and Trio RhoGEF. Knocking out either effector alone only partially blocks $G\alpha_q$ signaling, while knocking out both appears to completely block $G\alpha_q$ signaling (Williams et al., 2007: PMID 17942708). Trio RhoGEF proved to be as or more important than the classical PLC β effector pathway for multiple effects of $G\alpha_q$ signaling, including effects on growth, locomotion, and egg laying. Concomitant with the discovery of Trio RhoGEF as a $G\alpha_q$ effector in *C. elegans*, biochemical and structural studies demonstrated that mammalian $G\alpha_q$ binds and activates the orthologous mammalian RhoGEF proteins (Lutz et al., 2007: PMID 18096806; Rojas et al., 2007: PMID 17606614).

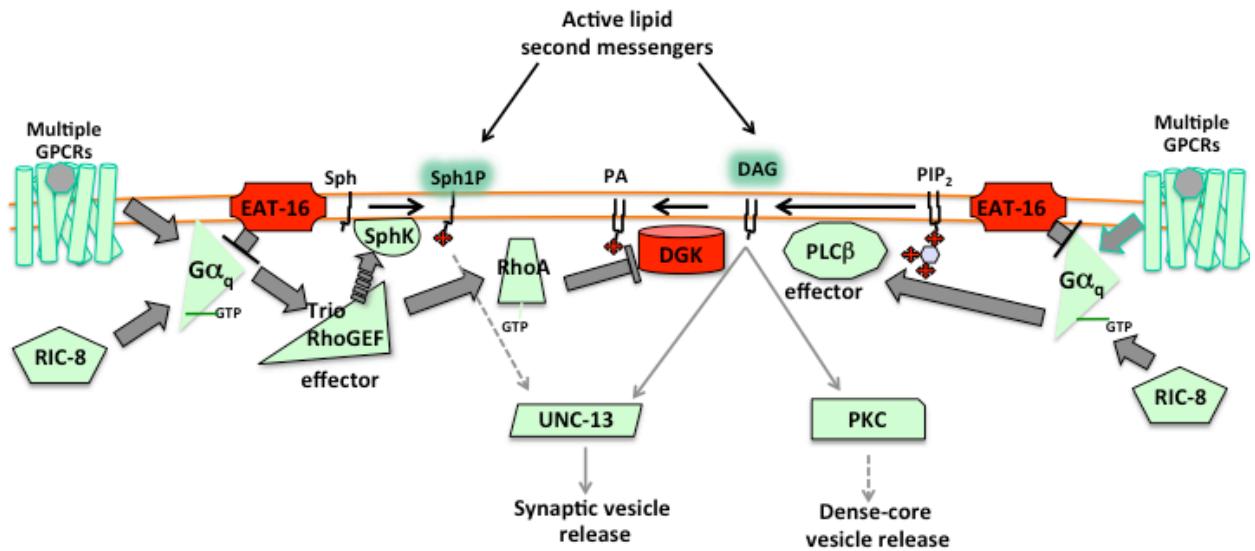


Figure 3. Mechanism of $G\alpha_q$ signaling. $G\alpha_q$ is induced to bind GTP to generate its active form by GPCRs, and induced to hydrolyze GTP to generate its inactive form by the RGS protein EAT-16. RIC-8 promotes $G\alpha_q$ signaling by acting as a chaperone to stabilize $G\alpha_q$ protein and/or by acting as a non-receptor activator to promote GTP binding by $G\alpha_q$. These proteins are drawn at both left and right to facilitate showing the two $G\alpha_q$ effector pathways at center. Left pathway: $G\alpha_q$ -GTP activates Trio RhoGEF, which in turn activates sphingosine kinase (SphK) to generate the lipid second messenger sphingosine 1-phosphate (Sph1P). Trio RhoGEF also increases levels of another lipid second messenger diacylglycerol (DAG) by activating RhoA to prevent diacylglycerol kinase (DGK) from converting DAG to phosphatidic acid (PA). Right pathway: $G\alpha_q$ -GTP activates phospholipase C β (PLC β) to generate the second messengers DAG and (not shown) IP $_3$. Sph1P and DAG each help UNC-13 to relocate to synaptic membranes and to promote synaptic vesicle release. DAG also activates protein kinase C (PKC) to promote dense-core vesicle release. Solid grey arrows (or bars), activation (or inhibition) via direct physical interactions. Dashed arrows or bars, indirect or poorly understood effects. Light green, proteins that promote $G\alpha_q$ signaling; red, proteins that inhibit $G\alpha_q$ signaling.

Two mechanisms for signaling downstream of the Trio RhoGEF effector of $G\alpha_q$ have been identified based on *C. elegans* genetics (Figure 3). First, while PLC β enzyme activity directly produces DAG, Trio RhoGEF can also increase DAG levels by an indirect mechanism. Trio RhoGEF activates the RhoA protein RHO-1, allowing it to directly bind and inhibit the diacylglycerol kinase DGK-1, blocking the first step in metabolic clearance of DAG, its phosphorylation to produce phosphatidic acid (PA) (McMullan et al., 2006: PMID 16391233). Thus both effectors for $G\alpha_q$ collaborate to increase DAG levels. Although Rho activity in mammals is principally known to regulate other processes, such as actin cytoskeleton dynamics and transcription, *C. elegans* genetics demonstrates that in its role mediating neurotransmitter signaling downstream

of $G\alpha_q$, a major physiologically significant role of Rho seems to be increasing DAG levels.

A second downstream mediator of the effects of $G\alpha_q$ /RhoGEF/RhoA signaling is the sphingosine kinase SphK (SPHK-1 in *C. elegans*). SphK catalyzes the phosphorylation of the membrane lipid sphingosine (Sph) to sphingosine-1-phosphate (Sph1P), a signaling lipid that among other functions promotes neurotransmitter release (Okada et al., 2009: PMID 18694820). In ventral cord cholinergic neurons, $G\alpha_q$ signaling via the Trio RhoGEF UNC-73 causes an increase in the perisynaptic localization of SphK, and this in turn appears to increase acetylcholine release (Chan et al., 2012: PMID 22588719). In a separate study, it was found that $G\alpha_q$ signaling recruits SphK to synapses via calcium influx using a mechanism involving the calcium binding protein CIB (CALM-1 in *C. elegans*) (Chan et al., 2012: PMID 22588719). The ability of SphK to increase neurotransmitter release depends on both its localization to perisynaptic regions and its ability to generate sphingosine 1-phosphate (Chan et al., 2012: PMID 22588719). At this point, the details of the mechanism by which Trio RhoGEF leads to recruitment of SphK to perisynaptic regions, and specifically the relationship between the calcium and Trio RhoGEF effects on SphK localization, remain to be determined.

How does the second messenger DAG produced by $G\alpha_q$ signaling lead to the ultimate effect of $G\alpha_q$ signaling, an increase in neurotransmitter release? Two DAG-binding proteins appear to mediate independent effects. One of these, UNC-13, is a protein essential for synaptic vesicle priming that facilitates neurotransmitter release by binding to the SNARE protein complexes that mediate vesicle fusion (James and Martin, 2013: PMID 24363652). Binding of UNC-13 to DAG in the membrane may help recruit it to synaptic membranes. The evidence for $G\alpha_q$ acting through UNC-13 includes the observations that in ventral cord motor neurons, $G\alpha_q$ activation of acetylcholine release depends on UNC-13, and that $G\alpha_q$ activation or addition of DAG analogs induces overexpressed UNC-13S::GFP to relocalize from a diffuse pattern in axons to punctate structures at synapses (Lackner et al., 1999: PMID 10571228). These effects can be abrogated by a mutation that blocks the ability of overexpressed UNC-13S::GFP to bind DAG. Recent electrophysiological studies provide perhaps the strongest evidence that UNC-13 functions in $G\alpha_q$ signaling to promote acetylcholine release from ventral cord motor neurons, with both the long and short isoforms of UNC-13 differentially affecting tonic and evoked release (Hu et al., 2015: PMID 25609620). Nevertheless, the extent to which UNC-13 mediates the behavioral effects of $G\alpha_q$ signaling has remained unclear. The recent development methods for precise genome engineering using CRISPR/Cas9 provide an opportunity to mutate the DAG binding site of native UNC-13 and potentially resolve this issue.

A second DAG binding protein that mediates effects of $G\alpha_q$ signaling in ventral cord motor neurons is protein kinase C (PKC) (Sieburth et al., 2007: PMID 17128266). PKC appears to affect release of neuropeptide-containing dense-core vesicles, although the targets via which PKC does this remain unknown. PKC does not appear to directly affect release of acetylcholine from small-clear synaptic vesicles. UNC-13 appears to affect small-clear vesicles, and there are differences of opinion as to whether it directly affects dense-core vesicles release (Sieburth et al., 2007: PMID 17128266; Speese et

al., 2007: PMID 17553987). Thus the current model is that $G\alpha_q$ signaling produces DAG to act via two different DAG-binding proteins, PKC and UNC-13, that together activate release of dense-core and synaptic vesicles (Figure 3).

A key observation about the role of DAG in $G\alpha_q$ signaling is that treating $G\alpha_q$ mutant worms with the DAG analogs known as phorbol esters rescues the paralysis of $G\alpha_q$ null and strong reduction-of-function mutants to wild-type levels of coordinated locomotion (Reynolds et al., 2005; PMID 15489511; Williams et al., 2007: PMID 17942708). The effectiveness of such non-localized phorbol ester treatment suggests that DAG is a “licensing factor” that promotes neurotransmitter release, i.e. that DAG need not be produced focally at synapses. Further, the fact that $G\alpha_q$ mutants can be rescued by phorbol ester treatment demonstrates that developmental defects do not contribute to the strong paralysis of $G\alpha_q$ strong loss-of-function or null mutants.

How does the other second messenger produced by $G\alpha_q$ signaling, sphingosine 1-phosphate, increase neurotransmitter release? An initial study suggested the effect of $G\alpha_q$ on neurotransmitter release via SphK appeared to be entirely independent and parallel to the ability of $G\alpha_q$ signaling to increase neurotransmitter release via increasing DAG levels (Chan et al., 2012: PMID 22588719). However, a subsequent study suggested SphK is essential for $G\alpha_q$ signaling to relocalize UNC-13S::GFP to synapses (Chan and Sieburth, 2012: PMID 22588719), and UNC-13S::GFP relocalization also appears to depend on DAG (Lackner et al., 1999: PMID 10571228). So, more work remains to clarify the relationship of DAG and SphK signaling downstream of $G\alpha$ in regulating neurotransmitter release.

Two upstream regulators of $G\alpha_q$ were identified through *C. elegans* genetic analysis. The Regulator of G protein signaling (RGS) protein EAT-16 behaves as a specific inhibitor of $G\alpha_q$ signaling (Hajdu-Cronin et al., 1999: PMID 10421631). The RIC-8 protein promotes $G\alpha_q$ signaling (Miller et al., 2000: PMID 11102364). I will elaborate on studies of RGS proteins and RIC-8 in sections 3.6 and 3.7 below.

3.4. The mechanism of $G\alpha_s$ signaling

The $G\alpha_s$ signaling pathway (Figure 4) has been intensively studied for decades in mammalian cells using biochemical approaches (Godinho et al., 2015: PMID 25859216). $G\alpha_s$ activated by GPCRs binds and activates its effector, the transmembrane enzyme adenylyl cyclase, which generates the second messenger cyclic AMP (cAMP). cAMP binds the regulatory subunits of protein kinase A (PKA), causing them to dissociate from and thus activate the catalytic subunit, which can then phosphorylate various target proteins. Signaling is terminated in part through the action of the enzyme phosphodiesterase to hydrolyze cAMP. $G\alpha_s$ is unique among $G\alpha$ isoforms in that no RGS protein is known to act on $G\alpha_s$ to help it hydrolyze GTP, so the slow intrinsic GTPase activity of $G\alpha_s$ is apparently used to terminate $G\alpha_s$ activity.

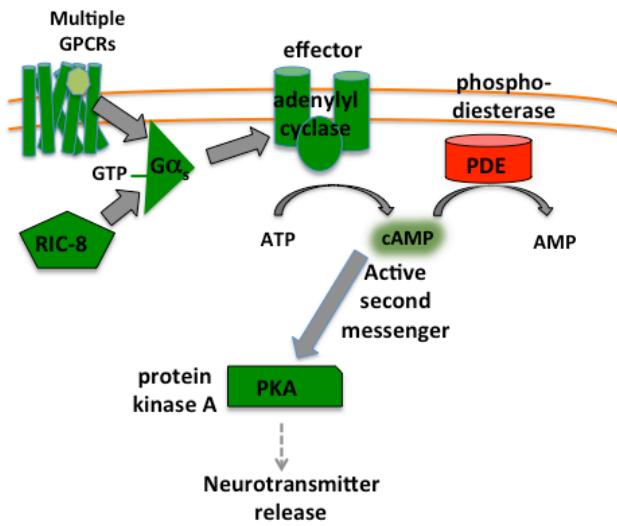


Figure 4. Mechanism of G α_s signaling. G α_s is induced to bind GTP to generate its active form by GPCRs. RIC-8 promotes signaling, either via its G α chaperone function or as a non-receptor activator. G α_s -GTP inactivates itself (not shown) via its slow intrinsic GTPase activity. G α_s -GTP activates the transmembrane enzyme adenylyl cyclase to generate the second messenger cyclic AMP (cAMP), which ultimately is hydrolyzed by phosphodiesterase. cAMP binds the regulatory subunits of protein kinase A (PKA), causing them to release the active subunit. PKA phosphorylation of unknown target proteins activates release of both synaptic and dense-core vesicles to increase neurotransmitter release. Solid grey arrows indicate direct physical interactions. Dashed arrow indicates a poorly understood effect. Green, proteins that promote G α_s signaling; red, proteins that inhibit G α_s signaling.

Genetic studies show that the same pathway delineated by biochemical studies in mammals also operates in *C. elegans* motor neurons (Figure 4; Schade et al., 2005: PMID 15489510). Initial studies showed that transgenic overexpression of constitutively-active G α_s kills neurons through a cAMP-dependent mechanism, as the killing could be suppressed with mutations in the adenylyl cyclase ACY-1 (Korswagen et al., 1997: PMID 9203577; Berger et al., 1998: PMID 9526004; Moorman and Plasterk, 2002: PMID 12019229). G α_s null mutants are lethal (Korswagen et al., 1997: PMID 9203577), but mutants with increased G α_s signaling are viable, and have provided important tools for genetic analysis of G α_s signaling. Mutations that increase G α_s signaling have been identified in G α_s itself (GSA-1 in *C. elegans*), adenylyl cyclase (ACY-1), protein kinase A (loss of the regulatory subunit KIN-2 causes constitutive activity of the catalytic subunit KIN-1), and in phosphodiesterase (PDE-4) (Schade et al., 2005: PMID 15489510; Charlie et al., 2006b: PMID 16624912). Increased G α_s signaling results in smoothly hyperactive sinusoidal locomotion, which is distinct from the hyperactive locomotion with abnormally deep body bends induced by loss of G α_o signaling or increased G α_q signaling (Schade et al., 2005: PMID 15489510). Epistasis analysis using G α_s signaling

pathway mutants supports the idea that $G\alpha_s$ in *C. elegans* functions in a pathway analogous to the mammalian $G\alpha_s$ signaling pathway that had been characterized biochemically (Schade et al., 2005: PMID 15489510; Reynolds et al., 2005: PMID 15489511).

$G\alpha_s$ pathway activity requires the guanine nucleotide exchange factor RIC-8, (Reynolds et al., 2005: PMID 15489511), although it remains unclear if this is due to the function of RIC-8 as chaperone or due to RIC-8 acting as a non-receptor nucleotide exchange protein.

The ultimate effect of $G\alpha_s$ signaling in neurons appears to be to promote neurotransmitter release. Schade et al. (2015: PMID 15489510) showed that activating $G\alpha_s$ signaling in both neurons and muscles using gain-of-function mutations appears to induce acetylcholine release from motor neurons, although Reynolds et al. (2005: PMID 15489511) showed that loss of *acy-1* just in neurons did not seem to affect neurotransmitter release. Separate studies in ALA neurons show that PKA activation can induce dense-core vesicle release (Zhou et al., 2007: PMID 18031683), so $G\alpha_s$ signaling may be able to activate neurotransmitter release via both synaptic and dense-core vesicles. The downstream targets of phosphorylation by PKA that increase vesicle release remain unknown.

$G\alpha_s$ signaling may operate in muscles, in addition to neurons, to control *C. elegans* behavior. Null mutants for the adenylyl cyclase ACY-1 show two separable defects: larval arrest and near-paralysis. In an *acy-1* null mutant, re-expression of *acy-1* in either muscles or neurons can rescue the larval arrest, but neither is sufficient to rescue paralysis, indicating that *acy-1* may be required in both neurons and muscles for proper locomotion (Reynolds et al., 2005: PMID 15489511). Rescue experiments also indicate that $G\alpha_s$ signaling is required for adult neural function, but is not required for neural development, since induced expression of *acy-1* in paralyzed animals lacking neural *acy-1* can restore locomotion behavior in these animals (Reynolds et al., 2005: PMID 15489511).

Genetic studies initiated to study $G\alpha_s$ signaling have led to a broader genetic analysis of the *in vivo* functions of cAMP in *C. elegans*. While the adenylyl cyclase ACY-1 mediates all known effects of $G\alpha_s$ signaling (Schade et al., 2005: PMID 15489510), there are other adenylyl cyclase isoforms in *C. elegans* that appear to generate a $G\alpha_s$ -independent pool of cAMP that also affect locomotion and larval growth (Charlie et al., 2006b: PMID 1461419). cAMP is an important negative regulator of sleep-like states throughout the animal kingdom (Zimmerman et al., 2008: PMID 18538867), and $G\alpha_s$ pathway activating mutants are being used to study sleep-like states in *C. elegans* (Belfer et al., 2013: PMID 23633751). Ghosh-Roy et al., (2010: PMID 20203177) has also used $G\alpha_s$ pathway mutants to study the role of cAMP in axon regeneration.

3.5. The mechanism of $G\alpha_o$ signaling

The mechanism of $G\alpha_o$ signaling (Figure 5) is poorly understood and remains an area ripe for further investigation using the *C. elegans* system. Biochemical studies in

mammals have shown that $G\alpha_o$ is expressed throughout the nervous system and is by orders of magnitude the most abundant $G\alpha$ protein in neurons, constituting ~1.5% of membrane protein in the brain (Robishaw and Sternweiss, 1984: PMID 6438083). Despite efforts to identify $G\alpha_o$ binding proteins that might function as effectors for $G\alpha_o$ or its paralogs (Chen et al., 1999: PMID 10480904; Takesono et al., 1999: PMID 10559191; Cuppen et al., 2003: PMID 18629017), no such protein has been validated as a *bona fide* effector of $G\alpha_o$. Activation of $G\alpha_o$ releases $G\beta\gamma$ subunits, allowing them to activate specific K^+ channels and inhibit specific Ca^{2+} channels, resulting in inhibition of neural activity (Reuveny et al., 1994: PMID 8022483; Herlitze et al., 1996: PMID 8637576).

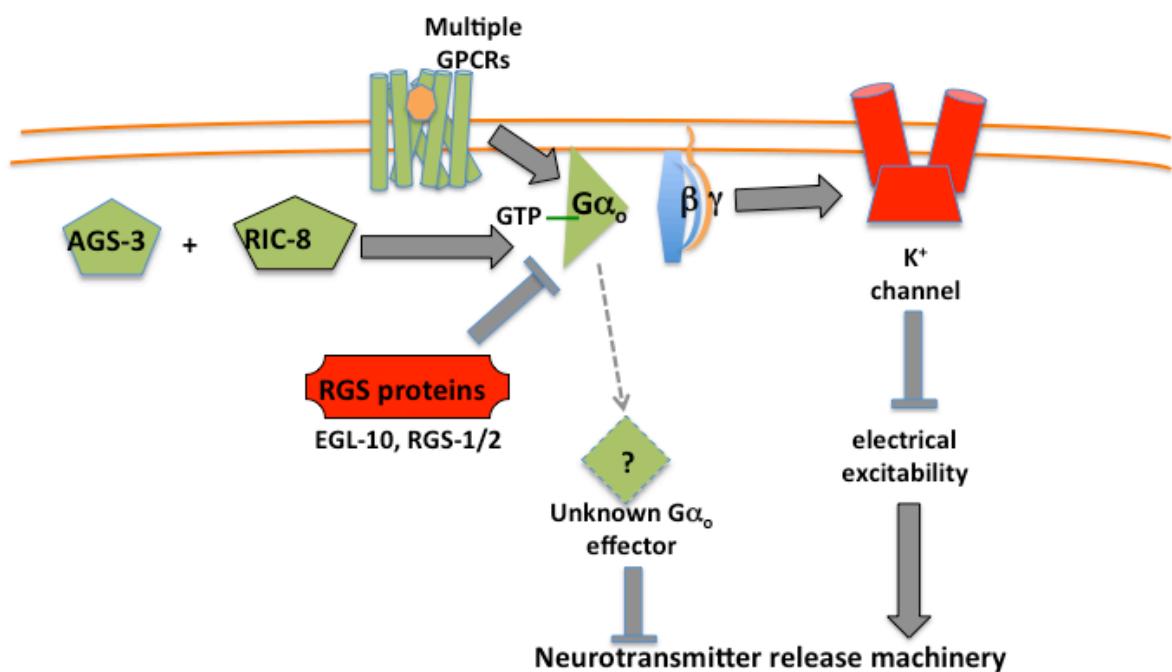


Figure 5. Mechanism of $G\alpha_o$ signaling. $G\alpha_o$ is induced to bind GTP to generate its active form by GPCRs, or by the non-receptor activators AGS-3 and RIC-8. Alternatively, or in addition, RIC-8 may function as a chaperone for $G\alpha_o$. $G\alpha_o$ -GTP may signal to inhibit the neurotransmitter release machinery by an unknown effector protein. The $G\beta\gamma$ subunits released from $G\alpha_o$ can activate specific K^+ channels to reduce electrical excitability, which is in turn facilitates neurotransmitter release. Green, proteins that promote $G\alpha_o$ signaling; red, proteins that inhibit $G\alpha_o$ signaling.

The ultimate effect of $G\alpha_o$ signaling in *C. elegans* neurons is to inhibit neurotransmitter release, and this has been studied in detail in ventral cord motor

neurons, where $G\alpha_q$ signaling inhibits acetylcholine release (Nurrish et al., 1999: PMID 10677040; Miller et al., 1999: PMID 10571227; Charlie et al., 2006a: PMID 16272411; Vashlishan et al., 2008: PMID 18466746), and in HSN neurons, where $G\alpha_o$ signaling inhibits release of serotonin and other neurotransmitters that stimulate egg laying (Tanis et al., 2008: PMID 18202365). $G\alpha_q$ and $G\alpha_o$ signaling have the opposite effects on locomotion, egg laying, and a variety of other behaviors (Movie 1), suggesting that in general, while $G\alpha_q$ signaling promotes neurotransmitter release (see section 3.2 above), $G\alpha_o$ has an opposing effect of inhibiting neurotransmitter release.

Activation of $G\alpha_q$ signaling in ventral cord motor neurons appears to increase the localization of the synaptic vesicle priming protein UNC-13S to presynaptic termini, as seen by visualizing a fusion of UNC-13S to the green fluorescent protein (Lackner et al., 1999: PMID 105712). A $G\alpha_o$ loss-of-function mutation also causes an increase in localization of this UNC-13S::GFP protein to presynaptic termini (Nurrish et al., 1999: PMID 10677). This result suggests that that $G\alpha_q$ and $G\alpha_o$ oppose each other at a mechanistic level. The effects of $G\alpha_q$ and $G\alpha_o$ on UNC-13S::GFP localization both depend on the diacylglycerol (DAG) binding site of UNC-13 (Lackner et al., 1999: PMID 105712; Nurrish et al., 1999: PMID 10677). Thus it is assumed that the increase in DAG levels produced by $G\alpha_q$ signaling cause the observed changes in UNC-13S::GFP localization. $G\alpha_o$ signaling could affect UNC-13::GFP and inhibit neurotransmitter release by decreasing DAG levels (Nurrish et al., 1999: PMID 10677). However, no biochemical experiments have demonstrated any effect of $G\alpha_o$ on DAG or on the enzymes that generate or degrade DAG (Jose and Koelle, 2005: PMID 15563467; Perez-Mansilla and Nurrish, 2009: PMID 19615533).

While genetic studies in *C. elegans* have not clarified the downstream mechanism of $G\alpha_o$ signaling, they have identified novel upstream regulators of $G\alpha_o$. The RGS protein EGL-10 is expressed in all neurons, and EGL-10 null mutants show sluggish locomotion and egg-laying defects due to increased $G\alpha_o$ signaling (Koelle and Horvitz, 1996: PMID 8548815). There are other RGS proteins (RGS-1, RGS-2, RGS-7) that additionally inhibit $G\alpha_o$ in specific cell types or under specific circumstances (see section 3.7 below). $G\alpha_o$ signaling is promoted by RIC-8, working in combination with a GPR-domain containing protein (see section 3.6 below). As in the case of RIC-8's effect on $G\alpha_s$ signaling (section 3.3), it is unclear whether RIC-8's effect on $G\alpha_o$ signaling reflects its nucleotide exchange activity, its G α chaperone function (Gabay et al., 2011: PMID 22114146; Chan et al., 2013: PMID 23431197), or both.

3.5.1. Does $G\alpha_o$ have an effector?

One view is that $G\alpha_o$ has no effector, and that $G\alpha_o$ signaling occurs simply by release of G $\beta\gamma$ subunits that would then be entirely responsible for further downstream signaling by regulating K $^+$ and Ca $^{2+}$ channels. The plausibility of such a scheme is bolstered by careful genetic studies of heterotrimeric G protein signaling in yeast, in which mating pheromone signaling through a heterotrimeric G protein is indeed carried out by activation of a G α protein whose principal function is simply to release G $\beta\gamma$ so

that $G\beta\gamma$ can induce further signaling events (Dohlman and Thorner, 2001: PMID 11395421).

Genetic studies in *C. elegans* strongly suggest that $G\alpha_o$ signaling does not proceed purely through $G\beta\gamma$ effectors, and that $G\alpha_o$ must also signal directly through its own yet-to-be-discovered effectors. The only study of $G\beta\gamma$ effectors in *C. elegans* showed that when the EGL-6 neuropeptide receptor activates $G\alpha_o$ in the HSN neurons, the resulting inhibition of egg laying requires IRK-1, a homolog of mammalian $G\beta\gamma$ -activated K^+ channels, and that no other such K^+ channel is required (Emtage et al., 2012: PMID 23152612). However, this same study also demonstrated that IRK-1 does not mediate inhibition of egg laying when other methods are used to activate $G\alpha_o$, suggesting that other effectors besides K^+ channels must mediate $G\alpha_o$ signaling. This study leaves open the possibility that these other effectors could be additional $G\beta\gamma$ effectors such as Ca^{2+} channels. However, other genetic results argue in favor of effectors for $G\alpha_o$ itself.

First, $G\alpha_q$ and $G\alpha_o$ release the identical $G\beta\gamma$ subunits, as there is only one $G\beta\gamma$ isoform generally expressed in *C. elegans* neurons, yet activation of $G\alpha_q$ and $G\alpha_o$, acting in the same neurons, have exactly opposite effects on neurotransmitter release and behavior (Lackner et al., 1999: PMID 10571228; Nurrish et al., 1999: PMID 10677040; Tanis et al., 2008: PMID 18202365). To argue that $G\alpha_o$ signaling principally results from released $G\beta\gamma$ inhibiting neural activity, one needs to explain how $G\alpha_q$ signaling does not give the same result. This discrepancy might result from the far greater abundance of $G\alpha_o$, or by supposing that $G\alpha_o$ and $G\alpha_q$ reside in different membrane microdomains, but so far there is no actual data to support either idea.

Second, the genetics of $G\alpha_o$ signaling in *C. elegans* does not match the genetics of $G\beta\gamma$ -mediated pheromone signaling pathway in yeast. In the yeast pheromone signaling pathway, a null mutant for the $G\alpha$ subunit results in constitutive signaling, as $G\alpha$ is no longer present to sequester $G\beta\gamma$ and inhibit its ability to signal (Whiteway et al., 1989: PMID 2536595). Conversely, transgenic overexpression of yeast $G\alpha$ inhibits pheromone signaling, apparently by increasing the pool of $G\alpha$ available to associate with and inhibit $G\beta\gamma$ (Cole et al., 1990: PMID 2105453). The analogous experiments in *C. elegans* with $G\alpha_o$ give precisely the opposite results. $G\alpha_o$ null mutations appear to result in a loss of signaling, not constitutive signaling (Mendel et al., 1995: PMID 7886455; Ségalat et al., 1995: PMID 7886454), and overexpression of wild-type $G\alpha_o$ appears to result in constitutive signaling, not loss of signaling (Mendel et al., 1995: PMID 7886455). These results are exactly analogous to those obtained from genetic studies of $G\alpha_q$ in *C. elegans* (Brundage et al., 1996: PMID 8630258), a case in which it is clear that downstream signaling is due to $G\alpha$ effectors, not $G\beta\gamma$ effectors.

Could it be that $G\alpha_o$ signals by directly binding to and inhibiting adenylyl cyclase? Adenylyl cyclase is the effector directly activated by $G\alpha_s$ (see section 3.3. above), and it can also be directly bound and inhibited by members of the $G\alpha_{i/o}$ family of $G\alpha$ proteins, at least in cultured cells. Indeed, $G\alpha_o$ overexpressed in cultured cells has been shown to inhibit one isoform of adenylyl cyclase (Näsman et al., 2002: PMID 12472880). While this hypothesis is intriguing, there is currently no genetic evidence that inhibition of adenylyl cyclase is a physiologically relevant mechanism for $G\alpha_o$ signaling. If $G\alpha_o$

signaled by inhibiting adenylyl cyclase, we might expect that $G\alpha_o$ and $G\alpha_s$ signaling would show precisely opposite effects in *C. elegans* genetic experiments. However, this is not the case (see section 3.3 above), and $G\alpha_o$ signaling rather shows far more precise opposition to the effects of $G\alpha_q$ signaling.

Could it be that $G\alpha_o$ signals by directly binding to and regulating one of the components of the $G\alpha_q$ signaling pathway, thus neatly solving the problem of identifying the $G\alpha_o$ effector and explaining how $G\alpha_o$ signaling opposes $G\alpha_q$ signaling (section 3.5)? Two specific $G\alpha_q$ signaling pathway proteins (Figure 3) have been proposed as $G\alpha_o$ effectors: DGK and EAT-16. First, all genetic studies are consistent with the possibility that $G\alpha_o$ could signal by activating diacylglycerol kinase (DGK), the enzyme that terminates $G\alpha_q$ signaling by destroying the $G\alpha_q$ second messenger diacylglycerol. However, all efforts to demonstrate a biochemical effect of $G\alpha_o$ on DGK activity have proven negative (Jose and Koelle, 2005: PMID 15563467; Perez-Mansilla and Nurrish, 2009: PMID 19615533). The second hypothesis is that the effector for $G\alpha_o$ could be the $G\alpha_q$ -specific RGS protein EAT-16. An elegant model based on both genetic analysis and structural features of the EAT-16 protein complex proposes that inactive $G\alpha_o$ sequesters EAT-16, and activation of $G\alpha_o$ releases EAT-16 to inhibit $G\alpha_q$ (Robatzek et al., 2001: PMID 11250160). However, no biochemical studies of either the *C. elegans* proteins nor their mammalian equivalents has produced any support for this model.

If $G\alpha_o$ indeed signals directly through its own effectors, why have these effectors not yet been identified? Known effectors for other G proteins are often multi-subunit membrane protein complexes, and the cDNA expression library screening methods that have been employed in attempts to identify $G\alpha_o$ effectors (Chen et al., 1999: PMID 10480904; Takesono et al., 1999: PMID 10559191; Cuppen et al., 2003: PMID 18629017) are not well-suited for finding such proteins. Genetic screens in *C. elegans* for $G\alpha_o$ signaling proteins would have failed to identify $G\alpha_o$ effectors if there are multiple redundant effectors, such that mutating a single effector gene causes little reduction in $G\alpha_o$ signaling. In addition, if a $G\alpha_o$ effector is essential for viability or reproduction, mutating such an effector would not produce animals that could be detected and recovered in a genetic screen. Because there are perfectly reasonable explanations why a $G\alpha_o$ may have eluded discovery, the absence of such a discovery at this point should not be taken as evidence that $G\alpha_o$ effectors do not exist.

3.6. The relationship between $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ signaling

$G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ signaling all operate at the same time in the same neurons, and Figure 2 presents a model showing them acting in parallel to each other until they intersect downstream by ultimately all regulating neurotransmitter release. However, genetic epistasis experiments have been used to examine the relationship between the three G protein signaling pathways, and other relationships between the G protein signaling pathways are also consistent with these genetic results.

Double mutants for $G\alpha_q$ and $G\alpha_o$ resemble $G\alpha_q$ single mutants, and this and results from other double-mutant combinations are consistent with the interpretation that $G\alpha_o$ signaling acts to inhibit $G\alpha_q$ signaling (Hajdu-Cronin et al., 1999: PMID 10421631;

Miller et al., 1999: PMID 10571227; Charlie et al., 2006a: PMID 16272411). More specifically, this means that $G\alpha_o$ signaling could inhibit the $G\alpha_q$ pathway at any level, upstream of $G\alpha_q$, at the level of $G\alpha_q$, or at any level downstream of $G\alpha_q$. Only molecular experiments will ultimately be able to distinguish between these possibilities.

The relationship between $G\alpha_s$ and $G\alpha_q$ signaling has been investigated by combining mutations that increase signaling in one pathway with mutations that block signaling in the other pathway (Reynolds et al., 2005: PMID 15489511; Charlie et al., 2006a: PMID 16272411). This was done in both directions (i.e. a $G\alpha_s$ pathway gain-of-function mutation combined with a $G\alpha_q$ null mutation, and also a $G\alpha_q$ gain-of-function mutation combined with a $G\alpha_s$ pathway null mutation). The results of these experiments do not yield a simple epistatic relationship between the two signaling pathways, but do yield several important insights. First, the $G\alpha_s$ pathway is virtually completely dependent on the $G\alpha_q$ pathway to exert its effects on locomotion (Reynolds et al., 2005: PMID 15489511). Second, the $G\alpha_q$ pathway can still promote locomotion in the absence of a functional $G\alpha_s$ pathway, but the resulting locomotion is uncoordinated (Reynolds et al., 2005: PMID 15489511). Third, phorbol esters (analogs of the DAG ultimately produced by the $G\alpha_q$ pathway) can also promote uncoordinated locomotion in the absence of a functional $G\alpha_s$ pathway, suggesting $G\alpha_s$ signaling regulates locomotion downstream of DAG production (Reynolds et al., 2005: PMID 15489511). Fourth, the $G\alpha_q$ and $G\alpha_s$ pathways have distinct effects in driving locomotion, one line of evidence for which is that loss of $G\alpha_q$ signaling appears to strongly reduce acetylcholine release from motor neurons while loss of $G\alpha_s$ signaling does not (Charlie et al., 2006a: PMID 16272411).

3.7. Receptor-independent activation of heterotrimeric G proteins

An important contribution of the *C. elegans* system was the discovery of the receptor-independent $G\alpha$ chaperone and activator RIC-8, which was first identified in a genetic screen for mutants with reduced acetylcholine release from ventral cord motor neurons (Miller et al., 1996: PMID 8901627). Genetic studies showed that reduction of function mutations in RIC-8 and $G\alpha_q$ have similar phenotypes and were consistent with RIC-8 acting upstream of $G\alpha_q$ to promote $G\alpha_q$ signaling (Miller et al., 2000: PMID 11102364). Subsequent genetic studies demonstrated that RIC-8 also appears to promote $G\alpha_s$ signaling (Reynolds et al., 2005: PMID 15489511) and $G\alpha_o$ signaling (Miller and Rand, 2000: PMID 11102364; Hofler and Koelle, 2011: PMID 21832186).

Biochemical studies of mammalian RIC-8 have identified two mechanisms by which RIC-8 can promote $G\alpha$ signaling. First, RIC-8 can act as a nucleotide exchange factor to convert inactive $G\alpha$ -GDP to active $G\alpha$ -GTP (Tall et al., 2003: PMID 12509430; Chan et al., 2011: PMID 21467038). Unlike GPCRs, which are transmembrane proteins that act as nucleotide exchange factors to activate $G\alpha\beta\gamma$ heterotrimers, RIC-8 is a soluble protein and will not act on the heterotrimer, but rather can only activate free $G\alpha$ -GDP. This leads to the model depicted in Figure 1 in which a $G\alpha\beta\gamma$ heterotrimer would need to initially be activated by a GPCR to generate $G\alpha$ -GTP, but after GTP hydrolysis the inactive $G\alpha$ -GDP produced might be reactivated by the nucleotide exchange activity of RIC-8, thus prolonging signaling.

The second mechanism by which RIC-8 can activate G α signaling is by simply stabilizing G α proteins. Knocking out RIC-8 in mammalian cells causes dramatic reductions in the levels of G α proteins (Gabay et al., 2011: PMID 22114146). Biochemical studies show RIC-8 acts as a chaperone to help fold nascent G α proteins (Chan et al., 2013: PMID 23431197). It has not yet been determined if RIC-8 mutations in *C. elegans* similarly cause reductions of G α protein levels. The genetics of RIC-8 are consistent with RIC-8 promoting G α signaling either by simply stabilizing G α proteins, by acting as a nucleotide exchange factor, or by both mechanisms.

Another type of receptor-independent activator for G α_0 are proteins containing a short motif that binds specifically to G α proteins of the G $\alpha_{i/o}$ subfamily and that is known either as the GPR (G protein regulatory) or GoLoco motif (Siderovski et al., 1999: PMID 10470031; Peterson et al., 2000: PMID 10969064). *C. elegans* has three such proteins. The two very similar and functionally redundant GPR-1 and GPR-2 proteins act in early embryonic cells with G α_0 to regulate spindle positioning during asymmetric cell divisions (Colombo et al., 2003: PMID 12750478; Gotta et al., 2003: PMID 12814548; Srinivasan et al., 2003: PMID 12730122). The AGS-3 protein acts with G α_0 to regulate function of adult neurons (Hofler and Koelle, 2011: PMID 218321860). Biochemical studies of mammalian GPR/GoLoco proteins emphasize the fact that when these proteins bind G α -GDP, they inhibit nucleotide exchange, which would suggest that they prevent formation of active G α -GTP and thus inhibit signaling (Siderovski et al., 1999: PMID 10470031; Peterson et al., 2000: PMID 10969064). However, *C. elegans* genetic studies clearly demonstrate that precisely the opposite is true, since GPR-1/2 promote G α_0 activity in embryonic cell divisions and AGS-3 also promotes G α_0 signaling in adult neurons (Colombo et al., 2003: PMID 12750478; Gotta et al., 2003: PMID 12814548; Srinivasan et al., 2003: PMID 12730122; Hofler and Koelle, 2011: PMID 218321860). The apparent conflict between these results can be resolved by the findings that all known functions of the GPR/GoLoco proteins in *C. elegans* also require RIC-8, along with the biochemical result that RIC-8 can act as a nucleotide exchange factor on the complex between G α_0 -GDP and a GPR/GoLoco protein to produce active G α_0 -GTP (Thomas et al., 2008: PMID 18541531). Thus an attractive model is that when active G α_0 -GTP hydrolyzes GTP to become G α_0 -GDP, the function of the GPR/GoLoco protein is to bind G α_0 -GDP, preventing it from re-associating with G $\beta\gamma$, which would have terminated signaling, and rather to present G α_0 -GDP to RIC-8 for nucleotide exchange to convert it back to active G α_0 -GTP (Figure 1). The net result would be to prolong signaling that had been initiated earlier by a GPCR.

The *C. elegans* GPR/GoLoco protein AGS-3 is of particular interest as it has a close mammalian brain ortholog, AGS3, and a role in mediating behavioral response to food deprivation. AGS-3 is expressed in most or all *C. elegans* neurons and is required for several different behavioral changes that normally occur in response to short-term food deprivation (Hofler and Koelle, 2011: PMID 218321860). Biochemical studies show that over the first few hours of food deprivation, AGS-3 protein from *C. elegans* lysates changes from a detergent-insoluble form to a detergent-solubilizable form, indicating that a physical change in the protein occurs. These results lead to a model in which food deprivation leads to a physical change in AGS-3, allowing it to activate G α_0 signaling to

induce behavioral changes appropriate for the food-deprived state of the animal. It will be fascinating to see if this role of AGS-3 in mediating response to food deprivation is conserved in mammals (Hofler and Koelle, 2012: PMID 24058824).

3.8. Regulators of G protein signaling inhibit $G\alpha_o$ and $G\alpha_q$ signaling

Another important contribution of the *C. elegans* system was the discovery and characterization of the family of Regulator of G protein Signaling (RGS) proteins. The first RGS protein, Sst2p, was identified in yeast (Dietzel and Kurjan, 1987: PMID 2830483), but it was not clear that it had any homologs in higher eukaryotes. Genetic studies identified the *C. elegans* RGS protein EGL-10 as an inhibitor of $G\alpha_o$ signaling, and comparison of Sst2p and EGL-10 allowed the discovery of an “RGS domain” conserved between the two and that is also found in a large family of proteins in *C. elegans* and mammals (Koelle and Horvitz, 1996; PMID 8548815). Biochemical studies showed that the RGS domain binds directly to $G\alpha$ proteins, and by stabilizing the transition state for GTP hydrolysis, inactivates signaling by driving the conversion of active $G\alpha$ -GTP to the inactive species $G\alpha$ -GDP (Berman et al., 1996: PMID 8910288; Tesmer et al., 1997: PMID 9108480). GTPase activators were previously known for the small ras-like G proteins, which have structural similarity to the GTPase domain of $G\alpha$ proteins but lack a catalytic arginine residue that $G\alpha$ proteins use for GTP hydrolysis. Thus small G proteins are unable to convert GTP to GDP at a meaningful rate without first binding a GTPase activating protein that inserts an arginine residue into their active site (Li and Zhang, 2004: PMID 15236956). In contrast, $G\alpha$ subunits of heterotrimeric G proteins were initially thought to not require help from any GTPase activating proteins to convert to their inactive GDP-bound state, since they have an intrinsic GTPase activity orders of magnitude faster than that of small GTPases. However, genetic studies in *C. elegans* show that *in vivo*, the $G\alpha_o$ and $G\alpha_q$ proteins require RGS proteins to appropriately terminate signaling (Koelle and Horvitz, 1996: PMID 8548815; Hajdu-Cronin et al., 1999: PMID 10421631). In contrast, neither genetic studies in worms nor biochemical studies of mammalian proteins have identified a GTPase activator for $G\alpha_s$. Thus $G\alpha_s$ may indeed simply use its slow intrinsic GTPase activity to terminate signaling.

The two most intensively studied RGS proteins in *C. elegans* are EGL-10 and EAT-16, which serve as specific inhibitors of $G\alpha_o$ and $G\alpha_q$ signaling, respectively (Koelle and Horvitz, 1996: PMID 8548815; Hajdu-Cronin et al., 1999: PMID 10421631). They are members of the R7 family of RGS proteins, of which there are four members in mammals that are expressed in the brain (Anderson et al., 2009: PMID 19521673). *In vitro*, these mammalian R7 proteins all preferentially inactivate $G\alpha_o$ rather than $G\alpha_q$, and it is unclear if any of them might actually be $G\alpha_q$ specific *in vivo*, like *C. elegans* EAT-16. Analysis of chimeras between EGL-10 and EAT-16 shows that the $G\alpha_o$ versus $G\alpha_q$ specificity of these proteins lies in an N-terminal domain that remains of unknown biochemical function (Patikoglou and Koelle, 2002: PMID 12354761). The *C. elegans* R7 RGS proteins, like their mammalian homologs, contain a $G\gamma$ -like domain that is constitutively bound to a $G\beta$ -like protein known as $G\beta_5$ in mammals and GPB-2 in *C.*

elegans (Chase et al., 2001: PMID 11250150; Robatzek et al., 2001: PMID 11250160; van der Linden et al., 2001: PMID 11333232). Thus the R7 RGS/G β complex in many respects resembles a G $\beta\gamma$ complex, but there is as yet no understanding of what this means or how it might be used. EAT-16, like the mammalian R7 RGS proteins, is anchored to the plasma membrane via a lipid-modified membrane anchoring subunit (Porter and Koelle, 2009: PMID 19923320). There is no known membrane anchor for EGL-10.

There are at least 13 RGS proteins in *C. elegans*, and the genes for all have been knocked out and studied (reviewed by Porter and Koelle, 2009). While the R7 family RGS proteins EGL-10 and EAT-16 are widely expressed, and mutants cause severe disruption of G protein signaling and behavior, the other RGS proteins appear to have much more specific functions. For example, RGS-3 is expressed only in a subset of sensory neurons, where it dampens signaling in response to strong stimuli, increasing the dynamic range for sensing these stimuli (Ferke et al., 2007: PMID 17196529). RGS-1, RGS-2, and RGS-7 are all inhibitors of G α_o , but have much more limited functions than does the main G α_o inhibitor EGL-10. RGS-7 is expressed in early embryos where it regulates the function of G α_o in controlling asymmetric cell divisions (Hess et al., 2004: PMID 154796380). RGS-1 and RGS-2 are very similar to each other and are functionally redundant (Dong et al., 2000: PMID 10950865). Knocking out both disrupts a specific behavioral response that occurs when starved animals are re-fed. Knockouts for number of additional RGS genes so far have shown no detectable defects, suggesting that these RGS genes, like RGS-1 and RGS-2, may have redundant and/or very specific functions that will be difficult to define.

3.9. Reconciling studies of neural G α signaling in *C. elegans* with those in more complex model organisms

The studies in *C. elegans* reviewed above show that the molecular details of the G α_q , G α_s , and G α_o signaling pathways, to the extent they are known, are the same in *C. elegans* versus the other invertebrate and vertebrate systems in which they have been studied, until we get down to the very most downstream outputs of these signaling pathways. The *C. elegans* work has been focused largely on the effects of the G proteins in ventral cord motor neurons on locomotion behavior and in the HSN neurons on egg-laying behavior. Using these two behaviors as readouts, the relevant outputs of signaling that have been identified are changes in the efficiency of the neurotransmitter release machinery. All the proteins involved in these changes are highly conserved across evolution, and functional studies in mammalian neurons support the idea that the very same mechanisms are used in mammalian brain to allow G protein signaling to modulate neurotransmitter release (Rhee et al., 2002: PMID 11792326; Wierda et al., 2007: PMID 17442248).

Studies of neural G protein signaling in more complex model organisms have focused on readouts of signaling very different from those used in *C. elegans*. When electrophysiological measurements have been used to measure signaling outcomes, the focus has been on the regulation of specific ion channels by G $\beta\gamma$ subunits (Reuveny

et al., 1994: PMID 8022483; Herlitze et al., 1996: PMID 8637576), and indeed some of the effects of neural G protein signaling in *C. elegans* are mediated by such ion channels (Emtage et al., 2012: PMID 23152612). When effects on long-term memories have been the readout of signaling, the focus has been on the lasting effects of neural G protein signaling on synaptic structure or on transcription (Kandel, 2004: PMID 16134023). It is not yet fully clear whether neural G protein signaling in *C. elegans* results in these sorts of lasting changes. We do know that $G\alpha_q$ signaling in worms, as in mammals, results in activation of the Rho GTPase, whose best-studied function is as a regulator of the actin cytoskeleton, but it remains to be seen if $G\alpha_q$ signaling through Rho leads to functionally significant and lasting structural changes in *C. elegans* synapses. In *Aplysia* and mammals, $G\alpha_s$ signaling produces cAMP that activates the transcription factor CREB to induce changes in transcription that may underlie long-term memories (Kida et al., 2002: PMID 11889468; Kandel, 2012: PMID 22583753), although the relevance CREB is less clear in *Drosophila* memory (Perazzona et al., 2004: PMID 15470148). *C. elegans* also has a CREB homolog that was shown to be essential for worms to form a long-term memory of a food-odorant association (Kauffman et al., 2010: PMID 20502519) and that mediates G protein regulation of gene expression (Suo et al., 2006: PMID 17021164). Thus it seems likely that the mechanisms of neural G protein signaling are deeply conserved across evolution, but that the different types of behavioral readouts used to study such signaling in different model organisms tends to focus attention on different downstream effects of the signaling pathways on neural function.

4. Concluding remarks and future directions

Finally, I return to the simplified model presented in Figure 2 to illustrate an overview of what has been learned from studies of neurotransmitter signaling through heterotrimeric G proteins in *C. elegans*. As described in section 2, a typical neuron expresses many GPCRs, and these receptors sense the levels of neurotransmitters that are released, not just from presynaptic partners to our typical neuron, but also from distant neurons and that are thus acting extrasynaptically. Each neurotransmitter and its receptor are expressed in very specific and small set of neurons, and the extrasynaptic communication between these cells is a crucial type of functional connection that goes beyond the physical synaptic connections between neurons and that is essential for understanding neural circuits. As described in section 3, three different G α proteins, $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$, integrate signaling by the many GPCRs that may be active on a neuron at any one time. The major outcomes of neural G protein signaling studied in *C. elegans* are the opposing effects that $G\alpha_q$ and $G\alpha_o$ have to activate and inhibit the synaptic vesicle release machinery, respectively. $G\alpha_s$ signaling collaborates with the $G\alpha_q$ pathway to promote synaptic output. Other outcomes of G α activation, such as regulation of K⁺ and Ca²⁺ channels, or effects on neural structure or transcription, also seem to occur but are not responsible for the most obvious effects of the G proteins on *C. elegans* behavior. The neurotransmitters, receptors, G proteins, downstream signaling molecules and mechanisms used in *C. elegans* are all conserved in mammals.

The contribution of *C. elegans* studies has been to focus attention on the ubiquity of extrasynaptic signaling through GPCRs, the effects of neural G proteins on the neurotransmitter release machinery, and on new signaling molecules such as RIC-8, TrioRhoGEF, and RGS proteins that were discovered in *C. elegans* using the power of forward genetic screens.

Opportunities for future progress using *C. elegans* include the chance to grapple with the huge family of GPCRs that activate neural G proteins. Perhaps the entire set of GPCRs expressed on some model neurons, such as the ventral cord motor neurons or the HSN egg-laying neurons, can be defined and used to study how these receptors act together to regulate activity of these neurons. More work remains to define the mechanisms by which the three major neural G proteins affect neurotransmitter release, and at what level they intersect. In particular, the effector for $G\alpha_0$, if it exists, still remains to be identified. Finally, it remains to be seen whether *C. elegans* can be used to successfully study the types of changes in synaptic structure and transcription that occur downstream of G proteins in neurons of more complex organisms.

5. Acknowledgements

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6. Table 1 and Table 2

Table 1. G protein coupling and pharmacological profiles of *C. elegans* G protein coupled small molecule neurotransmitter receptors

Serotonin receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
SER-1	Binding studies, heterologous cell signaling, mutant resistant to serotonin	$G\alpha_q$ in heterologous cells	Hamdan et al., 1999, PMID 10098838	Hamdan et al., 1999, PMID 10098838	Serotonin (0.72 and 0.8 μ M), lisuride (partial, 0.2 and 0.24 μ M), DOI (partial, >100 μ M). No activity with dopamine or octopamine.	Hamdan et al., 1999, PMID 10098838
SER-4	Binding studies, heterologous cell signaling, mutant resistant to serotonin	$G\alpha_{i/o}$ in heterologous cells	Olde and McCombie, 1997: PMID 9061615	Olde and McCombie, 1997: PMID 9061615	Serotonin (~0.1 μ M), tyramine	Olde and McCombie, 1997: PMID 9061615; Petrascheck et al., 2007: PMID 18033297

SER-5	Mutant resistant to serotonin	G_{α_s} predicted from sequence analysis	Harris et al., 2009: PMID 19193891			
SER-7	Binding studies, heterologous cell signaling, mutant resistant to serotonin	G_{α_s} in heterologous cells, G_{α_s} in MC neurons, $G_{\alpha_{12}}$ in M4 neuron	Hobson et al., 2003: PMID 12969249; Hobson et al., 2006: PMID 16204223; Song and Avery, 2012: PMID 22323705	Hobson et al., 2003: PMID 12969249; Hobson et al., 2006: PMID 16204223	Serotonin (30 nM), tryptamine (4.7 μ M)	Hobson et al., 2003: PMID 12969249; Hobson et al., 2006: PMID 16204223

Dopamine receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
DOP-1	Binding studies, mutation affects dopamine response	G_{α_q} in ventral cord motor neurons and touch response neurons	Chase et al., 2004: PMID 15378064; Kindt et al., 2007: PMID 17698017	Suo et al., 2002: PMID 11814642		
DOP-2	Binding studies, mutant resistant to dopamine, heterologous cell signaling	$G_{\alpha_{i/o}}$ in heterologous cells, GOA-1 in SIA neurons, GPA-14 in yeast interaction assay and ADE neurons. GOA-1 and GPA-7 in male mating.	Suo et al., 2003: PMID 12887685; Suo et al., 2009: PMID 19609300; Correa et al., 2012: PMID 23166505; Pandey and Harbinder, 2012: PMID 22280843; Mersha et al. 2013: PMID 23607404	Suo et al., 2003: PMID 12887685	dopamine (75 nm)	Suo et al., 2003: PMID 12887685

DOP-3	Mutant resistant to dopamine, heterologous cell signaling	G _{a_o} in ventral cord motor neurons, G _{a_{i/o}} in heterologous cells	Chase et al., 2004: PMID 15378064; Sugiura et al., 2005: PMID 16001968		dopamine (27 nM), tyramine (500 nM), octopamine (250 μM)	Sugiura et al., 2005: PMID 16001968
DOP-4	Mutant resistant to dopamine, heterologous cell signaling	G _{a_s} in heterologous cells	Sugiura et al., 2005: PMID 16001968		dopamine (9.3 μM), >1 mM for dopamine, octopamine, tyramine and serotonin	Sugiura et al., 2005: PMID 16001968

Octopamine receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC ₅₀) in heterologous cells	References for heterologous cell studies
OCTR-1	Binding studies, heterologous cell signaling, mutant resistant to octopamine	G _{a_o} in ASH neurons, G _{a_o} in heterologous cells	Harris et al., 2010: PMID 20534837	Wragg et al., 2007: PMID 18057198	octopamine (0.39 μM in <i>Xenopus</i> oocytes; but binding studies suggest higher affinity)	Mills et al., 2012: PMID 22124329
SER-3	Mutant resistant to octopamine, heterologous cell signaling	G _{a_q} in ASH neurons, G _{a_q} in heterologous cells	Suo et al., 2006: PMID 17021164		octopamine (24 nM in mammalian cells; 0.33 μM in <i>Xenopus</i> oocytes), tyramine (26 μM in mammalian cells)	Petrascheck et al., 2007: PMID 18033297; Mills et al., 2012: PMID 22124329
SER-6	Mutant resistant to octopamine	G _{a_s} in ADL neurons	Mills et al., 2012: PMID 22124329;		octopamine (2 μM in <i>Xenopus</i> oocytes) > tyramine. No response to dopamine or serotonin.	Mills et al., 2012: PMID 22124329;

Tyramine receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
SER-2	heterologous cell signaling, mutant resistant to tyramine	$G\alpha_{i/o}$ in heterologous cells, $G\alpha_o$ in VD neurons	Rex and Komuniecki, 2002: PMID 12354282; Donelly et al., 2013: PMID 23565061	Rex and Komuniecki, 2002: PMID 12354282	tyramine (360 nM, 403 nM)	Rex and Komuniecki, 2002: PMID 12354282; Rex et al., 2004: PMID 15569254
TYRA-2	Binding studies, heterologous cell signaling	$G\alpha_{i/o}$ in heterologous cells	Rex et al., 2005: PMID 15953361	Rex et al., 2005: PMID 15953361	tyramine (50 nM), octopamine and dopamine had little/no effect	Rex et al., 2005: PMID 15953361
TYRA-3	Binding studies, mutant resistant to tyramine	$G\alpha_q$ in heterologous cells	Hapiak et al., 2013; PMID 23986246	Wragg et al., 2007: PMID 18057198	tyramine (70.8 nM)	Hapiak et al., 2013; PMID 23986246

Acetylcholine receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
GAR-1	heterologous cell signaling	$G\alpha_i$ in <i>Xenopus</i> <td>Park et al., 2000: PMID 10679207</td> <td></td> <td>acetylcholine (~100 nM), carbachol, no effect of oxotremorine</td> <td>Park et al., 2000: PMID 10679207</td>	Park et al., 2000: PMID 10679207		acetylcholine (~100 nM), carbachol, no effect of oxotremorine	Park et al., 2000: PMID 10679207
GAR-2	heterologous cell signaling	$G\alpha_{i/o}$ in <i>Xenopus</i> G\alpha_o in ventral chord cholinergic neurons	Lee et al., 2000: PMID 11032868; Dittman and Kaplan 2008: PMID 18614679		acetylcholine (~35 nM), carbachol, no effect of oxotremorine	Lee et al., 2000: PMID 11032868; Suh et al., 2001: PMID 11700045
GAR-3	heterologous cell signaling, mutant resistant to acetylcholine agonist arecoline	$G\alpha_q$ and $G\alpha_{i/o}$ in heterologous cells, $G\alpha_q$ in pharyngeal muscles, $G\alpha_q$ in male spicule protraction	Min et al., 2000: PMID 10854271; Park et al., 2003: PMID 12927813; Steger and Avery 2004; PMID 15238517; Park et al., 2006: PMID 16631594; Liu et al., 2007: PMID 17287516		carbachol (13.0 and 11.5 μ M), oxotremorine	Hwang et al., 1999: PMID 10635059; Min et al., 2000: PMID 10854271; Park et al., 2003: PMID 12927813

GABA receptors	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
GBB-1	Mutant insensitive to optogenetically released GABA	$G\alpha_o$ in cholinergic motor neurons	Dittman and Kaplan 2008: PMID 18614679			
GBB-2	Mutant insensitive to optogenetically released GABA	$G\alpha_o$ in cholinergic motor neurons	Dittman and Kaplan 2008: PMID 18614679			

Glutamate receptor homologs	Experimental evidence for ligand assignment	G protein coupling	References for G protein coupling	References for binding studies	Agonists (EC_{50}) in heterologous cells	References for heterologous cell studies
MGL-1	Mutant resistant to mGluR agonists <i>trans</i> -ACPD and LCCG-I					
MGL-2	Heterologous cell signaling	$G\alpha_q$ in heterologous cells	Tharmalingam et al., 2012: PMID 22652059		glutamate (8.61 μM), quisqualate (169 μM), DHPG (partial agonist),	
MGL-3	None - inferred to be a glutamate receptor from sequence homology.					
Poorly-studied putative small-molecule GPCRs T02E9.3 C24A8.1 C24A8.6 F35H10.10 F59D12.1 T21B4.4						

Table 2. Genetically-established functions and expression patterns of *C. elegans* G protein coupled small molecule neurotransmitter receptors

Serotonin receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
SER-1	Exogenous serotonin-stimulated egg laying, feeding, and slowing of locomotion, α-methyl-5-HT stimulated egg laying, male ventral tail curling, food-induced slowing, food modulation of aversive response, heat shock response, longevity	Carnell et al., 2005: PMID 16291940; Dempsey et al., 2005: PMID 15654117; Xiao et al., 2006: PMID 16890216; Dernovici et al., 2007: PMID 17443782; Murakami and Murakami, 2007: PMID 17559503; Srinivasan et al., 2008: PMID 18522834; Harris et al., 2011: PMID 21814562	Pharyngeal muscle (pm3, pm4, pm5, pm6, pm7, pm8), CEP, RMG, RMH, RMF, RMD, RIA, RIC, URY, additional head neurons, vulval muscle and epithelial cells, tail neurons (PVT, PVQ, possibly DVC) ventral nerve cord, excretory cell, uterine cells, ray sensory neurons in male	Cho et al., 2000: PMID 11167007; Tsalić et al., 2003: PMID 14568548; Carnell et al., 2005: PMID 16291940; Dempsey et al., 2005: PMID 15654117; Xiao et al., 2006: PMID 16890216; Dernovici et al., 2007: PMID 17443782
SER-4	Egg laying induced by imipramine but not by fluoxetine or serotonin, inhibition of locomotion by serotonin, effects of ethanol on gustatory plasticity and locomotion, thermotaxis memory behavior	Cho et al., 2000: PMID 11167007; Dempsey et al., 2005: PMID 15654117; Murakami and Murakami, 2007: PMID 17559503; Wang et al., 2011: PMID 21889959; Gürel et al., 2012: PMID 23023001; Li et al., 2013: PMID 24223727	NSM, RIB or AIB, RIS, pharyngeal neuron, pair of sublateral interneurons or motorneurons, retrovesicular ganglion, PVT tail neuron, vm2 vulval muscles, DBA or DVC tail interneuron	Tsalić et al., 2003: PMID 14568548; Gürel et al., 2012: PMID 23023001
SER-5	Exogenous serotonin-stimulated egg laying, food and serotonin-dependent increase in sensitivity of ASH neurons to octanol and decrease in sensitivity to Cu ²⁺ , reduced sensitivity to serotonin and fluoxetine induced paralysis.	Hapiak et al., 2009: PMID 19001289; Harris et al., 2009: PMID 1919389; Kullyev et al., 2010: PMID 20739712; Harris et al., 2011: PMID 21814562; Guo et al., 2015: PMID 25585042	Neurons including AWB and ASH, body wall muscles, vulval muscles	Hapiak et al., 2009: PMID 19001289; Harris et al., 2009: PMID 19193891
SER-7	Exogenous serotonin-stimulated egg laying, pharyngeal pumping, and food intake. Regulation of pharyngeal pumping. Egg laying. Hypoxia regulation of gustatory and, effects of ethanol on gustatory plasticity, thermotaxis memory behavior	Hobson et al., 2006: PMID 16204223; Donohoe et al., 2009: PMID 19447297; Hapiak et al., 2009: PMID 19001289; Pocock and Hobert, 2010: PMID 20400959; Wang et al., 2011: PMID 21889959; Song and Avery, 2012: PMID 22323705; Song et al., 2013: PMID 23390589; Li et al., 2013: PMID 24223727; Gomez-Amaro et al., 2015: PMID 25903497; Leiser et al. 2015: PMID 26586189	Pharyngeal neurons MC, M4, I2, I3, M5, M3, I4, I6, and M2. Vulval muscles.	Hobson et al., 2006: PMID 16204223

Dopamine receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
DOP-1	Antagonizes DOP-3 to control locomotion, a behavioral decision making paradigm, and acetylcholine release. Regulates swim-to-crawl transition, fat reservoirs, local food search behavior. Delays habituation to mechanical stimulation. Promotes nicotine approach behavior.	Chase et al., 2004: PMID 15378064; Sanyal et al., 2004: PMID 14739932; Kindt et al., 2007: PMID 17698017; Allen et al., 2011: PMID 21515580; Vidal-Gadea et al., 2011: PMID 21969584; Sellings et al., 2013: PMID 23351035; Barros et al., 2014: PMID 24465759; Bhattacharya et al., 2014: PMID 25167143; Wang et al., 2014: PMID 25536037	DVA, PLM, PHC, ALN, ALM, AVM, PLN, PVQ, AUA, RIB, RIM, RIS, unidentified head neurons, head muscles, labial and amphid sheath/socket cells, excretory gland cells, cholinergic ventral cord motor neurons	Tsalik et al., 2003: PMID 14568548; Sanyal et al., 2004: PMID 14739932; Chase et al., 2004: PMID 15378064; Bhattacharya et al., 2014: PMID 25167143
DOP-2	Antagonizes octopamine signaling, inhibits CRE-mediated gene expression in SIA. Mediates swimming-induce paralysis. Inhibits unproductive male mating behavior. Promotes nicotine approach behavior. Regulates touch habituation and chemosensory associative learning. Affects a decision-making paradigm.	Suo et al., 2009: PMID 19609300; Carvelli et al., 2010: PMID 20410438; Correa et al., 2012: PMID 23166505; Sellings et al., 2013: PMID 23351035; Mersha et al. 2013: PMID 23607404; Wang et al., 2014: PMID 25536037; Correa et al., 2015: PMID 26156999	All dopaminergic neurons of hermaphrodite (CEP, ADE, PDE) and probably of male (R5A, R7A, R9A). RIA, SIA, SIB, RID, PDA, HOA.	Suo et al., 2003: PMID 12887685; Tsalik et al., 2003: PMID 14568548; Suo et al., 2009: PMID 19609300; Correa et al., 2015: PMID 26156999
DOP-3	Antagonizes DOP-1 to control locomotion, a behavioral decision making paradigm, and acetylcholine release. Mediates swimming induced paralysis. Inhibits unproductive male mating behavior. Antagonizes octopamine signaling and inhibits CRE-mediated gene expression in SIA. Regulates avoidance of octanol and 2-nonenone.	Chase et al., 2004: PMID 15378064; McDonald et al., 2007: PMID 18094261; Suo et al., 2009: PMID 19609300; Ezak and Ferkey, 2010: PMID 20209143; Carvelli et al., 2010: PMID 20410438; Kimura et al., 2010: PMID 21123582; Omura et al., 2012: PMID 22719914; Correa et al., 2012: PMID 23166505; Wang et al., 2014: PMID 25536037	Ventral cord GABAergic and cholinergic neurons, ASK, PVD, SIA, RIC, head neurons, tail neurons, body wall muscles, male tail cells	Chase et al., 2004: PMID 15378064; Suo et al., 2009: PMID 19609300; Ezak and Ferkey, 2010: PMID 20209143; Correa et al., 2012: PMID 23166505;
DOP-4	Mediates swimming induced paralysis, swim/crawl transition, food enhancement of ASH response to repellents, alcohol-induced disinhibition of crawling.	Carvelli et al., 2010: PMID 20410438; Ezcurra et al., 2011: PMID 21304491; Topper et al., 2014: PMID 24681782; Vidal-Gadea et al., 2011: PMID 21969584	I1, I2, ASG, AVL, CAN, PQR, vulva, intestine, rectal glands, rectal epithelial cells, male ray 8, additional head neurons, ASH (inferred indirectly).	Sugiura et al., 2005: PMID 16001968; Ezcurra et al., 2011: PMID 21304491

Octopamine receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
OCTR-1	Mediates effect of exogenous octopamine on response to octanol. Controls innate immunity by regulating the unfolded protein response.	Wragg et al., 2007: PMID 18057198; Harris et al., 2010: PMID 20534837; Sun et al., 2011: PMID 21474712; Mills et al., 2012: PMID 22124329; Sun et al., 2012: PMID 22791024	ASI, ASH, AIY, ADE, CEP, spermatheca, uterine toroid cells, head and tail neurons	Wragg et al., 2007: PMID 18057198
SER-3	Mediates octopamine- and starvation-induced expression of a CREB-dependent reporter gene in SIA neurons. Antagonizes OCTR-1 in the control of octanol response by exogenous octopamine. Mediates inhibition of ASI sensory neuron by ASH sensory neuron via a circuit involving octopamine released from RIC.	Suo et al., 2006: PMID 17021164; Mills et al., 2012: PMID 22124329; Guo et al., 2015: PMID 25585042	ASH, ASG, ASI, SIA, PHA, PHB, PVQ, neurons in the head and tail, head muscles, intestine, phasmid socket cells, spermatheca	Suo et al., 2006: PMID 17021164; Mills et al., 2012: PMID 22124329; Guo et al., 2015: PMID 25585042
SER-6	Mediates octopamine- and starvation-induced expression of a CREB-dependent reporter gene in SIA neurons. Mediates exogenous serotonin-stimulated fat reduction. Mediates exogenous octopamine mediated inhibition of response to 100% octanol.	Srinivasan et al., 2008: PMID 18522834; Mills et al., 2012: PMID 22124329; Yoshida et al., 2014: PMID 24446241	SIA, RIC, AWB, ADL, ASI, head neurons, tail neurons, posterior ventral cord neurons, intestine	Srinivasan et al., 2008: PMID 18522834; Yoshida et al., 2014: PMID 24446241

Tyramine receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
SER-2	Mediates paralysis and suppression of pharyngeal pumping by exogenous tyramine. Suppresses head movements during backing. Facilitates execution of omega turns.	Rex et al., 2004: PMID 15569254; Donelly et al., 2013: PMID 23565061	Neurons: AIY, AVH, AUA, RIC, SAB, RID, RIA, SAB, SDQ, CAN, DA9, LUA, ALN, PVC, NSM, AIZ, DVA, BDU, SIA, RME, PVT, OLL, PVD, VD. Excretory gland cells. Muscles: pm1, pm6, head muscles, male posterior body wall muscles and diagonal muscles. Possibly male CP neurons. Uterine toroid cells ut1 and ut2.	Tsalik et al., 2003: PMID 14568548; Rex et al., 2004: PMID 15569254; Donelly et al., 2013: PMID 23565061
TYRA-2			MCL, NSM, ASE, ASG, ASH, ASI, PVD, CAN, ALM	Rex et al., 2005: PMID 15953361
TYRA-3	Mediates effect of exogenous tyramine on response to octanol; affects decision to leave bacterial lawn. Antagonizes the effect of serotonin to sensitize aversive responses.	Wragg et al., 2007: PMID 18057198; Bendesky et al., 2011: PMID 21412235; Hapiak et al., 2013: PMID 23986246	ADE, CEP, ASK, ADL, AIM, AUA, BAG, DEP, OLQ, SDQL, AFD, AWC, RIC, ASI, spermatheca, distal tip cell, vulval muscles, additional head and tail neurons	Carre-Pierrat et al., 2006: PMID 17082916; Unpublished results cited in Wragg et al., 2007: PMID 18057198; Bendesky et al., 2011: PMID 21412235
Acetyl-choline receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
GAR-1			head neurons, PVM	Lee et al., 2000: PMID 11032868
GAR-2	Mediates inhibition of egg laying. Mediates feedback inhibition of cholinergic ventral cord motor neurons to regulate locomotion.	Bany et al., 2003: PMID 12954868; Dittman and Kaplan 2008: PMID 18614679	head neurons, ventral cord GABAergic and cholinergic, neurons, cell near vulva	Lee et al., 2000: PMID 11032868; Dittman and Kaplan 2008: PMID 18614679
GAR-3	Regulates multiple calcium-dependent processes in pharyngeal muscle. Sensitizes nicotinic signaling in male spicule neurons and muscles to facilitate mating. Regulates head movements by compartmentalizing axon activity in the RIA interneuron. Promotes acetylcholine release from cholinergic motor neurons.	Steger and Avery 2004; PMID 15238517; Liu et al., 2007: PMID 17287516; Hendricks et al., 2012: PMID 22722842; Chan et al., 2013: PMID 23986249	I3, pharyngeal muscle, body wall muscles, anal depressor muscle, cholinergic ventral cord neurons, VD, DD, male diagonal muscles, male ray 8 neuron, SPC, PCB, and PCA neurons and the spicule protractor muscles	Steger and Avery 2004; PMID 15238517; Liu et al., 2007: PMID 17287516; Chan et al., 2013: PMID 23986249

GABA receptors	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
GBB-1	With GBB-2, decreases acetylcholine release from ventral cord motor neurons to regulate locomotion. Affects lifespan.	Dittman and Kaplan 2008: PMID 18614679; Schultheis et al., 2011: PMID 21613582; Chun et al., 2015: PMID 26537867	Expression in ventral cord cholinergic neurons in inferred indirectly.	Dittman and Kaplan 2008: PMID 18614679; Schultheis et al., 2011: PMID 21613582
GBB-2	With GBB-1, decreases acetylcholine release from ventral cord motor neurons to regulate locomotion.	Dittman and Kaplan 2008: PMID 18614679; Schultheis et al., 2011: PMID 21613582	Expression in ventral cord cholinergic neurons in inferred indirectly.	Dittman and Kaplan 2008: PMID 18614679; Schultheis et al., 2011: PMID 21613582
Glutamate receptor homologs	Functions revealed in mutants	References for mutant phenotype	Expression pattern	References for expression pattern
MGL-1	Regulates starvation response, perhaps by directly sensing amino acids from food. Pharmacological activation of MGL-1 inhibits pharyngeal pumping. Mediates reduction in pharyngeal pumping when animals are removed from food.	Kang and Avery, 2009: PMID 19136622; Dillon et al., 2015: PMID 25869139	Expression in AIY and AIB is inferred indirectly. Head neurons, tail neurons, pharyngeal neurons including NSM.	Kang and Avery, 2009: PMID 19136622; Dillon et al., 2015: PMID 25869139
MGL-2	Regulates starvation response, perhaps by directly sensing amino acids from food.	Kang and Avery, 2009: PMID 19136622	Expression in AIY and AIB is inferred indirectly. Head neurons, tail neurons, pharyngeal neurons including NSM, I5. Intestine, pharyngeal-intestinal valve, pharyngeal muscle.	Kang and Avery, 2009: PMID 19136622; Dillon et al., 2015: PMID 25869139
MGL-3			Head neurons, pharyngeal neurons including NSM.	Dillon et al., 2015: PMID 25869139

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