A Novel and Functionally Diverse Class of Acetylcholine-gated Ion Channels

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Abstract

Fast cholinergic neurotransmission is mediated by pentameric acetylcholine-gated ion channels; in particular, cationic nicotinic acetylcholine receptors play well-established roles in virtually all nervous systems. Acetylcholine-gated anion channels have also been identified in some invertebrate phyla, yet their roles in the nervous system are less well-understood. Here we describe the functional properties of five previously-uncharacterized acetylcholine-gated anion channels from *C. elegans*, including four from a novel nematode specific subfamily known as the diverse group. In addition to their activation by acetylcholine, these diverse group channels are activated at physiological concentrations by other ligands; three, encoded by the *lgc-40*, *lgc-57* and *lgc-58* genes, are activated by choline, while *lgc-39* encoded channels are activated by octopamine and tyramine. Intriguingly, these and other acetylcholine-gated anion channels show extensive co-expression with cation-selective nicotinic receptors, implying that many cholinergic synapses may have both excitatory and inhibitory potential. Thus, the evolutionary expansion of cholinergic ligand-gated ion channels may enable complex synaptic signalling in an anatomically compact nervous system.

Keywords

Choline / *C.elegans* / Co-expression / Polymodal / Synaptic localisation

Introduction

Rapid signalling through neuronal networks is essential for producing coordinated behaviours in animals. At the fundamental level, fast neurotransmission is mediated through neurotransmitter release, resulting in activation of ion channels on the postsynaptic neuron. In the textbook view, based predominately on mammalian systems, there are two major excitatory

neurotransmitters, glutamate, and acetylcholine, and two inhibitory neurotransmitters, GABA and glycine. Glutamate acts through a family of tetrameric ligand-gated cation channels, while the remaining neurotransmitters activate pentameric receptors from the Cys-loop receptor superfamily of ligand-gated ion channels (LGICs). Although LGICs are highly conserved across phyla, ligand binding properties and ion selectivity diverge significantly, resulting in a large diversity of mechanisms by which small molecules acting via LGICs can influence the activity in neuronal circuits.

Despite their conserved general structure, Cys-loop LGICs vary significantly in their functional properties, particularly when channels from invertebrate phyla are considered. For example, insects and nematodes express inhibitory glutamate receptors from the Cys-loop superfamily, localised both in neurons and muscles, which are targets of the anthelmintic drug ivermectin (Cully *et al*, 1996, 1994). Many animals including insects, nematodes and mammals also express LGICs that can be gated by aminergic ligands, including histamine-gated chloride channels in *Drosophila* (Gisselmann *et al*, 2002), various nematode channels gated by dopamine, tyramine and serotonin (Morud *et al*, 2021; Pirri *et al*, 2009), as well as the excitatory mammalian 5-HT₃ receptors (Kondo *et al*, 2014; Lombaert *et al*, 2018). Even more divergent roles for LGICs have been identified in marine species, where LGICs gated by terpenes and chloroquine function as chemoreceptors in octopus (van Giesen *et al*, 2020).

Cys-loop channels can be subdivided into two large clades, the first containing the nicotinic receptors and their paralogues (including the 5HT-3 receptors), and the other containing channels more closely related to GABA_A receptors (Jones & Sattelle, 2008). The *C. elegans* genome contains several subfamilies which appear to have diversified independently from vertebrate channels during evolution, leading to the existence of several nematode

specific LGIC subfamilies. The *C. elegans* genome contains a number of GABA_A-like subfamilies, including genes encoding both anion and cation-selective channels (Ranganathan *et al*, 2000; Jobson *et al*, 2015; Ringstad *et al*, 2009; Putrenko *et al*, 2005; Margie *et al*, 2013; Yassin *et al*, 2001). One of these subgroups consists of genes for monoamine-gated channels (both anion and cation selective), and a second subgroup consists of eight channels, four of which are acetylcholine-gated anion channels and four that are still uncharacterized. A third subfamily, known as the “diverse group” (Hobert, 2013), is largely uncharacterised; though one member, LGC-40, has been reported to be a low affinity serotonin-gated channel also gated by acetylcholine and choline (Ringstad *et al*, 2009), the properties of the remaining eight channels in this subgroup, including their ligands, ion selectivity, and expression patterns, are currently unknown.

Here we describe the deorphanisation and characterisation of five new *C. elegans* LGICs activated by acetylcholine, including four members of the diverse subgroup. One of these, LGC-39, forms a polymodal homomeric anion channel activated by acetylcholine as well as by monoamines, while three others are activated by both choline and acetylcholine. Using public single cell RNAseq expression data together with our new electrophysiological data, we also predict the polarity of synapses in the worm connectome, as well as intracellular localisation patterns for uncharacterized LGICs. These results highlight the unexpected functional diversity of cholinergic signalling in the *C. elegans* nervous system.

Results

Deorphanisation of Uncharacterised LGICs Reveals Diversity of Cholinergic Channels

The *C. elegans* genome encodes a diverse superfamily of pentameric ligand-gated ion channels (LGICs), of which several subfamilies are poorly characterised. In particular the

diverse group, consisting of the GGR-1 (here renamed to LGC-57) and the LGC-45 subfamilies, together includes nine LGICs (Figure 1B), whose activating ligand and function are largely unknown. To investigate the properties of these channels, we first expressed each channel gene in *Xenopus* oocytes and tested for current activation during the perfusion of a panel of neurotransmitters. Despite their homology to vertebrate GABA_A and glycine receptors (the source of the name GGR-1), we observed no activation of any members of the GGR-1 group (or LGC-45 group) by either GABA or glycine. Instead, we found three closely related channels LGC-57 (aka GGR-1), LGC-58 (aka GGR-2) and LGC-40 that were specifically gated by choline and acetylcholine (Figure 1A). All three channels showed a preference for choline, with EC₅₀ values 2.5 to 3-fold lower for choline than acetylcholine (Figure 1C). These findings parallel a previous report that LGC-40 forms a choline and acetylcholine-gated channel, although in contrast to that report we did not observe serotonin responses (Ringstad *et al*, 2009). We did not observe currents in response to any of the tested compounds for the remaining members of the LGC-57 group (LGC-41 and LGC-42) nor for any members of the LGC-45 group (Figure S1A).

In addition to the LGC-57 and LGC-45 groups, many other *C. elegans* LGICs lack identified ligands. While several members of the so-called ACC group of LGICs have been shown to form acetylcholine-gated chloride channels, four members of this subfamily (*acc-4*, *lgc-47*, *lgc-48*, and *lgc-49*) had not previously been characterised (Figure 1B). Upon expression in *Xenopus* oocytes, we found that one of these channels, LGC-49, formed an acetylcholine gated channel with an EC₅₀ of 19μM (Figure 1C). Unlike the members of the LGC-57 group, LGC-49 showed no significant activation by choline. No ligand-induced currents were observed for ACC-4, LGC-47 or LGC-48, either alone, or in combination with closely related and previously characterised LGCs, such as ACC-1 (Figure S1A-B) (Putrenko *et al*, 2005).

We next investigated the ion selectivity of the newly-deorphanised channels by carrying out ion substitution experiments in oocytes expressing LGC-57, LGC-58, LGC-40, or LGC-49. For all channels we observed significant reversal potential shifts following substitution of standard high NaCl buffer for low Cl (Na Gluconate), but not for substitution with Na⁺-free (NMDG) solution, indicating selectivity for anions over cations for all the tested channels (Figure 2A-B). We also tested the previously deorphanised channel LGC-46 (Liu *et al*, 2017), which to date lacked ion selectivity data; this likewise showed reversal shifts characteristic of an anion selective channel (Figure 2A-B). Interestingly, all members of the LGC-57 group possess a PAR motif (Proline-Alanine-Arginine), located in the M2-3 intracellular loop, which has been shown to impart anion selectivity to LGICs (Wotring *et al*, 2003). Although several uncharacterised members of the ACC group have sequences that diverge from the PAR motif, both LGC-49 and LGC-46 contain the PAR motif sequence. Thus, the PAR motif appears to correlate with anion selectivity in both the LGC-57 and ACC groups of nematode acetylcholine-gated LGICs.

Cholinergic Channels Display Diverse Antagonist Binding Properties

In order to understand if there are further functional differences between the channels deorphanised in this study, we exposed each channel to three cholinergic antagonists, mecamylamine, strychnine and d-tubocurarine. Strychnine and d-tubocurarine have been shown to compete with the full agonist for the ligand binding domain, although their binding mechanisms vary between LGICs of different classes (Brams *et al*, 2011); in contrast, mecamylamine has been shown to interact with the transmembrane regions of mammalian nAChRs (Bondarenko *et al*, 2014). We saw that the antagonistic profile differed significantly between the newly-deorphanised channels. For example, within the LGC-57 group the two

smallest antagonists, mecamylamine and strychnine, had a similar IC_{50} values for LGC-57, LGC-58a and LGC-40 (Figure 2D). However, tubocurarine, the largest molecule of the antagonists, displayed an 11-fold shift in IC_{50} for LGC-57 compared to LGC-58 and LGC-40 (Figure 2D). Thus, the binding capabilities of tubocurarine on LGC-57 differs substantially from that of its closest family members LGC-58 and LGC-40. Likewise, in the ACC group, LGC-46 and LGC-49 could both be blocked by mecamylamine and strychnine (Figure 2D); LGC-49 could additionally be blocked by tubocurarine and picrotoxin. These dissimilarities again highlight the discrete differences between channels from the same subfamily, which may have similar ligand binding profiles for endogenous ligands. Interestingly, tubocurarine was the most potent blocker for the ACC group channels LGC-46 and LGC-49, whereas this antagonist was the least effective of those tested for the LGC-57 group channels.

We also tested the channels' responses to repeated stimulation by the endogenous primary ligand. We found LGC-40 to be sensitive to repeated stimulation, displaying a significant difference in ratio between the first and second pulse after both 10s of and 60 s of washing intervals (Figure 2C). In contrast, all other channels were capable of fast activation intervals as they did not display any decrease in peak amplitude after repeated stimulation (Figure 2C). Thus, LGC-40 appears to desensitize more than the other channels following activation by its ligand.

LGC-39 is a Novel Polymodal Channel Activated by Cholinergic and Aminergic

Ligands

One channel from the LGC-57 subfamily, LGC-39, showed distinct ligand binding properties from the rest of the group. Unlike the other LGC-57 subfamily members, LGC-39 showed relatively little activation by choline. Moreover, while acetylcholine activated LGC-

39 channels strongly (with an EC_{50} of $1290\mu M$), the most potent ligands for LGC-39 were the monoamines octopamine and tyramine (with EC_{50} values of $921\mu M$ and $686\mu M$ respectively; Figure 3A-B). In addition, LGC-39 also displayed small currents in response to dopamine (Figure 3A). Like other members of the LGC-57 family, LGC-39 contains the PAR sequence, and LGC-39-expressing oocytes showed reversal potential shifts in response to chloride but not sodium substitution (Figure 3C). Thus, *lgc-39* appears to encode a homomeric anionic, polymodal channel, capable of being activated by both aminergic and cholinergic neurotransmitters (Figure 3A-C).

We tested the effects of antagonists on LGC-39 currents evoked by different activating ligands. In the presence of acetylcholine, LGC-39 could be blocked by the antagonists mecamylamine, strychnine and tubocurarine. In contrast, the octopamine response could not be blocked by mecamylamine or strychnine. Surprisingly, strychnine, in the presence of octopamine or without an activating ligand, acted as a partial agonist, inducing a small current with an EC_{50} of $7.5\mu M$ (Figure 3D). These data suggest that the putative endogenous ligands of LGC-39, acetylcholine and octopamine, may interact with and activate the receptor in different manners. To further separate the functionality of the ligands, we also investigated if repeated activation by the different ligands influenced the ability for reactivation of LGC-39 differently (Figure 3E). No difference was seen for any wash interval between the ligands, which could suggest that all ligands occupy the binding site in a similar time frame or that the recovery time for the receptor is independent of the activating ligand.

Cholinergic Channels show Broad and Varied Expression in The *C. elegans* Nervous System

To gain insight into the roles of cholinergic LGICs in the nervous system, we generated reporter lines to characterise their neural expression patterns. We used a similar set of fluorescent reporter lines to characterise the expression pattern of the newly deorphanised LGICs based transcriptional reporter transgenes in which the upstream promoter of the *lgc* gene drives the expression of a fluorescent protein. We then identified transgene-expression based upon location, morphology and known marker lines. Using such a transcriptional reporter, we observed expression of *lgc-39* in a range of interneurons and motor neurons, including the AVA premotor interneurons (Figure 4E). In addition to receiving extensive cholinergic input, these neurons are the major synaptic target for the only octopaminergic neurons, the RICs, suggesting that LGC-39 may be exposed to both octopamine and acetylcholine *in vivo* (Figure 4F) and may be involved in both cholinergic and octopaminergic synaptic transmission. For the rest of the LGC-57 group, we also observed primarily neuronal expression, with little overlap observed in the neurons expressing reporters for *lgc-40*, *lgc-49*, *lgc-57* and *lgc-58* (Figure 4A-D). This was particularly striking in motoneurons, with *lgc-40* expressed in many pharyngeal neurons (M2, M3, MC, MI, I2), *lgc-49* in sensory neurons, including posterior sensory neurons such as ALN and PLN, *lgc-57* in the A-class and B-class motoneurons of the ventral cord, and *lgc-58* in the egg-laying motoneurons (VCs; *lgc-57* was also observed in a subset of VCs) This suggests that these channels are likely to exist primarily as homomers *in vivo* and function in distinct target neurons.

We also used reporters to analyse the expression pattern of several still-orphan LGICs, including *lgc-42*, *lgc-47*, *lgc-48*, *lgc-43* and *lgc-45*, as well as the previously deorphanised ACh-gated channel *lgc-46* (Takayanagi-Kiya *et al*, 2016) (Figure S2A). These reporters also showed diverse and distinct patterns of expression, primarily in neurons. For example, *lgc-46* was broadly expressed in several neurons, mostly in the head (Figure S2C). Most of the orphan

channels were also expressed specifically in neurons; for *lgc-47* this expression was unusually broad, encompassing sensory, motor, and interneurons, while *lgc-48* was expressed only in a single pair of ADL chemosensory neurons. Interestingly, two the orphan channels, *lgc-43*, and *lgc-45*, which lack a PAR sequence and may thus encode cationic channels, did not appear to be expressed in neuronal tissue, but instead in the hypodermis (Figure S2A). Together, these data suggest that these channels play various roles in, and outside, the nervous system.

Excitatory and Inhibitory ACh Receptors are Co-expressed in Many Neurons

Our expression analysis indicated that many of the inhibitory ACh receptors newly deorphanised in this study are expressed in neurons previously shown to also express excitatory ACh receptors. These results imply that the role for acetylcholine as an inhibitory neurotransmitter may be greater than previously assumed, and that acetylcholine contributes to both inhibitory and excitatory events in many neurons. To determine the extent to which excitatory and inhibitory receptors for the same neurotransmitter are expressed in individual neural classes, we made use of single cell RNAseq dataset from *C. elegans* neurons (Taylor *et al*, 2021). We first generated a complete list of ionotropic receptors for each of the three classical neurotransmitters ACh, GABA and glutamate (Table S1). Since missing receptors would have the potential to bias predictions, we predicted the ligand and ion selectivity of orphan channels based upon homology with characterised channels and the presence or absence of a PAR motif in the M2-3 intracellular loop (see Methods).

From this analysis, we found a remarkable frequency of neuron classes co-expressing both inhibitory and excitatory receptors for the same neurotransmitter. This was particularly notable for acetylcholine, with over 60% of neuron classes expressing both excitatory and inhibitory ACh receptors. In contrast, GABA receptors were more biased toward inhibition,

with over 40% of neuron classes only expressing inhibitory GABA receptors (Figure 5A, S3). To make generalised predictions of synaptic polarity, we summed expression of inhibitory and excitatory receptors for each neurotransmitter in each neuron class and assigned synapse polarity based on the most prevalent receptors in each neuron class, assuming that all receptors in a cell are present equally at all synapses (Figure 5C, S4). This analysis suggested that the majority of ACh and Glu synapses are excitatory and most GABA synapses inhibitory, though this varied significantly for individual connections (Figure 5B).

Determining Synaptic Localisation of LGICs

We reasoned that the single cell RNAseq dataset (Taylor *et al*, 2021) might also be useful for predicting the intracellular localization of cholinergic LGICs, as presynaptic receptors would be predicted to be expressed in cholinergic neurons while postsynaptic receptors should be expressed in neurons receiving cholinergic input. To assess the correlation between the number of cholinergic synapses a neuron makes ('ACh out'), or receives ('ACh in'), with the expression level of cholinergic LGICs, we produced two heatmaps representing either ACh out (Figure 6A), or in (Figure 6B), correlated to transcript level (Figure S5). This analysis identified some receptors, such as *lgc-49*, that were primarily expressed in neurons that receive a high number of cholinergic synapses (VD, DD; Figure 6C), consistent with a postsynaptic receptor. Other channels were primarily expressed in neurons that make many cholinergic synapses, such as *acc-1* in SMB and SAB (Figure 6D), consistent with presynaptic localization. Others, such as *lgc-40* showed little correlation to cholinergic in- or out-degree (Figure 6E), suggesting extrasynaptic localization and function or as for *lgc-46* a similar correlation between in or out degree (Figure 6F).

To empirically assess the synaptic localization of cholinergic LGICs, we generated endogenous GFP-tagged CRISPR lines for members of the LGC-57 subgroup, including *lgc-39*, *lgc-40*, *lgc-57*, and *lgc-58* and (Figure 7A-D). In all cases GFP was inserted in the intracellular M3/4 loop and the function of the resulting chimeric protein was verified in *Xenopus* oocytes (Figure S6A). We observed a clear difference in the localisation patterns for these channels. LGC-39::GFP was localised in distinct punctate structures both in the nerve ring and along the ventral cord, suggestive of synaptic localisation (Figure 7A) and consistent with the positive correlation between *lgc-39* expression and cholinergic in-degree. In contrast, LGC-40::GFP appeared to have a diffuse nerve ring and touch receptor neuron GFP signal (Figure 7B), with cell bodies often being visible, while LGC-57::GFP was found to be very lowly expressed and little protein expression could be localised (Figure 7C). LGC-58::GFP showed a clear nerve ring and VC4/5 localisation, including some punctate structures; since LGC-58 showed a small but significant positive correlation with cholinergic out-degree, this may indicate presynaptic localization (Figure 7D). Together, these results suggest that LGC-39 receptors may be postsynaptic, while LGC-40, LGC-57 and LGC-58 may be primarily extrasynaptic and/or presynaptic.

Discussion

A Novel Family of Cholinergic LGICs

This study highlights the diversity among cholinergic LGICs in *C. elegans*. Nematodes have previously been shown to contain excitatory LGICs related to nicotinic receptors, as well as inhibitory acetylcholine-gated chloride channels of the ACC family. Here we describe a new subfamily of cholinergic LGICs, four of which form homomeric acetylcholine-gated anion channels. In contrast to the previously described ACC subfamily of acetylcholine-gated anion channels, three of these newly orphanised channels (LGC-57, LGC-58 and LGC-40) are

gated preferentially by choline, the metabolite of acetylcholine which is abundant at cholinergic synapses.

These results add to the already-extensive catalogue of acetylcholine-gated channels in *C. elegans* (Yassin *et al*, 2001; Putrenko *et al*, 2005; Takayanagi-Kiya *et al*, 2016), highlighting the expansion and importance of cholinergic transmission in nematodes. These newly deorphanised channels display subtle variations in their ability to bind ligands and antagonists, which translates into physiologically relevant differences that may increase the fine tuning in the control of neuronal transmission and contribute to complex neuronal signalling within a relatively minimal neuronal network. Interestingly, the electrophysiologically similar channels LGC-57, LGC-58, and LGC-40 show largely distinct patterns of expression within the nervous system of *C. elegans*, suggesting they may form homomeric channels with distinct functions *in vivo*. When tagged with a fluorescent protein, these three channels also showed a diffuse pattern of localisation within the neuron, suggesting that in contrast to ACC group channels such as LGC-46 (Takayanagi-Kiya *et al*, 2016), they may not be synaptically localised. This suggests a possible distinct extrasynaptic role for choline acting via these channels in the modulation of the nervous system.

Our results raise the intriguing possibility that choline itself may function as a neuromodulator. The idea that choline could activate cholinergic receptors independently from acetylcholine has been discussed for other cholinergic receptors based on choline's ability to dose-dependently either block or activate nicotinic channels (Purohit & Grosman, 2006). However, the importance of choline activation to the *in vivo* functions of these channels has remained unclear. Here we have identified three cholinergic receptors, LGC-40, LGC-57 and LGC-58, in which choline acts as a full agonist, showing preference in binding and activation

degree for choline over acetylcholine. Previous reports show that in mammalian neuromuscular AChRs, aromatic residues in the extracellular domain play a vital part in stabilising the binding of acetylcholine over the binding to choline (Bruhova & Auerbach, 2017); interestingly, at least some of these aromatic residues are lacking in the choline-gated receptors identified here. This suggests that LGC-40, LGC-57 and LGC-58 may in fact be optimized for activation by choline rather than acetylcholine. Thus, it is not unreasonable to hypothesise that choline could be an authentic endogenous ligand for these LGC-57-group channels *in vivo*.

A Polymodal LGIC Activated by Both Aminergic and Cholinergic Ligands

In this study we also identified a novel polymodal channel, LGC-39, which is gated not only by acetylcholine but also by the aminergic ligands tyramine and octopamine. The expression pattern of *lgc-39* suggests that the channel might be exposed to all these ligands *in vivo*. For example, *lgc-39* is highly expressed in the AVA premotor neurons, which receive a many synapses from acetylcholine producing neurons (White, 1986) as well as from the RICs, the only octopamine producing cells in the nervous system (Alkema & Horvitz, 1999). The AVA neurons also receive some input from tyraminergeric and dopaminergic neurons, transmitters which we also found can activate LGC-39. Interestingly, in contrast to the choline-gated channels LGC-40, LGC-57, and LGC-58, we observe clear punctate localisation of LGC-39 in both the nerve ring and ventral cord, with no fluorescence visible in the cell bodies. This may suggest a role for LGC-39 channels as postsynaptic receptors for both cholinergic and aminergic neurotransmission.

The concept of a truly polymodal receptor has not before been investigated in great detail, though previous observations have described roles for receptors that can use dual ligands. For example dopamine exhibits a pseudo competitive ability to antagonise GABA_A

currents (Hoerbelt *et al*, 2015), while D-serine can function as an allosteric modulator of NMDA receptor activity (Wolosker & Balu, 2020). Based on their capability to achieve dose-dependent activation, both amines and acetylcholine appear to be true ligands of LGC-39, most likely binding the ligand-binding domain. Understanding the mechanisms by which these multiple neurotransmitters can activate LGC-39 and potentially affect different behavioural outputs will be of interest in future studies.

Functional Insights into The *C. elegans* Connectome

With increasing molecular and physiological characterisation of neurotransmitter receptors in *C. elegans*, it is becoming feasible to predict the functionality of synapses more accurately in the *C. elegans* connectome. In this study we used the expression pattern of newly and previously deorphanised LGICs for the three classical neurotransmitters, ACh, glutamate and GABA, to predict the polarity of synapses in the *C. elegans* connectome. By assigning synapse polarity based on the relative expression levels of anionic and cationic receptors, we have provisionally predicted the sign of chemical synapses involving classical neurotransmitters. Although similar attempts have been made in the past to assign polarity to *C. elegans* synapses (Fenyves *et al*, 2020), these predictions were based upon incomplete or incorrect ligand assignment for many LGICs. These revised predictions correlate well with experimental data for many well-characterised circuits, such as the excitatory connections between the ASH nociceptors and the AVA interneurons, as well as between the AVAs and the VA and DA motorneurons (Mellem *et al*, 2002; Piggott *et al*, 2011). Predicted inhibitory connections between AVB and AVA interneurons also correspond with empirical data on the locomotor circuit (Kawano *et al*, 2011; Qi *et al*, 2012). Other predictions were counterintuitive; for example, inhibitory connections were predicted between the AVDs and AVAs, two

interneuron pairs thought to be co-ordinately active during reverse locomotion (Faumont *et al*, 2012). Our predictions thus generate interesting functional hypotheses for future investigation.

A surprising outcome of the expression analysis was the high frequency with which neurons expressed cationic and anionic receptors for the same neurotransmitter. This was especially prevalent for acetylcholine; our analysis indicated that 60% of neuron classes express both inhibitory and excitatory receptors for acetylcholine, 30% for glutamate and 10% for GABA. One explanation for this apparent paradox is that excitatory and inhibitory receptors might be differentially localised in neurons, with some found extrasynaptically and others enriched in at synapses. Various *C. elegans* LGICs are known to act in regions other than the post-synapse; for example *lgc-35* has been shown to mediate GABA spill-over transmission (Jobson *et al*, 2015), while *lgc-46* appears be localised to the pre-synapse as an autoreceptor (Takayanagi-Kiya *et al*, 2016). The choline-sensitive channels from the LGC-57 group likewise appear to be extrasynaptic in their localization (Figure 7). In addition, postsynaptic sites might themselves contain a mixture of excitatory and inhibitory receptors, which could differ in their ligand affinity, desensitization kinetics and regulation. Indeed, LGIC localisation is not static; for example, glutamatergic AMPA receptors have been shown in many species to increase their synaptic localisation during learning (Malinow & Malenka, 2002), and recent evidence indicates that *C. elegans* LGICs also display regulated membrane trafficking upon learning (Morud *et al*, 2021). Thus, *C. elegans* may contain large numbers of complex cholinergic synapses with the potential to be excitatory or inhibitory depending on context or experience.

Materials and Methods

C. elegans culture

Unless otherwise specified, *C. elegans* worms were cultured on NGM agar plates with OP50 (Stiernagle, 2006). A full list of strains used in this study can be found in table S1.

Xenopus laevis oocytes

Defolliculated *Xenopus laevis* oocytes were obtained from EcoCyte Bioscience (Dortmund, Germany) and in ND96 (in mM: 96 NaCl, 1 MgCl₂, 5 HEPES, 1.8 CaCl₂, 2 KCl) solution at 16° C for 3-7 days.

Molecular biology

Unless otherwise specified, cDNA sequences of *C. elegans* genes were cloned from wildtype N2 worm cDNA (generated by reverse transcription PCR from total worm RNA using Q5 polymerase (New England Biosciences)). Where multiple isoforms are present isoform a was used. LGC-39 cDNA was generated by gene synthesis (ThermoFischer). For expression in *Xenopus* oocytes, ion channel cDNA sequences were cloned into the KSM vector downstream of a T3 promoter and between *Xenopus* β -globin 5' and 3'UTR regions using the HiFi assembly protocol (New England Biosciences). *C. elegans* expression constructs were also generated using the HiFi assembly protocol (New England Biosciences) into the pDESTR4R3II backbone. *C. elegans* gDNA sequences were cloned from wildtype N2 gDNA and expression verified by the addition of GFP or mKate2 introduced on the same plasmid after an intercistronic splice site (SL2 site). Unless otherwise specified promoter sequences consist of approximately 2kb of gDNA upstream of the start codon.

CRISPR/CAS9-mediated gene manipulation

Endogenous tagging of the M3/4 cytosolic loop of *C. elegans* LGIC proteins with GFP was carried out either using the SapTrap protocol (Schwartz & Jorgensen, 2016; Dickinson *et al*,

2018) for *lgc-39(lj121)*, or by SunyBiotech (Fuzhou, China) for *lgc-57(syb3536)*, *lgc-58(syb3562)*, and *lgc-40(syb3594)*.

RNA synthesis and microinjection

cRNA was synthesised *in vitro* using the T3 mMessage mMachine transcription kit according to manufacturer's protocol to include a 5' cap (ThermoFischer Scientific). Prior to injection RNA was purified using the GeneJET RNA purification kit (Thermo Fischer Scientific). Size sorted and defolliculated *Xenopus* oocytes (Ecocyte) were placed individually into 96-well plates and injected with 50 nL of 500 ng/ μ L RNA using the Roboinject system (Multi Channel Systems GmbH). When two constructs were co-injected the total RNA concentration remained 500 ng/ μ L, with a 1:1 ratio of the components. Injected oocytes were incubated at 16°C in ND96 until the day of recording, typically between 3-6 days post injection.

Two-Electrode Voltage Clamp (TEVC) recording and data analysis

Two-electrode voltage clamp recordings were carried out using either the Robocyte2 system or a manual set up with an OC-725D amplifier (Multi Channel Systems GmbH). Glass electrodes with a resistance ranging from 0.7-2 M Ω were pulled on a P1000 Micropipette Puller (Sutter). Electrodes contained AgCl wires and backfilled with a 1.5M KCl and 1 M acetic mixture. Unless otherwise stated, oocytes were clamped at -60mV. Continuous recordings at 500Hz were taken during application of a panel of agonists or antagonists. Data was recorded using the RoboCyte2 control software, or with WinWCP for manual recordings, and filtered at 10 Hz.

Dose response protocols used 10s agonist application pulses with 60s of wash in ND96 between each dose. Data was gathered over at least two occasions, using different batches of oocytes.

Peak current for each dose was normalised to the oocyte maximum current using a custom-built python script (Morud *et al*, 2021). Normalised data was imported into Graphpad (Prism) and fitted to either a three or four parameter nonlinear Hill equation to obtain the highest degree of fit and calculate the EC_{50} . Antagonist dose responses and ion selectivity recordings were carried out using the EC_{50} concentration of the primary agonist. Antagonist dose response protocols used 10s agonist + antagonist windows, with 60s of ND96 washes between doses. The agonist concentrations remained constant. Antagonist IC_{50} values were calculated using a second custom built python script (Morud *et al*, 2021). Normalised data was imported into Graphpad (Prism) and fitted to either a three or four parameter nonlinear Hill equation to obtain the highest degree of fit and calculate the IC_{50} .

Ion selectivity was detected using a voltage ramp protocol from -80mV to +60mV (20mV/s) in the presence of the primary agonist in three different solutions: ND96, NMDG (Na^+ free) and Na Gluconate (low Cl-) solutions. Data was normalised to max current and ΔE_{Rev} was calculated using a custom-built python script (Morud *et al*, 2021). The resulting individual values or mean, SD and n for each construct was imported in GraphPad for further plotting and statistical analysis. Statistically significant differences in ΔE_{Rev} were calculated in GraphPad using a two-way ANOVA with Tukey's correction for multiple comparisons. A representative normalised trace for each construct was also generated in Graphpad.

Confocal and Cell ID

Worms were prepared and immobilised with 75 mM NaAzide in M9 and mounted onto 2% agarose in M9 pads. Image stacks were acquired with a 63x water immersion lens on a Leica SP8 or STED or using a 40x oil immersion objective on a Zeiss LSM780. Collapsed z-stack images were generated in Fiji/Image J. Neurons expressing fluorescent reporters were

identified by cell shape, position and crossing with the multicolour reference worm NeuroPAL (Yemini *et al*, 2020).

Synaptic polarity prediction

Inhibitory and excitatory chemical synapse prediction for ACh, Glu and GABA synapses were made based upon expression levels of appropriate LGICs in postsynaptic cells. Chemical and electrical connectome data was obtained from Wormweb (<http://wormweb.org/details.html>), LGIC expression data was taken from the Cengen project using threshold level 4 (Taylor *et al*, 2021), ligand and selectivity for each channel was based upon this work, previous work and predictions, a full list can be found in Table 1. Binary expression of LGICs for each neurotransmitter in each neuron class were made based upon expression and characterised in four groups: only excitatory, only inhibitory, both excitatory and inhibitory or none. These binary values were used to make the binary expression heatmap. Overall polarity of a synapse was calculated by summing the expression of all inhibitory and all excitatory LGICs for a given neurotransmitter in each cell class. The sum inhibitory was then taken from the sum of excitatory expression, resulting in an overall positive or negative signed expression in each neuron class for each neurotransmitter. The ratio of these sums was also calculated to indicate the strength of polarity. It was assumed that each LGIC in each neuron class is present equally in all synapses, therefore each incoming connection could be assigned a polarity based upon its receptor expression for that neurotransmitter. The resulting network with polarity was imported into cystoscope (Shannon *et al*, 2003) for plotting and further analysis. Analysis scripts can be found on GitHub at [hiris25/Worm-Connectome-Polarity](https://github.com/hiris25/Worm-Connectome-Polarity).

Expression and cholinergic synapse analysis

The total number of cholinergic input or output synapses was calculated for each neuronal class by summing the number of presynapses for each cell that received a synapse from an ACh-producing neuron (receiving synapses), or the total number of post-synapses an ACh-producing neuron class makes (sending synapses). Acetylcholine producing cells were described by (Pereira *et al*, 2015), the assumption was that all synapses made by an ACh-producing cell also release ACh, even when this cell co-transmits another neurotransmitter. Synapse number for each neuron was taken from (White, 1986). Expression data was obtained from (Taylor *et al*, 2020) using a stringent threshold of 4. Neuronal classes were sorted by ACh in or out degree and the expression of each gene was mapped using a heatmap with an upper threshold of 500. Correlation between expression level and ACh in, or out, degree was mapped using relplot in python's seaborn package, confidence intervals were placed at 68%, corresponding to the standard error of the estimate.

Data analysis

Unless otherwise specified data was imported into GraphPad for further analysis and plotting. Unless otherwise specified, one-way ANOVA was used to calculate significant differences, using the Bonferroni (food leaving & dispersal) or Tukey's (food leaving time course, chemotaxis) correction for multiple comparisons. *= $P < 0.05$, **= $P < 0.005$, ***= $P < 0.001$, ****= $P < 0.0001$.

Data Availability

Python scripts can be found at on GitHub at [hiris25/TEVC-analysis-scripts](#) and [hiris25/Worm-Connectome-Polarity](#). Aggregated data used for analysing TEVC data are available upon request from the Lead Contact. Further information and requests for *C. elegans* strains and

plasmids is to be sent to and will be fulfilled by the Lead Contact William R Schafer,
wschafer@mrc-lmb.cam.ac.uk

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Author Contributions

I.H., J.M. and W.R.S. designed the experiments. I.H., J.M., performed experiments and analysed data. I.H., J.M. and W.R.S. wrote the manuscript and all authors read and critically revised the manuscript to its final form.

Conflicts of Interest

The authors declare no competing interests.

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Figure Legends

Figure 1. Deorphanisation of Cholinergic Ligand Gated Ion Channels. (A) Continuous current traces of *Xenopus* oocytes expressing LGC-57, LGC-58a, LGC-40, and LGC-49, oocytes were perfused for 10s with a panel of ligands each at 1mM: ACh (acetylcholine), Ch (choline), Bet (betaine), TA (tyramine), DA (dopamine), 5-HT (serotonin), OA (octopamine), GA (GABA), GLU (glutamate), His (histamine), MA (melatonin). (B) Phylogenetic tree of a subgroup of Cys-loop ligand gated ion channels in *C. elegans*, groups of interest are highlighted by colour: ‘ACC’ group of acetylcholine gated channels (red), orphan ‘cationic diverse’ group (yellow), orphan LGC-57 group (green). Gene names in bold are deorphanised in this study. (C) Dose response curves for LGC-57, LGC-58a, LGC-40 and LGC-49 in response to their major ligand(s). Current is normalised by I/I_{max} for each oocyte, for LGC-49 current is raw un-normalised current. Error bars represent SEM of 5-14 oocytes. Curves are fitted with a four-parameter variable slope, inserts show EC_{50} in μM for each ligand.

Figure 2. Ion Selectivity and Antagonistic Characterisation of Cholinergic LGICs. (A) Representative current voltage plots of newly de-organised channels in ND96, Na gluconate or NMDG solutions. Current was normalised by subtraction of leak current (in absence of activating ligand) and to the peak current for each oocyte. (B) Box plot of ΔE_{rev} of NMDG and Na Gluconate vs. ND96 in oocytes expressing LGC-57, LGC-58a, LGC-40, LGC-39, LGC-46, and LGC-49, E_{rev} was calculated in the presence of the major agonist of each channel and

leak subtracted. N=6-11 oocytes. (C) Current ratio of oocytes expressing LGC-57, LGC-58, LGC-46, LGC-49 or LGC-39 undergoing repeated agonist application with 10s, 30s and 60s wash intervals. Error bars represent SEM. N=5-9 oocytes per condition. (D) Antagonist application in the presence of ligands using mecamylamine, strychnine, tubocurarine and picrotoxin. Current is normalised by I/I_{max} for each oocyte, curves are fitted with a three-parameter variable slope, error bars represent SEM of 2-7 oocytes, inserts show IC_{50} in μM for each antagonist.

Figure 3. LGC-39 Forms a Polymodal Ligand-Gated Ion Channel. (A) Continuous current trace of a *Xenopus* oocyte clamped at -60mV expressing LGC-39, perfused during 10s pulses with a panel of ligands each at 1mM: ACh (acetylcholine), Ch (choline), Bet (betaine), Tyr (tyramine), DA (dopamine), 5-HT (serotonin), Oct (octopamine), GA (GABA), Glu (glutamate), His (histamine). (B) Dose response curve for LGC-39 in response to ACh, Oct and Tyr. Current is normalised by I/I_{max} for each oocyte. Error bars represent SEM of 8-12. Curves are fit with four-parameter variable slope, inserts show EC_{50} in μM for each ligand. (C) Current-voltage relationship during recordings in NMDG, Na Gluconate or ND96 in oocytes expressing LGC-39. Insert shows ΔE_{rev} vs. ND96 in mV +/- SEM of 5 oocytes. (D) Antagonist dose response curves for LGC-39 expressing oocytes activated by either ACh, Oct or no ligand at a constant dose and varying doses of mecamylamine, strychnine and tubocurarine. Error bars represent SEM of 3-7 oocytes. Curves are fit with X, inserts show IC_{50} in μM for each ligand. (E) Three different agonists do not differ in how they influence the ability for LGC-39 to be stimulated with short time intervals after repeated stimulation.

Figure 4. Newly Deorphanised LGICs are Expressed Broadly in the Nervous System. (A-E) Fluorescent reporters of interictally spliced mKate2 or GFP driven under the promoter

and/or genomic sequence of reveals broad neuronal expression of cholinergic channels with little overlap. (F) Schematic depicting a subset of the synaptic connections received by the *lgc-39* expressing neuron class, AVA, numbers in brackets show the total number of synapses for each connection.

Figure 5. Predicting Synapse Polarity Based on LGIC Expression. (A) Bar chart depicting the percentage of total neuron classes expression inhibitory, excitatory, both or no receptors for GABA, glutamate (Glu) and acetylcholine (ACh). (B) Bar chart depicting the percentage of total synapses for a given neurotransmitter that are predicted to be inhibitory, excitatory or have no prediction. (C) Network diagram depicting the polarity of synaptic connections between neuron classes, connection colour shown polarity: teal (inhibitory), pink (excitatory), grey (no prediction). Gap junctions are represented by dashed lines. Line weight represents number of synapses and nodes are coloured by the major neurotransmitter a class release. Diagram made with cytoscape using the EntOpt clustering package. (D) Network diagram depicting the predicted polarity of the locomotion circuit, connection colour shows polarity: teal (inhibitory), pink (excitatory), grey (no prediction). Inserts show the fold magnitude of expression of the major receptor type for each neurotransmitter in each neuron class. Gap junctions are represented by dashed lines. Line weight represents number of synapses and nodes are coloured by the major neurotransmitter a class release. Diagram made with cytoscape.

Figure 6. Correlation of cholinergic synapses with expression pattern of cholinergic ion channels. (A-B) Heatmaps showing expression level of newly deorphanised LGICs in each neuron class. Neurons are sorted by the total number of cholinergic synapses they make (top, 'ACh Out') or receive (bottom, 'ACh In'). (C-F) Scatter plots showing correlation between

the total number of cholinergic synapses made (orange) or received (red) by a neuron and expression of *lgc-49*, *acc-1*, *lgc-40* or *lgc-46*.

Figure 7. Protein expression pattern of cholinergic ion channels. (A-D) Localisation of endogenously GFP tagged LGC-39, LGC-40, LGC-57 and LGC-58. White arrows highlight areas of interest, represented in higher magnification below.

Expanded View Figure Legends

Supplementary Table 1: The list shows ligand identity and ion selectivity for LGICs in *C. elegans*. For orphan receptors the ligand has been predicted based on homology, this has been noted in the list.

Supplementary Table 2: List of *C. elegans* strains used in this study

Supplementary Figure 1: Negative traces for still orphan LGICs, ion selectivity representative curves and channel kinetics.

A. Continuous TEVC traces from oocytes clamped at -60mV expressing LGC-32, LGC-33, LGC-34, LGC-42, LGC-44, and LGC-48, exposed to 10s of a panel of ligands. B. LGC-47 and ACC-1 do not form a heteromeric channel in *Xenopus* oocytes. Left: Acetylcholine induced dose response curves for oocytes expressing ACC-1 alone or in combination with LGC-47. Error bars represent SEM of 7-12 oocytes per construct, insert shows EC₅₀ values. Right: Continuous TEVC traces from oocytes clamped at -60mV expressing LGC-47, ACC-1 or a combination, exposed to 10s of a panel of ligands.

Supplementary Figure 2: Expression patterns of still orphan LGICs, and *in vitro* data for functional GFP tagged LGICs.

A. Expression of fluorescent reporters for still orphan LGICs. B. Acetylcholine induced dose response curve, continuous trace, and expression pattern of LGC-46. Error bars represent SEM of 6 oocytes. Insert shows EC₅₀. Dose response curves for GFP tagged LGICs in response to their major activating ligands. Error bars represent SEM of 4-5 oocytes per construct, inserts show EC₅₀. C. Expression of fluorescent reporters for *plgc-46* shows a broad neuronal expression pattern with expression in e.g., AIZ, RIH and AVE.

Supplementary Figure 3: Binary heatmap of synaptic sign prediction for the three major neurotransmitters; ACh, glutamate and GABA. The heatmap shows the summed expression level of all LGICs in *C. elegans* per neuron and transmitter, a net sum of excitatory channels is displayed in red, inhibitory in green, equal expression in peach and no expression of LGICs in white.

Supplementary Figure 4: Expression heatmap for the three major neurotransmitters

The heatmap shows the summed expression level, and ion selectivity, for LGICs separated by transmitter (ACh, glutamate and GABA) and neuron class. Net excitatory channel expression is represented in pink and inhibitory in green.

Supplementary Figure 5: Correlation graphs between cholinergic synapses and expression of selected LGICs.

Scatter plots showing gene expression level vs total no. cholinergic synapses received (red) and made (orange). Lines fit using relplot with shaded areas representing the standard error of the line fit.

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745 **Supplementary Figure 6: GFP tagging of LGICs does not influence channel function.**

746 Acetylcholine or choline induced dose response curves for oocytes expressing GFP tagged
747 version of LGC-40, LGC-57 and LGC-58 shows that EC₅₀ values are not influenced by the
748 insertion of the GFP tag. Error bars represent SEM of 7-12 oocytes per construct, insert shows
749 EC₅₀ values.

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752 **Figures:**

753 **Figure 1**

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