

Caenorhabditis elegans Glutamate Transporters Influence Synaptic Function and Behavior at Sites Distant from the Synapse^{*[5]}

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To ensure precise neurotransmission and prevent neurotoxic accumulation, L-glutamate (Glu), the major excitatory neurotransmitter in the brain, is cleared from the synapse by glutamate transporters (GluTs). The molecular components of Glu synapses are highly conserved between *Caenorhabditis elegans* and mammals, yet the absence of synaptic insulation in *C. elegans* raises fundamental questions about Glu clearance strategies in the nematode nervous system. To gain insight into how Glu clearance is accomplished and how GluTs impact neurotransmission, we probed expression and function of all 6 GluTs found in the *C. elegans* genome. Disruption of each GluT impacts multiple Glu-dependent behaviors, with GluT combinations commonly increasing the severity of behavioral deficits. Interestingly, the sole GluT that we find expressed in neurons is localized predominantly in presynaptic neurons, in contrast to the postsynaptic concentration of neuronal GluTs typical in mammals. Moreover, 3 of the 6 GluT genes appear strongly expressed on the capillary excretory canal cell, where they affect Glu-dependent behaviors from positions distal to glutamatergic circuits. Indeed, our focused study of GLT-3, one of the distally expressed GluTs, shows that despite this distance, GLT-3 function can balance the activity mediated by synaptic release and synaptic receptors. The effects of distal GluTs on glutamatergic circuits support that Glu diffusion outside the vicinity of the synapse is a critical factor in *C. elegans* neurotransmission. Together with the presynaptic localization of neuronal GluTs, these observations suggest an unusual strategy for Glu clearance in *C. elegans*.

L-Glutamate (Glu)⁴ is the neurotransmitter that mediates most excitatory signaling in the mammalian brain and plays a pivotal role in neuronal communication in development, basic physiology, and synaptic plasticity. Aberrant Glu signaling, especially exaggerated stimulation by Glu, is involved in a range of neurodegenerative conditions (1). To terminate Glu signaling and clear the synapse in preparation for the next neuronal impulse, Glu released in mammalian synapses during synaptic activity is rapidly removed from the extracellular space by specialized high affinity Glu transporters (GluTs) (2, 3). In mammals, most Glu clearance is accomplished by GluTs expressed on the surface of glial cells that surround the synapse with a smaller contribution mediated by neuronal transporters that are primarily postsynaptic.

We study glutamatergic neurotransmission in *Caenorhabditis elegans*, a model organism with established advantages for elaborating conserved cellular processes (4, 5) and a record of providing unique insights into many areas of neurobiology, including axon guidance (6), neurotransmitter packaging (7), synaptic release (8), and mechanosensation (9). Some aspects of glutamatergic neurotransmission are well described in *C. elegans*, and studies have shown that the molecular components of Glu synapses are highly conserved between *C. elegans* and mammals. These include presynaptic vesicular Glu transporters (vGluTs), of which at least 3 family members are found in the genome and one of which, *eat-4*, has been characterized (10), and postsynaptic Glu receptor/channels (GluRs) (11). Previous studies have mapped all *C. elegans* neurons that express GluRs and are therefore postsynaptic in glutamatergic synapses (12). Moreover, excitatory glutamatergic circuits have been described at the identified neuron level; known presynaptic polymodal neurons respond to diverse sensory stimuli by releasing Glu and activating postsynaptic GluRs in command interneurons, leading to motor responses (13, 14). In addition to the conserved excitatory Glu signaling system, Glu also has an important inhibitory role in *C. elegans* by gating hyperpolarizing Cl⁻ channels such as those that regulate the activity of the pharynx by inducing muscle relaxation (15, 16). Though much less is known about Glu clearance in *C. elegans*, molecular cloning of one nematode GluT and uptake studies in *Xenopus* oocytes supported that, like the other molecular components of

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental information on methods and supplemental Figs. S1–S3.

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⁴ The abbreviations used are: Glu, L-glutamate; GluT, glutamate transporter; GluR, glutamate receptor; vGluT, vesicular glutamate transporter; GFP, green fluorescent protein; WT, wild type; KO, knock-out.

glutamatergic synapses, GluTs are structurally and functionally conserved between nematodes and humans (17–19).

Despite the availability of detailed information on glutamatergic signaling in *C. elegans* from the description of the molecular mediators of Glu release and response to the identification of synapses and circuits that mediate specific behaviors, a major gap remains in our understanding of glutamatergic neurotransmission in this animal. The organization of the nematode nervous system in which synaptic connections are made by neurons *en passant* without insulation by glia (20) raises important questions concerning the principles that govern the accuracy of neurotransmission in *C. elegans* and especially concerning the overall strategy of Glu clearance in this animal. In this study we combine data on the expression patterns and on the phenotypes of likely null mutants for all 6 GluT genes encoded by the *C. elegans* genome (designated as Δ *glt-1*; Δ *glt-3*– Δ *glt-7*) to provide a whole organism view of Glu clearance in this powerful animal model. We focus on the function of one GluT to show in detail how GLT-3 modulates nematode neurophysiology while being expressed at some distance from the affected circuits. We find that in *C. elegans* there is a significant reliance on Glu clearance at a distance, a strategy that is often overlooked in “canonical” understanding of synaptic function.

EXPERIMENTAL PROCEDURES

The following *C. elegans* strains were obtained from the *C. elegans* Genetic Center or from their original producers: wild type; Bristol N2; *eat-4*: MT6308 *eat-4(ky5) III*; *glr-1*: CX3019 *mut-2(r459) I*; *dpy-19(n1347) glr-1(ky176) III*; *nmr-1*; *glr-2 glr-1: nmr-1(ak4) II*; and *glr-2(ak10) glr-1(ky176) III*. We constructed the following strains: Δ *glt-3*: ZB1096 *glt-3(bz34) IV*; Δ *glt-4*: ZB1098 *glt-4(bz69) II*; Δ *glt-5*: ZB1099 *glt-5(bz70) II*. The following GluT deletion mutants were obtained from knockout consortia: Δ *glt-1(ok206)* was received from the *C. elegans* Gene Knockout Project (R. Barstead, Oklahoma Medical Research Foundation); Δ *glt-6(tm1316)* and Δ *glt-7(tm1641)* were received from the National Bioresource Project for the nematode (S. Mitani, Department of Physiology, Tokyo Women's Medical University School of Medicine).

The *C. elegans* laboratories at Rutgers/University of Medicine and Dentistry of New Jersey collaborated to construct deletion libraries according to a standard protocol (21). The deletion genotype was detected by PCR using primers that flank the deletion site, yielding a PCR product that is smaller than that of WT, and verified with primers internal to the deletion segment, which are designed to produce an even shorter PCR fragment in WT, thus ensuring its detection in a mixed reaction. Deletion strains we made in this study were isolated as described in detail in the supplemental information and outcrossed 6 times against wild-type N2 before analysis. As a rule, each strain was obtained in at least two independent outcrosses for our original GluT deletion strains or crossed when combining multiple mutations, and the phenotypes were checked separately before data were combined. Crosses to create mutant combinations were followed by PCR to check for the presence of deleted genes. For double mutants with *eat-4*, *glt-3* deletion, strains were first made homozygous with linked *sma-2*, which was later removed by crossing with *eat-4*.

Construction of reporter GFP transgenic animals (22, 23) and behavioral assays (13, 24–26) were performed according to standard methods. Statistical significance is given according to *z* score or Student's *t* distribution. For more details on methods, see the supplemental information.

RESULTS

The C. elegans Genome Encodes 6 Genes with High Homology to Mammalian Glu Transporters—The *C. elegans* genome encodes 6 GluT homologous genes. Previous studies established that two GluT-encoding cDNAs (initially denoted as CeGlt-1 and CeGlt-2) originate from alternative splicing of a single gene now designated as *glt-1* (17, 18) and established that GLT-1 is a Na⁺-dependent Glu transporter when expressed in *Xenopus* oocytes (19). The other 5 genes found in the genome are designated *glt-3* to *glt-7*. All 6 nematode GluTs exhibit a high degree of homology to mammalian GluTs (supplemental Fig. S1). Primary sequence conservation is particularly strong in regions of high functional significance, including residues that determine the substrate selectivity of GluTs (2, 27). For example, all 6 *C. elegans* GluTs encode an arginine at the site associated with the binding of the γ -carboxyl group of Glu in mammalian GluTs (27), as opposed to a cysteine found at this site in neutral amino acid transporters, and are therefore expected to transport acidic amino acids. *glt* genetic interactions and impacts on glutamatergic signaling *in vivo* (see Figs. 2 and 3 below) further support their identification as glutamate transporters.

C. elegans GluTs Are Expressed in Distinctive Patterns in Diverse Cell Types—To gain insight into the overall strategy of Glu clearance in *C. elegans*, we determined the likely cellular expression patterns of GluTs by analyzing transgenic animals expressing GFP fusion reporters (22, 23) (Fig. 1). We find *glt-1* is strongly expressed in body wall muscles from early developmental stages (Fig. 1A). Early in development we also observe *glt-1* expression in hypodermal cells (not shown). Toward adulthood, the GLT-1-GFP signal becomes more restricted to the head muscles, where bright GFP punctae seem to project from the cell soma toward the center of the nematode head (Fig. 1B), indicating localization to the muscle arms. *C. elegans* muscle arms are unusual extensions of body wall muscles that project toward the motoneurons that innervate them; hence muscles, rather than neurons, reach out to make neuromuscular junctions. In the head, the muscle arms extend from the muscle cell soma, penetrate the nerve ring, and wrap circumferentially to line the inner side of the nerve ring (20). The positioning of GLT-1 on muscle arms puts this GLT near the nerve ring though not directly facing the sensory neuron command interneuron glutamatergic synapses, which are typically found on the outer side of the ring. The relative proximity of this GluT to the relevant synapses might serve to absorb Glu in the central area of *C. elegans* synaptic connection.

Unexpectedly, we found that a full-length *glt-3::gfp* fusion, later shown to be capable of rescuing the phenotypes of a *glt-3* mutant strain, is expressed at increasing levels from late embryogenesis to adulthood throughout the body-spanning excretory canal cell (Fig. 1C), an H-shaped capillary cell that contributes to excretion functions and electrolyte balance. The expression

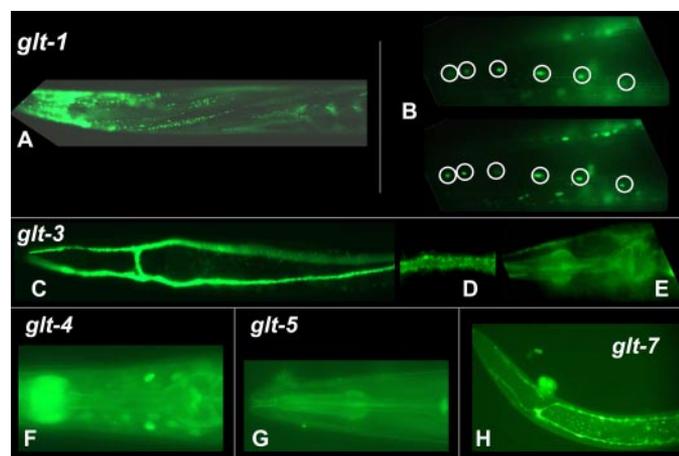


FIGURE 1. *C. elegans* GluTs are expressed in pharynx, excretory canal cell, and head neurons. Study of the expression patterns of nematode GluTs using GFP fusion constructs in transgenic animals. In all panels, anterior is on the left. *A*, expression of *glt-1* (using the GFP reporter $P_{glt-1:glt-1(exons\ 1-3)::gfp}$) in an adult animal. *glt-1* is heavily expressed in the body wall muscles of the head. *B*, expression of *glt-1* (using the GFP reporter $P_{glt-1:glt-1(exons\ 1-3)::gfp}$) in an adult animal. Closer examination of the expression in head muscle suggests localization of *glt-1* to the muscle arms that descend from the outer perimeter of the rodlike animal to its center. The upper panel shows a superficial focal plane (close to the cuticle), and the lower panel shows a deep focal plane (toward the center of the animal) at the same position. The surface of the muscle cell shows typical muscle stripes with light GFP expression. White circles indicate areas of heavy GFP signal that can be followed from the surface of the muscle cell toward the center of the head, and seem to stretch along the muscle arms. *C*, expression pattern of GLT-3 using the deletion-rescuing GFP reporter $P_{glt-3:glt-3(full)::gfp}$. A representative L3 animal showing strong GLT-3 expression throughout the excretory canal cell. This strong expression is maintained throughout adulthood. *D*, expression pattern of GLT-3 using the deletion-rescuing GFP reporter $P_{glt-3:glt-3(full)::gfp}$. At higher magnification and by focusing up and down, expression appears punctuate and cylindrical. This pattern likely reflects a subcellular localization on the abluminal (basolateral) side of the cell, because the luminal membrane is shaped like a slit with increased folding at the center of the body. We compared this expression with the expression of a luminally expressed protein (41) that shows a staining pattern distinct from that observed here. *E*, expression pattern of GLT-3 using the deletion-rescuing GFP reporter $P_{glt-3:glt-3(full)::gfp}$. Weak expression of *glt-3::gfp* is also seen in the pharynx (an overexposure is shown here). *F*, expression of GLT-4 using the GFP reporter $P_{glt-4:glt-4(exons\ 1-3)::gfp}$ is strong in the metacarpus of the pharynx and in some head neurons. *G*, expression of GLT-5 using the GFP reporter $P_{glt-5:glt-5(exons\ 1-2)::gfp}$. Weak expression is seen in the pharynx. *H*, an L3 animal showing expression of GLT-7 using the GFP reporter $P_{glt-7:glt-7(exons\ 1-3)::gfp}$ in the excretory canal cell. Expression is observed from embryonic stages to L3-L4. Only a few adults show faint expression of this construct. Expression of *glt-6* was studied in the course of a global expression pattern project by Hope *et al.* (42), who found that *glt-6* is strongly expressed in the canal cell and in the marginal cells of the pharynx. Potential caveats for the study expression pattern by translational GFP fusion constructs are discussed in the methods section of WormBook (29).

of GLT-3-GFP appears localized to the abluminal (basolateral) side (Fig. 1D), suggesting that GLT-3 serves to transport Glu from the body fluid into the canal cell. We find that *glt-3* is also weakly expressed in the pharynx (overexposure image in Fig. 1E).

The *glt-4::gfp* reporter is expressed strongly in the metacarpus region of the pharynx and in a few head neurons (Fig. 1F). Because in our survey of GFP-tagged transporters *glt-4* is the only GLT that appears to be expressed in neurons, we carefully studied the expression of *glt-4* in head neurons in comparison to known postsynaptic glutamatergic neurons, which have been previously identified by studying the expression of all GluRs encoded by the *C. elegans* genome (11). After comparing *glt-4::gfp* animals to animals expressing the GluR reporter

glr-1::gfp (11), we concluded that *glt-4* is not expressed in the postsynaptic command interneurons, a set of neurons postsynaptic to glutamatergic synapses that are critical for Glu signaling in *C. elegans* (11). Although low intensity signals precluded unambiguous identification of all neurons that express *glt-4::gfp*, consultation of the detailed description of *C. elegans* neuroanatomy confirmed that none of the unidentified *glt-4::gfp*-expressing neurons could be postsynaptic command interneurons.

We also compared *glt-4* expression with presynaptic glutamatergic neurons, some of which were identified by the expression of the presynaptic marker *eat-4*, one of three release vGluT genes in *C. elegans*, the other two having not yet been characterized (10). We identified AUA, RIA, and the set of six IL-2 neurons as expressing *glt-4::gfp*. Interestingly, some of the neurons that do express *glt-4::gfp* are exclusively presynaptic (IL-2), whereas others are both pre- and postsynaptic to glutamatergic synapses (AUA). We verified that the IL-2 neurons, which do not express any GluR (11), express both *glt-4* and the presynaptic marker *eat-4* by staining animals expressing *glt-4::gfp* or *eat-4::gfp* with DiI in the presence of calcium acetate (data not shown), a staining procedure that specifically visualizes the IL-2 neurons. Thus, *glt-4* is expressed in neurons that are exclusively presynaptic, in dual function (pre and postsynaptic) neurons, as well as in unidentified neurons, but is not expressed in the critical postsynaptic glutamatergic command interneurons. Consequently, our data indicate that expression of *glt-4*, the only neuronally expressed GluT we identify in *C. elegans*, correlates mainly with a presynaptic localization in Glu synapses, rather than the generally postsynaptic neuronal GluT expression typically found in most mammalian brain regions.

The *glt-5::gfp* fusion is weakly expressed in the pharynx (Fig. 1G). According to data from a global expression pattern project from the Hope laboratory (42), *glt-6* is expressed in the excretory canal cell and the pharynx throughout development. We observe here that the *glt-7::gfp* fusion also shows strong expression in the excretory canal cell from embryonic to larval stages (an animal at the L3 stage is shown in Fig. 1H). Strikingly, we noticed a complete disappearance of the *glt-7::gfp* signal in most adult animals, suggesting a possible development-specific role for this transporter. The colocalization of *glt-3*, *glt-6*, and *glt-7* expression to the excretory canal cell suggests a critical role for this body-spanning capillary cell in keeping ambient Glu concentration low throughout the nematode body.

Disruption of GluT Genes Confers Synaptic Glu Elevation Phenotypes—We obtained *C. elegans* stains carrying deletions in each of the 6 GluT genes by conducting PCR screens of ethyl methane sulfonate-mutagenized animals in our laboratory (*glt-3(bz34)*, *glt-4(bz69)*, and *glt-5(bz70)*) (21) or by receiving strains from *C. elegans* knock-out consortia (*glt-1(ok206)*, *glt-6(tm1316)*, and *glt-7(tm1641)*). Deletion was verified by PCR using a number of different primers and by sequencing (supplemental Fig. S2). All *glt* deletions appear likely to be null alleles and do not confer profound phenotypic abnormalities, such as lethality or uncoordination, as individual gene mutations.

We investigated the involvement of each of the *C. elegans* GluTs in the physiology of excitatory synapses by testing the effect of GluT gene deletions on four well characterized behaviors

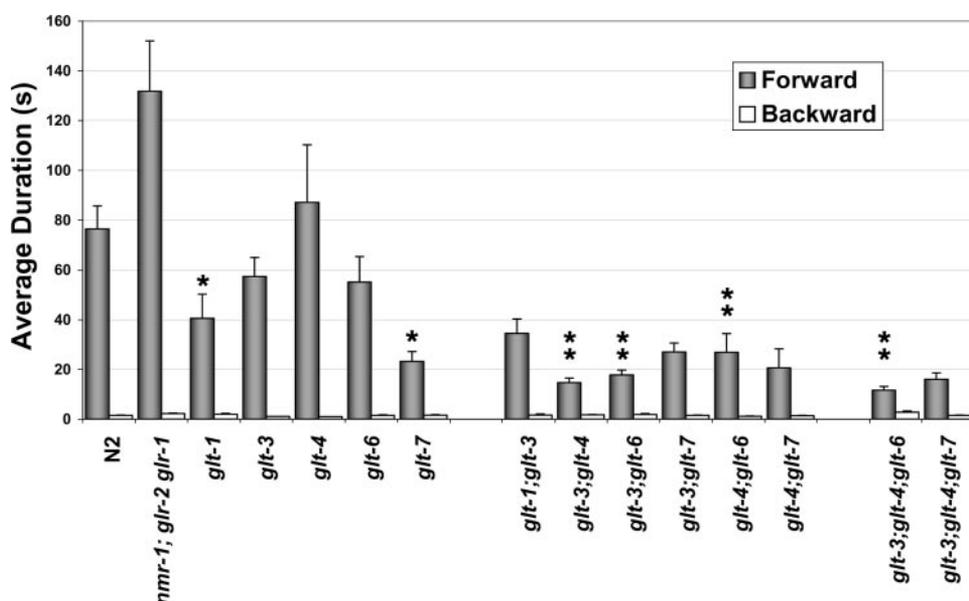


FIGURE 2. Spontaneous mobility: duration of forward runs is reduced in GluT mutants. We examined spontaneous mobility in wild-type (N2) and GluT mutants under conditions favoring forward movement (~10–30 min after transfer to a plate without food). We also examined a triple GluR mutant ($\Delta nmr-1; \Delta glr-2 \Delta glr-1$) (14) and compared the effect of Glu understimulation with the expected hyper-stimulatory effect of GluT KO mutations. For each strain 10–50 animals (average: 23) were tested in 2–4 sessions. Data show mean \pm S.E., with data combined for 2–3 independent outcrossed lines or combination strains for each mutant. *, statistically significant difference (using z test) between wild-type and the indicated GluT KO strain, $p < 0.01$. **, statistically significant difference (using z test) between the indicated combination mutant strain and strains carrying each of the single mutations included in the combination mutant, $p < 0.02$. Although all double and triple mutants are statistically different from wild-type ($p < 0.01$), the combination strains indicated by double asterisks also show a statistically significant difference from both their parental single deletion strains and a more severe defect, suggesting a cumulative effect.

that are mediated by glutamatergic synapses: (i) the dynamics of switching between forward and backward movement during spontaneous mobility (14, 26, 28), (ii) chemotaxis toward isoamyl alcohol (24), (iii) timely aversive reaction to octanol (25), and (iv) evasive reaction to nose touch (13).

Previous studies concluded that the activity of nematode GluRs in command interneurons causes the animals to reduce the time length of forward runs, while reducing Glu activity lengthens the time of forward runs (14, 26, 28). In line with these observations, we find that mutations in GluTs (especially *glt-1* and *glt-7*) reduce the time length of forward runs in spontaneous mobility (Fig. 2). The combination of two or three GluT knock-out (KO) mutants (especially the triple or any of the pairwise combinations of *glt-3*, *glt-4*, and *glt-6*) has a particularly strong effect on this behavior. In *glt-3,-4,-6* and in *glt-3,-4,-7* triple mutants we also observed an increased tendency to wobble in the same area before initiating a run (data not shown). However, even triple GluT KO mutants did not show the extremely high reversal rate reported for a strain expressing a hyperactive GluR (26).

In our analysis of three glutamate-dependent behaviors that depend on sensory stimuli (chemotaxis, nose touch aversion, or octanol aversion), we found that single Δglt mutants show moderately reduced responses to each specific stimulus (Fig. 3). For example, *glt-4* and *glt-7* mutations affect isoamyl chemotaxis and octanol aversion, whereas *glt-3* and *glt-6* mutations affect nose touch. Because we later focus on the role of *glt-3*, we verified that the reduced response to nose touch in $\Delta glt-3$ animals is rescued by the full-length *glt-3::gfp* reporter construct, con-

firmed that defects are exclusively attributed to the GluT deficiency (data not shown).

It may be noteworthy that the phenotypes of *glt-7* are different from those of the other two excretory canal cell-expressed GluTs (*glt-3* and *glt-6*), and defects are somewhat paradoxically observed in assays of adult animals, whereas the GFP reporter construct suggests only residual expression of *glt-7* at this stage. Adult phenotypes might be explained by residual *glt-7* expression in the adult not apparent from our GFP reporter or by long term changes induced when *glt-7* is missing in larval development.

In summary, our analyses of multiple Glu-dependent behaviors for each GluT in the genome strongly support that all GluT knock-outs confer phenotypes consistent with increased Glu signaling (Fig. 2) and demonstrate that individual GluTs can have a strong influence in specific neuronal circuits (Fig. 3). Moreover, our analysis of multiple combinations of glutamate trans-

porter knock-outs establishes that functional overlap of GluT activity impacts many glutamate-dependent behaviors in *C. elegans* (Fig. 2).

A Distal Transporter Is an Important Regulator of Synaptic Signaling—In addition to the observation that elimination of the excretory canal cell GluT *glt-3* contributes strongly to multiple Glu-dependent behavioral defects, we found in a related study that this distal GluT, but not the proximal GluTs *glt-1* or *glt-4*, plays a significant role in Glu-induced neuropathology.⁵ To define the role of the distal transporter *glt-3* in Glu clearance from excitatory synapses more rigorously, we examined the balance between Glu release, response, and clearance using strains that carry combinations of $\Delta glt-3$ with other mutations that affect Glu signaling at the sensory neuron-command interneuron synapses. We tested the ability of $\Delta glt-3$, which is expected to increase overall levels of Glu signaling, to compensate for the signaling-reducing effect of mutations in *eat-4*, a vGluT that facilitates Glu packing into release vesicles in presynaptic neurons (10) or *glr-1*, the principal postsynaptic GluR in these synapses (11, 14) (Fig. 3). Although the signal-reducing *eat-4(ky5)* mutant is dramatically defective in the response to isoamyl, octanol, and nose touch, the *eat-4; \Delta glt-3* double mutant shows a partially restored response in all of these assays. A similar compensatory effect is seen in isothermal tracking, a simple form of learning in *C. elegans* (supplemental Fig. S3). Furthermore, although the signal-reducing *glr-1(ky176)*

⁵ I. Mano and M. Driscoll, submitted for publication.

Glutamate Clearance Strategies in *C. elegans*

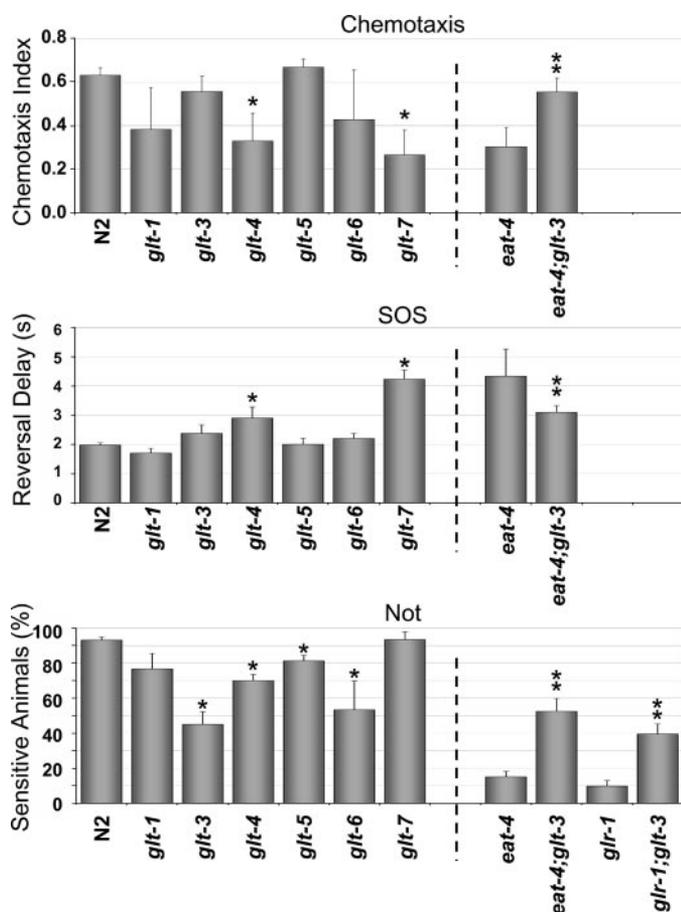


FIGURE 3. *glt-3* deletion impacts multiple Glu-dependent behaviors and can partially compensate for the synaptic weakening effects of mutations in a vGluT or a GluR subunit. These graphs depict the analysis of the behavioral responses to sensory stimuli that are known to be mediated by Glu signaling. *Left section of each panel* describes responses in GluT single mutants (Δ *glt-1*, Δ *glt-3*– Δ *glt-7*). *Right section of each panel* describes responses in *eat-4*; Δ *glt-3* and, when relevant, in *glt-1*; Δ *glt-3* double mutants. Data show mean \pm S.E. Data were combined for 2–3 independent outcrossed lines or combination strains for each mutant. Asterisks indicate statistical difference between GluT deletion mutants and wild type. Double asterisks are used in strains combining Δ *glt-3* and a mutation in *eat-4* or *glt-1*, and indicate a statistically significant difference from the originating *eat-4* or *glt-1* mutant strains using *z* test. *Upper panel* shows chemotaxis to isoamyl alcohol, average of 6 sessions. *, statistical difference between wild type and Δ *glt-4*, $p < 0.01$; **, statistical difference between *eat-4* and *eat-4*; Δ *glt-3*, $p < 0.02$. *Middle panel* shows time delay of aversive response to presentation of octanol (a test known as Smell on a Stick, or SOS), average of 10–20 animals. *, statistical difference from WT, $p < 0.001$; **, statistical difference between *eat-4* and *eat-4*; Δ *glt-3*, $p < 0.1$. *Lower panel* shows percentage of animals backing away from nose touch (head-on collision with an eyelash hair, a test known as NOT), average of 80–120 animals. *, statistical difference from WT, $p < 0.01$; **, statistical difference between *eat-4* and *eat-4*; Δ *glt-3* or statistical difference between *glt-1* and *glt-1*; Δ *glt-3*, $p < 0.001$). Across behavioral assays, the data show moderate Glu signaling defects in single GluT mutants and more severe cumulative defects in multiple GluT mutants. The defects caused by *eat-4* and *glt-1* mutations that reduce Glu signaling are partially suppressed when combined with the GluT mutation Δ *glt-3*, which appears to enhance Glu signaling in these backgrounds.

mutant is severely defective in the response to nose touch, but not in chemotaxis, (11), the nose touch response of the *glt-1*; Δ *glt-3* double mutant is partially restored. The observed inability to fully reconstitute normal neuronal signaling in these double mutants is expected, because Δ *glt-3* not only elevates Glu signaling levels, but is also likely to impose a condition of lingering Glu in the synapse that is probably suboptimal for syn-

aptic transmission. However, the effect of elimination of this distal GluT is not limited to a general “blurring” of Glu neurotransmission, because it can also have the opposite effect and augment synaptic efficacy if Glu signaling is weaker than normal. Consequently, our data support that elimination of *glt-3* results in an increased synaptic Glu concentration. When intact synapses experience elevated Glu concentrations because of transporter inactivation, synaptic function is impaired, whereas under conditions in which Glu signaling is reduced by mutation, elevated Glu concentrations resulting from GluT deficiency actually boost synaptic activity. Therefore, GLT-3 can increase the efficacy of specific weakened synapses despite being expressed away from the affected circuit, demonstrating a functional connection between Glu activity inside the synapse and the activity of a distal Glu uptake system.

Nematode GluTs Regulate Inhibitory Glu Synapses That Control Pharyngeal Pumping—Because the pharynx harbors Glu synapses affecting muscle contraction, we also examined the effect of GluT KO on the pharyngeal pumping rate, previously characterized to be inhibited by Glu acting on Glu-gated Cl^- channels (15, 16). We found that mutations in *glt-1*, *glt-3*, and *glt-6* result in a significantly reduced pharyngeal pumping rate (Fig. 4A). Δ *glt-3* mutants expressing a WT copy of *glt-3* from a transgene (P_{glt-3} *glt-3*(full)::*gfp*) are rescued for the pumping defect, confirming the pumping phenotype is a direct consequence of *glt-3* deficiency. Adding exogenous Glu to WT animals can phenocopy the effect of GluT KO, supporting that this phenotype can result from elevating Glu concentrations (Fig. 4A). Moreover, our extracellular recording of the pharyngeal muscle electrical activity indicates that the activity profile of the Δ *glt-3* strain bears the electrophysiological signature of excess Glu (Fig. 4, B and C), as it is virtually identical to that of wild-type animals saturated with exogenously applied Glu (16). These observations establish that GLT-3 normally functions to clear Glu from glutamatergic synapses in the pharynx and independently demonstrates that elimination of the GLT-3 transporter causes an endogenous elevation of Glu concentration.

Taken together, our genetic disruption of glutamate transporters in *C. elegans* demonstrates how GluTs maintain appropriate neuronal signaling affecting behavior, and unexpectedly emphasizes the central importance of transporters situated at a distance from the synapse that regulate overall Glu availability.

DISCUSSION

In this study we address a major gap in our understanding of glutamatergic neurotransmission in *C. elegans* by providing a whole organism overview of Glu clearance strategies in this animal model. Given the absence of glia at *C. elegans* synapses, we analyzed how and where GluTs regulate neurotransmission in the nematode. We report the first genetic and behavioral analysis of GluT functions in *C. elegans*, compiling data on the role of all six GluTs in maintaining balanced synaptic activity. Our analysis of GluT expression sites and GluT disruption phenotypes underscores the importance of distal Glu clearance and suggests that diffusion outside the vicinity of the synapse is a particularly significant mechanism of Glu clearance in the “open” architecture of the *C. elegans* nervous system.

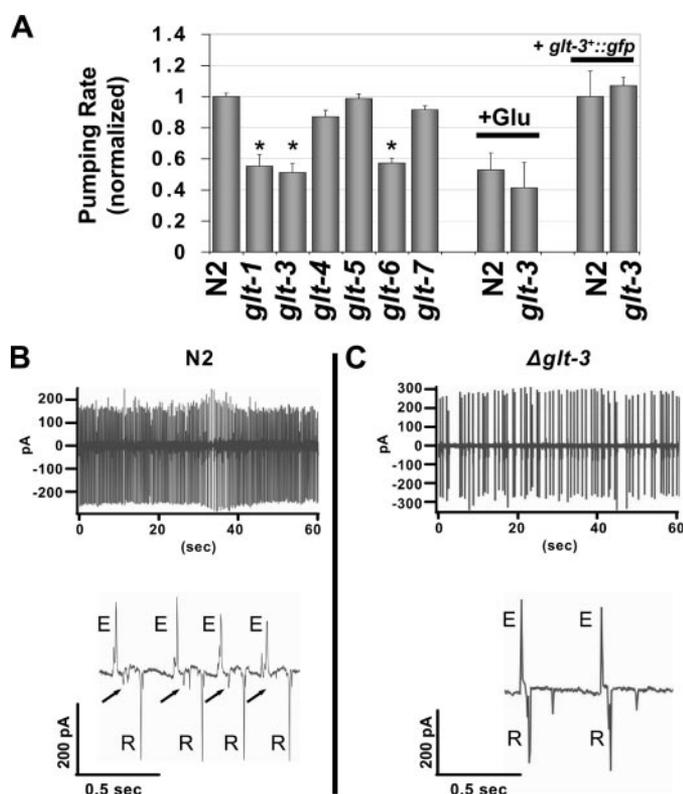


FIGURE 4. Pharyngeal pumping in *glt-3* shows excess inhibition consistent with elevated Glu signaling. A, pumping rate of WT and GluT deletion mutants presented as a fraction of the WT rate (average N2 pumping rate is 138 pumps/30 s). Data show mean \pm S.E. Δ *glt-3* rate is \sim 50% of WT. *, statistical difference from wild type, $p < 0.001$. Horizontal bar labeled +*glt-3::gfp* indicates pumping rate of animals transgenic for Ex[*P_{glt-3}*:*glt-3::gfp*;rol-6(*su1006*)], a transgene consisting of a WT full-length copy of the gene fused in frame to GFP plus a cotransformation marker that induces a rolling behavior. Because the head bending associated with the roller marker interferes with pharyngeal pumping, we assayed the subpopulation of nonrolling but *gfp*⁺ animals. Data under this bar are normalized to an average N2 + *glt-3::gfp* rate of 101 pumps/30 s. The *glt-3*⁺ copy rescues the pumping defect in Δ *glt-3* mutants. Horizontal bar labeled +Glu indicates pumping rate on plates soaked with 20 mM Glu, pH 7, in 5% dimethylformamide (5% dimethylformamide alone did not change pumping rate, not shown). WT pumping rate in the presence of excess Glu is similar to the rate of Δ *glt-3* on regular plates. $n = 55$ (N2); 16 (Δ *glt-1*); 60 (Δ *glt-3*); 31 (Δ *glt-4*); 16 (Δ *glt-5*); 30 (Δ *glt-6*); 30 (Δ *glt-7*); 20 (N2 + *glt-3::gfp*); 20 (Δ *glt-3* + *glt-3::gfp*); 8 (N2 + Glu); 5 (Δ *glt-3* + Glu). B and C, representative traces of electrophysiological recordings from WT and Δ *glt-3* animals. Top panel traces show activity during 1 min. Lower panel traces zoom in on a few pumping cycles. E, excitation; R, relaxation; arrows, relative plateau phase with small inhibitory postsynaptic potentials. The Δ *glt-3* trace (lower panel) shows elimination of the small inhibitory postsynaptic potentials and increased duration of silent intervals between pumping cycles. The difference in current amplitude between B and C is not considered to be meaningful because these can result from chance variations (animal size, tightness of seal, etc.).

C. elegans GluTs Act to Clear Glu and Keep a Balance in Glu Neurotransmission—Analysis of spontaneous mobility (Fig. 2) reveals that the effect of GluT KO is similar to that of a GluR hyperactivating mutation and opposite to that of GluR loss-of-function mutations. Together with the high conservation of GluT gene sequences, these observations support that *C. elegans* GluT gene disruption elevates Glu signaling and that these GluTs normally function to clear Glu in the nematode. In contrast to the expression of some mammalian GluTs on the luminal side of the kidney where they mediate Glu reabsorption, and their elimination therefore causes Glu loss (30), at least one of the nematode GluTs on the excretory canal cell,

GLT-3, seems abluminal. Moreover, mutant phenotypes indicate that all three excretory canal cell GluTs mediate Glu removal because their elimination causes phenotypes consistent with excess Glu signaling. Both behavioral and electrophysiological data (Fig. 4) support that *glt-3* normally acts to clear synaptic Glu in the pharynx. GluT deletions also cause overstimulation in excitatory sensory Glu synapses of the head ganglia. This is particularly evident by the partial restoration of Glu balance (Fig. 3, right panels) when a GluT mutation is combined with mutations that reduce Glu release (vesicle-loading *eat-4*) or Glu response (glutamate receptor *glt-1*).

glt-4 Encodes a Probable Presynaptic Neuronal GluT—Our analysis of GluT-GFP fusion proteins suggests that only one GluT, *glt-4*, is expressed in neurons seemingly concentrated in the head ganglia. Moreover, it is striking that the neuronally expressed *glt-4* has a primarily presynaptic localization in characterized glutamatergic synapses. The command interneurons, the most important postsynaptic Glu excitatory targets in the nematode (11), do not appear to express *glt-4* or any other GluT. In contrast, at least some of the neurons that are exclusively presynaptic to glutamatergic synapses (*i.e.* the set of six IL-2 neurons) do express *glt-4*. Although there are caveats to generalization, our evidence better supports a presynaptic, rather than a postsynaptic, concentration of the only neuronal GluT in *C. elegans*.

In mammalian brains, immunohistochemical studies localize neuronally expressed GluTs primarily to the postsynaptic side (3, 31) with less contribution of presynaptic neuronal GluTs (32, 33). Therefore, the mostly presynaptic neuronal localization of *glt-4* expression suggests an uncommon presynaptic emphasis in the functional organization of transmitter clearance in glutamatergic synapses in *C. elegans*. Mammalian presynaptic GluTs are particularly well studied in the retinal ribbon synapses (34), synapses that carry some interesting lines of functional similarity to glutamatergic synapses in the nematode. In the retinal ribbon synapses, particularly large numbers of vesicles are released, and synaptic release is tonic and graded. Tonic and graded neuronal communication are also the hallmarks of synaptic activity in *C. elegans* (35), although no ribbon structure is observed. Possibly, presynaptic GluT function confers some advantage for high volume, continuous clearance requirements.

Glu Clearance at a Distance: A Central Mechanism in C. elegans Physiology—Another notable distinction in *C. elegans* GluT organization is the prominent positioning of nonneuronal GluTs at some distance from the glutamatergic synapses and the demonstrated ability of GluTs expressed at different locations to affect the same distant synapses (Fig. 5). The transporters we found to exhibit the strongest GFP expression are located on relatively large structures: head muscles for *glt-1* and the capillary canal cell for *glt-3*, *glt-6*, and *glt-7*. The expression of *glt-1* in the head muscles and their muscle arms places these GluTs close to, but not directly facing, the glutamatergic synapses between sensory neurons and command interneurons and suggests that the large muscles can serve as a Glu sink. Importantly, our behavioral data and our data on Glu-induced toxicity⁵ show that a major contribution to Glu clearance in the nematode is mediated by *glt-3*. Although the faint *glt-3* expres-

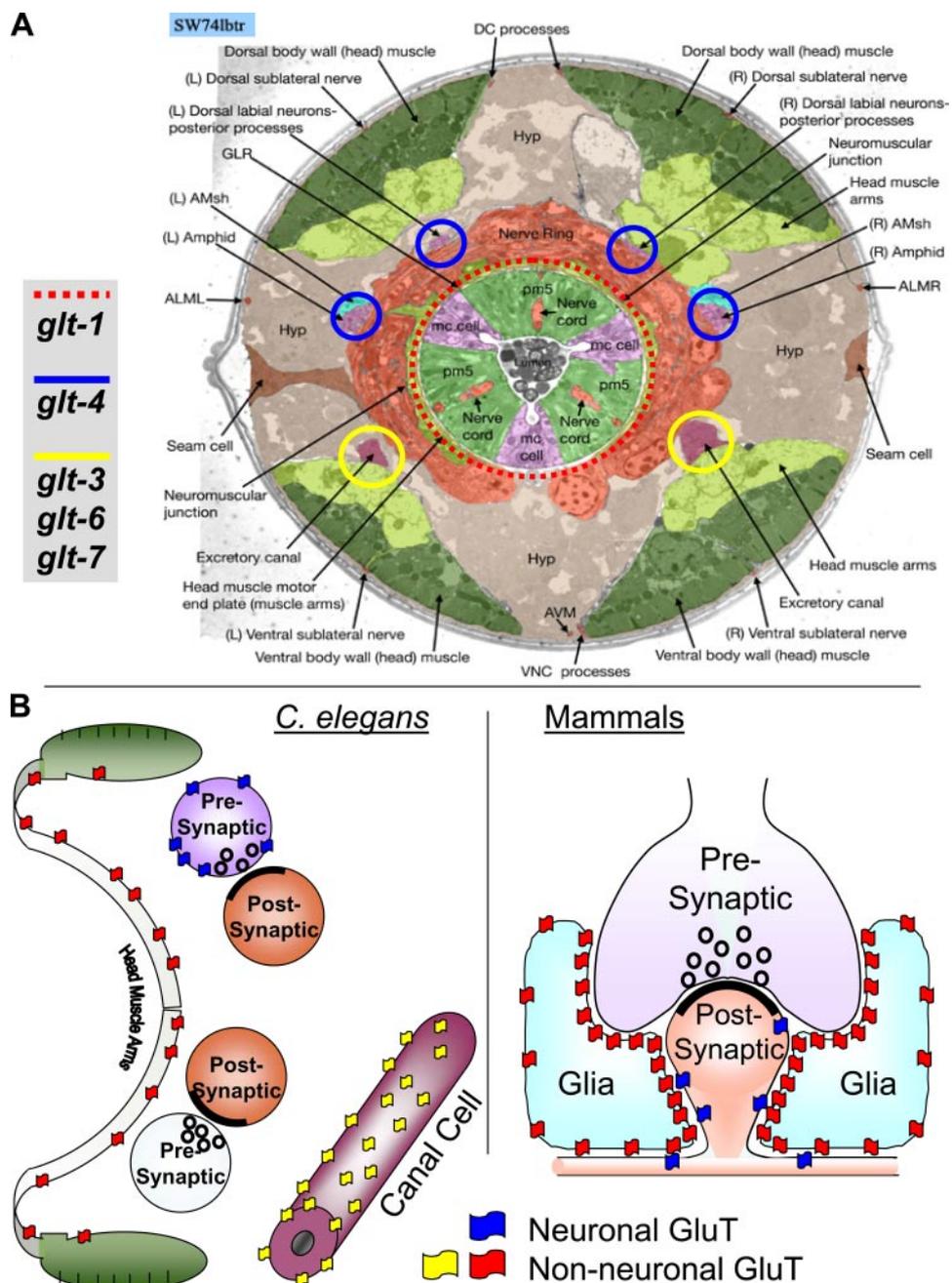


FIGURE 5. Strategies of Glu clearance in mammalian and nematode synapses. *A*, representation of areas of GluTs expression on a modified WormAtlas image of a pseudocolored transmission electron micrograph, a transverse section of *C. elegans* at the nerve ring level (Z. F. Altun and D. H. Hall, SW-Worm Viewer, Slice No. 74). The sensory neurons that come into the nerve ring and synapse on the command interneurons are labeled with blue circles. Some of the presynaptic neurons express *glt-4*; the two capillaries on the excretory canal cell, which expresses *glt-3*, *glt-6*, and *glt-7*, are labeled with yellow circles. The *glt-1*-expressing body wall muscles send extensions that wrap around the pharynx on the inner side of the nerve ring. This area is labeled with a dashed red circle. *B*, schematic representation of Glu clearance strategies in *C. elegans* and mammals. The current description of mammalian glutamatergic synapses (right panel, adopted from Danbolt (31)) emphasizes synapses encapsulated by glia, where GluTs are expressed in close proximity to the left. Neuronal GluTs are expressed mainly by the postsynaptic cell, on the “shoulders” of the spine (black arc represents postsynaptic density). In *C. elegans* (left panel), synaptic contacts occur *en passant* and are not separated by glia. GluTs are expressed in the presynaptic cell and on large nonneuronal structures such as the head muscles and the more distal excretory canal cell. This open organization is more permissive to Glu diffusion outside the immediate vicinity of the releasing synapses. Images are not drawn to proportion, and subcellular localization of nematode GluTs have not yet been experimentally defined.

sion in the pharynx could be responsible for the effect of GLT-3 on pharyngeal pumping, the much stronger expression in the canal cell is probably responsible for the majority of the effects

of the *glt-3* mutation. The GLT-3-GFP fusion protein is localized on the abluminal (basolateral) side of the canal cell, which is $\sim 7 \mu\text{m}$ away from the critical sensory neuron to command interneuron glutamatergic synapses that we have documented to be impacted by GluT deletion (Fig. 5A). Our observations on the localization and function of GLT-3 suggest that this transporter mediates Glu transport between body tissue/fluid and the canal cell to keep a low ambient Glu concentration in the pseudocoelomic body fluid. Low extracellular levels of neurotransmitter should enable Glu released at the synapse to be cleared by rapid diffusion out of the synaptic cleft (31, 36) followed by swift dilution in the pseudocoelomic body fluid and an eventual uptake and removal by the more distant canal cell. In this scenario, GLT-3 does not need to affect directly the concentration of Glu in the synapse and immediately remove the Glu that has just been released. Rather, by determining ambient Glu concentration throughout the body GLT-3 influences the base-line concentration against which clearance by diffusion can work. Possible alternative mechanisms, including an increase in the activity load on other transporters that are closer to the synapse and a shift in the overall balance of substrates and products of the glutamine-glutamate cycle (37, 38), also emphasize the overall importance of distant glutamate clearance in *C. elegans*. It is worth noting that special features of the nematode nervous system make clearance at a distance particularly effective in these animals. The *C. elegans* nervous system features an open architecture expected to be relatively permissive to diffusion. Neurites are loosely packed and synapses are formed *en passant* and are not wrapped by glia (20), allowing the rapid dilution of neurotransmitters that escape the synapse.

In conclusion, our analysis of the *C. elegans* GluT family provides the first whole organism view of Glu clearance strategies in this important animal model. We identify unconventional

emphasis on presynaptic and distal Glu clearance that is likely to facilitate Glu diffusion outside the vicinity of the releasing synapse (Fig. 5B). Although this strategy diverges from the prevalent view of highly localized Glu uptake in the mammalian brain, a few mammalian studies (39, 40) show that under specific circumstances at certain developmental stages and in certain brain areas, some of the principles that govern Glu clearance in the nematode are also operative in higher organisms. Thus, Glu clearance strategies highlighted in the nematode nervous system may also participate in Glu regulatory systems that contribute to mammalian physiology.

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