Synaptic Physiology of Glutamate Clearance in the Nematode Caenorhabditis elegans

Jenny (Chan Ying) Wong
CUNY City College
Synaptic Physiology of Glutamate Clearance in the Nematode *Caenorhabditis elegans*

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By
Jenny (Chan Ying) Wong

Thesis advisor:

Dr. Itzhak Mano
Physiology, Pharmacology, and Neuroscience
the Sophie Davis School of Biomedical Education

Thesis committee:

Dr. Itzhak Mano, Dr. Chris Li, Dr. Jonathan Levitt

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Abstract

L-Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian brain. Glu is normally cleared from the synapse by glutamate transporters (GluTs), but when these GluTs fail, it ultimately leads to Glu accumulation and brain damage in a process called excitotoxicity. The activity of GluTs is therefore a critical key in maintaining accurate synaptic signaling and in preventing excitotoxic accumulation of Glu and neurodegeneration. Much of the Glu clearance capacity is mediated by peri-synaptic GluTs expressed close to the glutamatergic synapses. However, a considerable fraction of Glu clearance is first achieved by diffusion of Glu away from the synapse and later uptake by distal transporters on glia and on the endothelialial cells of the blood capillaries. It is therefore important to study the mechanism of both proximal and distal Glu clearance. Taking advantage of the high conservation of Glu signaling from nematodes to mammals and the availability of strong research tools available in this system, we use *C. elegans* to study GluTs and strategies of Glu clearance in a whole-animal model. We found that accuracy in synaptic transmission and Glu clearance in *C. elegans* depends on a combination of GluTs located either near (proximally) or at a distance (distally) from the synapse. Our research now focuses on the molecular underpinning of GluT function, on understanding the mechanisms involved in Glu clearance by proximal and distal GluTs and on the effect of Glu clearance on transmission fidelity and the precision of neural circuitry. A better understanding of GluT clearance strategy in nematodes and its possible implications for mammalian systems might suggest possible future therapeutic interventions to prevent/reduce excitotoxic damage.
Introduction

Glutamate is an evolutionary ancient neurotransmitter that contributes to excitotoxicity but only became a common excitatory neurotransmitter as organisms evolved. L-Glutamate (Glu), a major excitatory neurotransmitter (NT) in the mammalian brain, is involved in many complex functions, including the development and physiology of the central nervous system. It is central to many processes, such as synapse induction and elimination, cell migration, differentiation and death. However, Glu only became a common excitatory NT as mammals developed a more complex nervous system. Instead, organisms during evolution used neuropeptides and NT such as Acetylcholine (Ach) to produce motor and sensory responses. This is somewhat surprising, however, in that many of the individual components of Glu signaling (i.e. receptors and transporters) can still be found in very simple organisms such as bacteria. A complete ensemble of the proteins involved in glutamatergic synapse is fully functional in cnidarians (alongside complete signaling systems that use other NTs). Nonetheless, it is only highly sophisticated invertebrates and vertebrates that depend on Glu as their main NT in the central nervous system, rather than just using Glu as a marginal NT.

To keep Glu signaling accurate and efficient the nervous system needs to prevent Glu spillover and exaggerated excitation. Glu, a common amino acid in all cells, is packed into synaptic vesicles by specific vesicular transporters and is released from presynaptic cells and diffuses across the synaptic cleft. Once it reaches the postsynaptic cell, Glu binds to cell-membrane glutamate receptors (GluRs). These receptors fall into two groups: metabotropic GluR (mGluRs) and ionotropic receptors (iGluR or simply GluRs). mGluRs are G-protein-coupled receptors (GPCR) with seven membrane-spanning domains and are linked to either cAMP or DAG/IP3 signaling. iGluRs are further divided into 3 subtypes according to their specific synthetic agonist, N-methyl-D-aspartate (NMDA-R), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA-R) and
kainate receptors (KA-Rs). These three iGluRs are Glu-gated membrane ion channels that are able to conduct sodium (Na⁺) and calcium (Ca²⁺) ions, which depolarize and stimulate the cell⁵-⁶.

The Glu released from the presynaptic cell binds to GluRs on postsynaptic cell and causes GluRs to become activated. The extent of activation of GluRs is dependent on the concentration of Glu in the synaptic cleft⁷. However, eventually Glu will dissociate from GluRs, diffuse away, and will be taken up by astrocytes through specialized cell-membrane Glu transporters (GluTs, also known as Excitatory Amino Acid Transporters, EAATs). This process is critical because it helps to maintain the Glu concentrations in the synapses at its very low resting level of submillimolar so that the GluRs are not desensitized. The Glu molecules that were taken up by the glia are converted to glutamine (Gln) by the enzyme glutamate synthase, to be reused and recycled between neurons and glia in a process called glutamate-glutamine cycle⁸-¹⁰. Therefore, most of the synaptically used Glu is not synthesized de novo. The Gln molecules are then released from the glia into the extracellular fluid, where it is taken up by the neurons and reconverted back to Glu by the enzyme, phosphate- activated glutaminase (PAG)¹¹-¹².

When GluTs clear the synapses for the next release event of Glu, it helps to limit ‘Glu spillover’ to neighboring synapses and allow synapses to function independently⁷,⁹. Glu that diffuses out of synapses can either activate the next synapse (if spillover is large), or desensitize it by inhibition of GluRs in neighboring synapses (if spillover is moderate/small). However, Glu spillover is not always an “undesirable” event, and is sometimes used to activate specific synapses to orchestrate the activity of a group of neurons. There are areas in the brain where the synapses are not completely separated by glia (e.g., hippocampus), allowing some Glu to leak out. Depending on the location and density of GluTs and GluRs, varying amounts of Glu may be able to diffuse away to neighboring synapses without being removed from the extracellular
fluid\textsuperscript{7,9,13}. The initial concentration of extracellular Glu and the cellular architecture determines the activation of distal GluRs by diffusion. It is important to note that GluRs are easily desensitized by low Glu concentrations, concentrations that are not high enough to activate the receptors\textsuperscript{14}. Thus, the fidelity and precision of synaptic activity depend on not only on transmitter release and response, but also clearance.

\textit{Excessive Glu signaling is toxic}

The regulation of Glu levels is extremely important because hyperactivity of GluRs due to prolonged exposure of Glu can lead to Glu-triggered neurodegenerative process called “excitotoxicity”\textsuperscript{2}. Excitotoxicity is mediated by excessive depolarization of the GluRs, on the postsynaptic membrane due to the influx of ions\textsuperscript{15}. The hyperactivity of the postsynaptic GluRs leads to the intracellular buildup of Ca\textsuperscript{2+} (Fig. 1). Exaggerated Ca\textsuperscript{2+} concentration has major toxic effects and drives many enzymes to be over-activated. The accumulation of Glu in synapse is caused by the failure of GluTs because energy depletion leads to a drop in Glu clearance. Eventually, the continuous cycle of Glu release and the failure of GluTs to clear the synapses, leads to shockwave-like propagation of neuronal death\textsuperscript{8}. Cell damage is also the result of increasing influx of other ions such as Na\textsuperscript{+} accompanied by chloride (Cl\textsuperscript{−}) and water, which also causes toxicity by creating an osmotic imbalance leading to cell swelling and eventually rupturing the cell membrane affecting its neighboring cells as well\textsuperscript{7,9,15-16}. 
Figure 1: Excessive accumulation of Glu is toxic to the cells

Molecular mechanisms of GluTs in the process of Glu clearance: structure of GluTs, GluT complex and stoichiometry of transport

The first crystallization of GluT homolog from bacteria, GltPh, allowed us to understand the mechanism of GluT clearance. GltPh are organized into a homotrimer bowl-shaped structure, with each subunit acting independently. Each protomer has eight transmembrane segments (TMs 1-8) and two helical hair-pins 1 and 2 (HP1 and HP2) (Fig. 2). The N-terminal of the protomer (TM1-TM6) surrounds the C-terminal as a scaffold for the transporter. The C-terminal (TM7, TM8, HP1 and HP2) is involved in substrate transport. It is through the movements of the hair-pins that GltPh is able to change its conformation from being extracellularly to intracellularly oriented and opening to one side or the other. This is because the tips of the two hairpins meet at the bottom of the bowl in a part of the protein that can move up or down (Fig. 2), thus allowing the opening of the Glu binding site face out or into the cell. While the whole core section (TM7-HP1-HP2-TM8) moves like an elevator in and out of the cell membrane, the tips of the two hairpins would act similarly to alternate gates, allowing Glu the access to its binding site at the transporter’s core. However, in some GluTs during the transition from outside to inside, there is an intermediate state where a small channel opens.
allowing Cl⁻ to flow through. GluTs therefore sometimes act like a Glu-gated Cl⁻ channel with an important function (see below).

**Figure 2:** **Left:** Schematic representation of Gltp̴ transmembrane topology. Source: Yernool et al. 2004. *Nature* **431**, 811-818. **Right:** The core domain of each subunit is alternating between outward and inward facing conformations. Source: Jiang & Amara. 2011. *Neuropharm.* **60**, 172-181

GluTs, are secondary active transporters since their activity depends on the close coupling of Glu transport with the co-transport of 3 Na⁺ ions and the counter-transport of potassium (K⁺) ions. The movement of 3 Na⁺ and K⁺ along their electrochemical gradients allows Glu influx against its concentration gradient³,⁷,¹⁷-¹⁹. This allows the GluT to indirectly use the energy-consuming cell membrane Na⁺/K⁺ ATPase to support the high-affinity reuptake of Glu against a sharp concentration gradient. This is especially important to maintain the synaptic Glu concentration in the nanomolar range, well below the concentration sensed by GluRs; In contrast, on the other side of the transporter, the intracellular Glu concentrations are as high as 10mM³,⁷,¹⁷,²⁰. This mechanism is responsible for the efficient removal of Glu from the synapse, with 95% of Glu going into glia and the remaining into the postsynaptic neuron. However, in some brain areas, presynaptic neuronal GluTs can also acts as a Cl⁻ channel, during the transition of transporting Glu from outside to inside²¹-²². Since the normal transport of GluT involves influx of net positive current, the channel-like Cl⁻ influx helps to prevent threshold
depolarization\textsuperscript{21}. The GluTs expressed on presynaptic neurons use this mechanism to monitor Glu release and prevent excessive release. One example of a presynaptic GluT that functions like Cl\textsuperscript{−} channel is the EAAT5 on the retinal bipolar neurons\textsuperscript{7,22}. The Glu release in one neuron causes a negative feed-back mechanism by inhibiting the release of Glu from the neighboring neuron\textsuperscript{22}. In contrast to EAAT5, GluTs on the postsynaptic neurons and glia uses this mechanism to remove Glu without being depolarized\textsuperscript{21}.

Glu signaling only became important in the brain of evolutionary-advanced animals with the appearance of the BBB and synapse-separating glia. This is needed for both circuit separation and prevention of excitotoxicity.

Glu is a common metabolite present in all cells at high concentrations. It is also present in the environment and diet of many animals. Therefore, the levels of Glu present in both intracellular and extracellular spaces must be regulated to ensure the accuracy of physiological synaptic communication and to prevent pathological accumulation of Glu and the resulting excitotoxicity. In order to prevent excessive Glu from penetrating the brain, invertebrates and vertebrates’ neurons and glia are protected and separated by either the Body-Nervous System Barrier (BdNSB) (in mammals provided by the arachnoid layer of the meninges) or the blood-brain barrier (BBB)\textsuperscript{3-4,23-24}. The BBB consists of capillary endothelial cells that come together to form tight junctions that seal the gaps between the endothelial cells that make the blood capillaries, and a layer of glia cells that wraps around the blood capillaries (Fig. 3)\textsuperscript{23-24}. These tight junctions divide the body fluids in the brain into two compartments of the BBB, the luminal (blood side) and abluminal (brain side), allowing certain substances to pass through the endothelial cell membranes. This is unlike the structure of capillaries outside of the brain, where small particles diffuse freely across the capillary wall through designated gaps between the endothelial cells (Fig. 3)\textsuperscript{23}. These barriers offer the passive isolation of the nervous system from
external sources of Glu while the presence of GluTs on the glia cells provides an active mechanism to remove Glu against a sharp concentration gradient\textsuperscript{23}. The appearance of BBB during evolution is correlated with the transition to the use of Glu as the main excitatory neurotransmitter\textsuperscript{23}. Although all animals make some use of Glu and have conserved proteins that function in Glu signaling, it is only in the more evolutionarily-advanced animals that have an effective BBB and method of Glu clearance, that use Glu as the main excitatory neurotransmitter in their nervous system. Therefore, it seems that the positioning of GluTs on insulating glia and on the BBB allows the transition to the extensive use of Glu in their nervous system.

\textbf{Figure 3:} The tight junctions between the endothelial cells in the BBB.


\textit{C. elegans a key invertebrate model, uses Glu as its main excitatory NT, even in the absence of a BBB}

We study neurotransmission in the nematode \textit{Caenorhabditis elegans} (\textit{C. elegans}), a very useful animal model system that has led to the discovery of cellular and molecular signaling pathways including processes in synaptic function, apoptosis, and RNAi\textsuperscript{25-28}. With a nervous system composed of only 302 neurons, the nervous system has been extensively studied and characterized in detail, making it relatively easy to determine the relationship between a gene and behavior\textsuperscript{29-31}. There are many differences between nematodes and mammals at the organ and system levels, but many cellular and biochemical pathways are highly conserved. One such
example is Glu signaling in *C. elegans*. Like all other animals, the molecular structure of the building blocks of Glu synapses in the nematode (i.e., GluRs, GluTs) is highly homologous in sequence and in function to their mammalian counterpart. However, unlike other simple invertebrates who use ACh as the main excitatory NT, the worm uses Glu as its most prominent excitatory NT (similarly to higher animals): At least 78 neurons release Glu (as they express eat-4)\(^3\), one of three vesicular GluTs found in the genome and 118 neurons express different excitatory GluRs\(^3\). While Glu is mostly used as an excitatory NT, the nematode, like other invertebrates also uses Glu as an inhibitory NT by expressing Glu-gated Cl\(^-\) channels (similar to GABA-Rs) in specific synapses and neuromuscular junctions (NMJs), such as in the pharynx\(^3\). However, the use of Glu as the central excitatory NT in this simple animal is particularly intriguing in light of the lack of synaptic isolation in the worm.

*C. elegans* also lacks a BBB and has only partial similarity to the BdNSB, exhibiting a much more open structure in comparison to the mammalian system\(^4,3\). This open BdNSB is formed by four of the glial cells, the CEP neuron sheath cells, which form a flat band around the nematode’s nerve ring and are open to diffusion of its body fluids\(^3\). Additionally, the synapses in the nematode’s nervous system are made *en passant*, with no presynaptic terminal or postsynaptic dendritic spine, making architecture open for NT diffusion. Therefore, this model system offers an intriguing functional neuronal architecture, suggesting an unusual ability to maintain Glu clearance, inviting us to study the physiological functions of the GluTs.
Specific circuits use Glu to drive behavior of different types: chemorepellents activate the ASH-AVA/AVD/AVE- backward- motorneurons, while chemoattractants activate the ASE-AIB-forward- motorneurons.

Figure 4: Two closely located glutamatergic circuits are functionally separate: ASE-AIB and ASH-AVA both uses Glu but stimulate opposite motor paradigms.

Behavior is the result of neuronal circuitry working together to respond to stimuli in order to produce an appropriate behavior. In C. elegans, the normal spontaneous movement is to first move forward for 16 secs and then briefly moves backwards. When the worms move in the forward direction during chemotaxis it may be due to the attraction towards the chemical cues found in the nematodes’ natural environment. These behaviors are controlled by two neuronal circuits that use Glu: ASH-AVA/D/E circuit and ASE-AIB circuit. These two circuits are anatomically close to each other, but are functionally separate (Fig. 4). In the first circuit, ASH sensory neurons are polymodal nociceptive neurons known to be involved in detecting both chemical and mechanical aversive stimuli. ASH is able to detect volatile repellants as well as
soluble repellants, including high concentrations of salt (NaCl, >200mM), and all concentrations of heavy metals, such as copper (Cu$^{2+}$) and cadmium (Cd$^{2+}$) also causing worms to reverse$^{38-39}$. It secretes Glu to activate postsynaptic interneurons like AVA, AVD and AVE causing the worm to reverse (Fig. 4)$^{37,40}$. In the other circuit, the ASE chemosensory neuron is the primarily detector of soluble attractant such as, low concentrations of NaCl, biotin and cAMP$^{36,41}$. The attractants activate the ASE neuron to release Glu on the AIB neurons and initiate forward movement (Fig. 4). In wild-type N2, the two circuits are functionally separated--- as far as we can tell from behavior, Glu stays in either the ASH-AVA/D/E or the ASE-AIB synapse and the signals do not mix, causing either forward or backward mobility. 

*Our previous studies in our lab identified six conserved GluTs that affect synaptic activity from both distal and proximal locations*

Given the extensive use of Glu in the nematode in the absence of BBB and synaptic isolation we ask how it is possible for the nematode to use Glu as its central excitatory NT and avoid Glu spillover and excitotoxic damage. We suspect the unique features of the nematode’s Glu clearance system might hold the key to this dilemma in allowing them to use Glu without causing spillovers between synapses and over-excitation of synaptic signaling by external Glu.
**Figure 5**: GFP-tagged constructs encoding proximal and distal GluTs in *C. elegans*.

In our lab, we have previously identified six GluT genes in *C. elegans*, which are similar to GluT genes in mammals. Many of the central synapses in the nematode are formed when the presynaptic sensory neurons extend a bundle of neuritis that wraps around the pharynx in a structure called the nerve ring, where they make synaptic connections with the postsynaptic command interneurons. Based on the cellular expression patterns using Green Fluorescent Protein (GFP) fusion reporter in *C. elegans*, the expression of *glt-1* and *glt-4* are found in the non-neuronal head cells and in neurons, respectively (Fig. 5). *glt-1* and *glt-4*, therefore, are inferred to be expressed proximal to the glutamatergic synapses because they are found in close proximity to the nerve ring (Fig. 5, proximal GluTs). Early in development, *glt-1* is strongly expressed in the body wall muscles, however, as the nematode matures, the expression becomes more restricted to the head muscles. On the other hand, *glt-4* is the only GluT that is expressed in the head neurons (with additional expression in the pharynx). In addition to those transporters expressed proximal to the synapses, the GluTs encoded by *glt-3*, *glt-6*, and *glt-7* are inferred to be expressed distal to the glutamatergic synapses because they are found on the excretory canal cell (Fig. 5), about 8 microns away from key Glu synapses (such as ASH-AVA). Nonetheless, the activity of the distal transporters has been found to have a major effect on Glu-dependent behaviors, which is why we believe these distal GluTs (*glt-3, -6, -7*) keep the concentration of Glu low throughout the nematode.
Protein sequences of distal GluTs illustrate a correlation between distal location and unusual structure of their active core suggesting a functional difference between distal and proximal GluTs.

![Crystal structure of bacterial GluT](image)

**Figure 6:** Crystal structure of bacterial GluT (homologous to *C. elegans* proximal GluTs)

The protein sequence of nematode’s GluTs has revealed an overall high degree of homology with mammalian GluTs. There is strong conservation of protein sequence especially at the core on the transporter sequence (comprised of HP1, HP2 and TM7). In addition, all six *C. elegans* GluTs encode an arginine at TM8, a binding domain which is substrate specific to γ-carboxy group of Glu in mammalian GluTs. This arginine in the active site allows the transport of acidic amino acids as opposed to a cysteine found at this site in the more distant relatives of the GluT gene family, which transport neutral amino acids. This suggests that all 6 nematode GluTs are acidic amino acid transporters. Surprisingly, some other features of the nematode’s GluTs show a few critical modifications that mirror the division of proximal and distal GluTs (the same changes are seen in two nematode species, *C. elegans* and *Caenorhabditis briggsae*). The nematodes’ proximal GluTs have the same active site as the GluTs seen in all other organisms, while the distal GluTs show nematode-unique active-site specific modifications. In nematode’s distal GluTs, the Na⁺ binding residue asparagine (Asn)310 is replaced with threonine (Thr); Glu-binding residue Thr(T)314 is replaced with Asn(N); Glycine (Gly) 357 at the external gate of the transporter is missing altogether (Fig. 6). These differences in the
catalytic core suggest a mechanistic difference in the nematodes distal GluTs function, while comparable changes have been shown in mammalian GluTs to be critical for transport activity\textsuperscript{7,17-19}. The role of these sequence modifications and how they fit with the function of distal GluTs in the nematode’s physiology remains a mystery. These observations are particularly intriguing paired with importance of distal GluTs in normal nematode physiology and their importance in preventing pathology\textsuperscript{42}.

**Hypothesis**

*C. elegans controls Glu concentration and circuit separation by utilizing a two-tier Glu clearance strategy.*

![Figure 7](image)

**Figure 7**: Glu Uptake maybe possibly divided into two tiers, proximal (high affinity) and distal (high capacity but low affinity) GluTs, in order to achieve sufficient Glu clearance.

A combination of high-affinity/low-capacity and low-affinity/high-capacity transporters is employed in diverse biological systems to handle a wide range of substrate availabilities, and is also used in mammals for clearance of neurotransmitters like serotonin\textsuperscript{45-46}. We therefore suspect that some of the differences between nematode proximal and distal GluTs might be related to such a division. A close examination of the sequence modifications in the distal GluTs on the canal cells suggest that the distal transporters might exhibit lower affinity and therefore a quicker turn-around transport rate than the classic proximal GluTs. We hypothesize that the
functional organization of Glu clearance in the nematode relies on a two-tier Glu uptake system. Based on their expression pattern, we have categorized \textit{glt-1} and \textit{glt-4} as proximal GluT and \textit{glt-3, glt-6, and glt-7} as distal GluTs. The distal GluTs are suggested to quickly mitigate the effect of large amounts of exogenous Glu and to provide an overall low ambient concentration of Glu that allows Glu clearance by diffusion to prevent inter-synaptic spillover. In contrast, proximal GluTs might provide the finer, more accurate mechanism of removing traces of Glu still present around the nerve ring to improve specificity in synaptic signaling. This clearance strategy provides a way for the organism to monitor and manipulate the levels of Glu in and around the synapses and handle a wide range of extracellular Glu concentrations (Fig. 7). We further believe that Glu increase in some synapses due to GluT mutation might also result in spillover of Glu between synapses and circuits and that these spillovers may alter the nematodes’ behaviors.

Our current findings propose that the mutation in the transporters indicates some differences between distal and proximal GluTs, with much greater effect seen by the mutations in the distal GluTs affecting the neuronal circuitry.
Materials and Methods

Strains, maintenance, and growth conditions

*C. elegans* strains were grown and maintained on MYOB plates containing *OP50 Escherichia coli* bacteria (*E. coli*) at 20° using methods as described unless noted\textsuperscript{42,47}. The following *C. elegans* strains were obtained from *C. elegans* Genetic Center or from their original producers:

**Wild-Type:** Bristol Var. N2; **eat-4:** MT6308 eat-4 (ky5) III; **nmr-1; glr-2 glr-1:** VM1268 nmr-1 (ak4) II; glr-2(ak10) glr-1(ky176)III, **glt-3:** ZB1096 glt-3(bz34) IV, **glt-1:** glt-1 (ok206) X, **glt-6:** glt-6 (tm1316) IV, **glt-7:** glt-7 (tm1641) IV, **glt-4:** ZB1098 glt-4 (bz69) II, **glt-3; glt-6:** glt-3(bz34) IV; glt-6 (tm1316) IV and **osm-9:** CX10 osm-9 (ky10)IV. All crosses were followed by Polymerase Chain Reaction (PCR) analysis and gel electrophoresis. $P_{nmr-1}::G\text{-CaMP}$ crosses were followed by monitoring the GFP expression on coelomycetes under imaging UV optics scope. We constructed the following crosses: **glt-3; glt-6; glt-7:** glt-3 (bz34) IV; glt-6 (tm1316) IV; glt-7 (tm1641) IV, **KD of glt-1 in ∆glt-4:** ZB1098 glt-4 (bz69) II and $P_{nmr-1}::G\text{-CaMP}xglt-3;glt-6;glt-7$: kyEx872 [$P_{nmr-1}::GCaMP$, Pcc::gfp]; glt-3(bz34) IV; glt-6 (tm1316) IV; glt-7 (tm1641) IV.

**Construction of full-length GLT-1::GFP reporter**

The gene encoding green fluorescent protein (GFP) in the pPD95.75 plasmid was obtained from A. Fire. The C12D12.2 cosmid (4.7 kb) obtained from Sanger Institute was divided into two separate parts. The first part that we labeled for convenience “the Red Segment” spans bases from #9718 to #12945, and the other which we labeled “the Yellow Segment” spans bases from #12947 to #14513 (Fig. 8). The oligos for the Red Segment contained both the *PstI* and *BamHI* site and the yellow segment contained both *BamHI* and *XmaI* site (Fig. 8).
Figure 8: Using the ApE program to construct GLT-1::GFP that was cut with PstI and Xmal.

The Red and Yellow Segments were amplified with VentR DNA Polymerase (NEB) and Phusion High-Fidelity DNA Polymerase (NEB) respectively. Amplified fragments were gel purified and inserted into TOPO Blunt vector (Invitrogen). The two segments were then ligated by first cutting the pTopo Red and pTopo Yellow segment with BamHI producing pTopo C12D12. pTopo C12D12.2 as then transformed into 5-alpha Competent E. coli (NEB) and then digested with PstI and Xmal. This C12D12.2 fragment was then introduced into pPD95.75 plasmid. Extrachromosomal transgenic lines were introduced into C. elegans by microinjecting the DNA into adult gonad using standard techniques with a rol-5(su1006) as a co-transformation marker48.

Synchronization of worm populations
All assays were performed on synchronized worm populations. To synchronize worm populations ~ 20 gravid adult worms were placed on to a plate containing OP50 E. coli and were allowed to lay eggs for 9 hours. The adults were then removed. Hatched worms were raised at
Developmental stage was determined by gonadal development. Only L4-young adult worms were assayed.

**RNA interference assays**

RNAi by feeding was performed as previously described with minor adaptations\(^47\). Day 1: RNAi clone picked from Ahringer library on Luria broth medium (LB) agar plates with ampicillin and tetracycline and incubated at 37°C. Day 2: RNAi single clone was then placed into 1 ml LB liquid culture containing 50 μg/ml of ampicillin and incubated at 37°C. Day 3: the 1-ml bacterial culture was transferred into 1-ml of LB liquid culture containing 50 μg/ml of ampicillin and incubated for 4 hrs at 37°C. A total of 150 μl of bacteria culture was spread onto MYOB plates containing 400 mM of βD-isothiogalactopyranoside (IPTG) and 50 μg/ml ampicillin. Day 4: Five L4 nematodes (P\(_0\)) were placed on the bacteria lawn that express double-stranded RNA of target gene or L440 (control, empty vector control) to knock-down expression levels of the targeted gene and allowed to lay eggs. Day 5: The P\(_0\) were removed and the eggs were allowed to grow. All nematodes were grown at 15° and behavioral assays were performed on day 9. Only the F\(_2\) population was scored.

**Behavioral Assays**

All freshly grown nematodes were washed three times with M9 buffer before beginning each assay unless otherwise stated. All behavioral analysis was based on standard methods\(^42\). Statistical significance of the difference in behavioral scores between strains is indicated for the data which we considered of critical importance, such as the mutations with a large effect. Statistical comparison between two strains was done using student’s t-test.
**Spontaneous Mobility**

Spontaneous mobility was assayed based on the protocol of Maricq group (U Utah)\(^49\). Duration of forward mobility was observed until the nematodes stops or reverses.

**Nose Touch**

Nose touch was assayed by placing an eyelash hair in the path of forward-moving worms and counting the number of animals that paused or backup in response to collision with the hair\(^37\). We repeated the assay in four sessions with 30 animals in each group at each session, a few times on each animal.

**IsoAmyl Alcohol Chemotaxi (IAA)**

Isoamyl alcohol chemotaxi was assayed according to Bargmann et al (Rockefeller U)\(^50\). Before beginning the assay, we applied 1 ul isoamyl diluted 1:100 in ethanol to a spot on a large non-seeded agar plate, and 1 ul ethanol to another spot on the opposite side of the plate (as a control). We placed 1 ul of Na\(^+\) azide to both spots to anesthetize worms that arrived there. Animals were placed equidistant from the two spots and allowed to move freely for 1 hr. We counted adult nematodes in equal areas around the two spots. The chemotaxis index was calculated as

\[
\frac{((\text{Number of animals at the test area}) - (\text{Number of animals at the control area}))}{\text{Total number of animals}}
\]

We repeated the assay in three to four sessions.

**Pharyngeal Pumping**

Pharyngeal pumping rate was measured by visually counting the movement of the pharyngeal grinder on a microscope for a period of 30 sec\(^42\). Only worms on the bacterial food source and with constant pharyngeal pumping were scored. The counts were repeated for three sessions with a total of 8 worms in each session and we took the average of the counts.
Drop test assays

The repellent drop test was performed based on the established protocol of Bargmann et al. (Rockefeller U) and Schafer et al. (Cambridge U) protocol\textsuperscript{38,51}. The advantage of the drop assay over the regular chemotaxis is that it allows us to characterize the phenotype of single animals of existing mutants and also allows us to study the mutant’s response to specific concentrations of either attractants or repellents. 10 nematodes were picked from a culture plate and placed on a plate without food for 15 min to avoid transferring food to the assay plates. The animals were then placed on the assay plate and settled for another 15 min. Then we assayed using a capillary to deliver the stimulus. A drop of stimulus was delivered near the tail of a forward moving animal. The mere mechanosensory stimulation of the drop will cause the worm to rush on forward, while a dissolved repellent (now enveloping the animal head to toe by capillary spread) will cause it to reverse. The response was recorded as either “1” (when the nematode stops moving forwards and reverses) or “0” (when the nematode continues to move forward). All response were observed within 4s interval after stimulation. All NaCl were dissolved in 1mM MgSO₄, 1 mM CaCl₂ and 5 mM KPO₄. Copper Chloride (CuCl₂) was dissolved in M13 buffer.

Microfluidics/Ca\textsuperscript{2+} Imaging
Figure 9: A worm microfluidic ‘chip’ used to measure G-CaMP signaling. The chip traps single worms and delivers a chemical stimulus to their nose. Streams 1 and 4 are used to redirect the stream 2 either towards or away from the worm’s nose.
Source: Chronis, N. (Frontier) Lab Chip, 2010, 10, 432-437

Microfluidics/Ca\(^{2+}\) imaging were performed in collaboration with a post-doc in the lab, Dr. KyungWha Lee, and with the support of the Bargmann, Shaham, and Chalasani labs. Recording was performed according to Chronis et al (Rockefeller U) using Nomarski Differential Interference Contrast (DIC), epifluorescence optics, and MetaMorph \(^{52}\). We used young adult worms expressing a Ca\(^{2+}\)-sensitive GFP (GCaMP) as a transgene in AVA/D/E neurons (using the \textit{nmr-1} promoter). To load a worm into the chip, we first placed single nematodes on an unseeded plate and pipette with a drop of S-Basal Medium (Fig. 9)\(^{44}\). The nematodes were then sucked into a polyethylene filled with S-Basal Medium that was maintained with vacuum. By manually controlling the pressure, we are able to flush the worm out we are able to situate the worm onto the chip and apply different chemical stimulus to the worm’s nose. The first generation G-CaMP signaling in our strain was too weak and the fluorescence bleached rather quickly. We, therefore, subtracted the effect of G-CaMP bleaching from the WT and mutant (Fig. 14). G-CaMP3 and G-CaMP5 reporters are currently being generated. Again, for clarification, the imaging data was obtained by Dr. KyungWha Lee, while I prepared the appropriate strains and observed Dr. Lee performing some of the imaging work.
Results

1) A full length GLT-1::GFP reporter suggest a wider expression than that seen previously

To learn more about the functions of *C. elegans* GluT, we first looked at the expression pattern of GluT by analyzing transgenic animals expressing Green Fluorescent Protein (GFP) fusion reporter\(^4^8,^5^3\). *GLT-1::GFP* was previously constructed; however, (unlike the *GLT-3::GFP* reporter construct) it did not include the full length protein \(^4^2\). The partial-length construct was previously found to be expressed throughout the length of the animal only in early developmental stages. Using our current full-length construct, the GFP expression was expressed more strongly throughout the hypodermal cells during all developmental stages, with more intensity of the GFP expression towards the nematode head and tail region in adult (Fig. 10a)\(^4^2\). *glt-1* is strongly expressed not only in the body wall muscles of the head but also throughout the body (Fig. 10a). The expression of *GLT-1::GFP* can also be seen now in the hypodermal cells (Fig. 10a and d). We have also stained the animals with DiI (a dye that diffuses from the outside into the externally-exposed amphid sensory neurons) in order to help us characterize the expression of *GLT-1::GFP* in the nerve ring (looking for potential expression in glia cells (Fig. 10b and c). However, DiI only helped us characterize a subset of glia and not all. Thus, further characterization of full-length *GLT-1::GFP* reporter is still in progress and would give us a better insight in the overall strategy of Glu clearance than previously has seen possibly.
a) GLT-1::GFP stained with DiI

b) Glt-1::GFP stained with Dil
Figure 10: *GLT-1::GFP* shows strong expression levels throughout in adult. a. Expression of *glt-1* in adult nematode. b and c. Adults were stained with vital dye Dii to visualize the amphid head neurons and inner labial glia. d. Expression of hypodermal and seam cells in adult nematode.
2) Behavioral assays can be used to follow Glu physiology in GluT KOs

To investigate the build-up of Glu in the synapses focusing on the roles of GluTs in nematodes and the neuronal circuitry involved, we used a genetic approach to create mutant with knock-out (KO) in all of the distal GluTs, and generated a triple KO strain: Δglt-3 (bz34); Δglt-6 (tm1316); Δglt-7 (tm1641) (glt-3,-6,-7). For proximal GluTs, we attempted combining deletions in Δglt-1 (ok206) and Δ glt-4 (bz69). However, we could not generate this double mutant, as Δglt-1/+; Δ glt-4/Δ glt-4 animals failed to segregate double homozygous animals. We could not point at the exact problem. As an alternative, and since (unlike glt-4) glt-1 is expressed in non-neuronal tissues, we used RNAi knockdown (KD) treatments against glt-1 in Δglt-4 (bz69) mutants to create a de facto double mutant in proximal GluTs (glt-1,-4)28. The activity of the ASH-AVA/D/E synapses was assayed by following spontaneous mobility and response to aversive stimuli, like nose touch and copper chloride (CuCl₂)37,38,50-51,54. The activity of the AWC-mediated response to volatile chemo-attractants was assayed using isoamylcohol (IAA)50. Glu’s ability to gate Cl⁻ channels in the pharynx was followed by determining pharyngeal pumping rate34. Glu has a dual role in NaCl sensation: at low concentrations it is known to activate the attractant ASE-AIB circuit, while at high concentration NaCl becomes a repellent by stimulating the ASH-AVA/D/E aversive circuit. The drop assay measures primarily the aversive properties of solutes like NaCl. In order to gain a better understanding of the overall Glu clearance strategy, we have also looked at Glu spillover in the synapses in vivo by using microfluidics and G-CaMP, a genetically-introduced Ca²⁺ fluorescent probe, to measure neuronal activity in distal and proximal synapses52,55-56.
3) Reversal behaviors mediated by the ASH-command interneuron synapses are affected by the distal GluT, glt-3

A number of scenarios involving backward movement use the repellent-sensory neuron ASH and its downstream command interneurons AVA/D/E. The activity of these synapses is apparent in both spontaneous mobility and aversive stimulus-triggered reversals. To test the synaptic functions in these critical Glu synapses, we observed the “spontaneous” mobility or forward runs, the switch from forward to shortbackward movement which, is controlled by the ASH-AVA/D/E avoidance circuit. The GluRs on the command interneurons (AVA/D/E) have been shown to affect the length of forward runs, as stimulation of these command interneurons triggers activation of the VA motorneurons and intitiation of the backward mobility program. It was therefore found that an increase in GluRs activity in these command interneuron caused the N2 to increase activity of the backward mobility program and reduce the time length of forward runs, while glr-1;glr-2;nmr-1 KO dramatically reduced probability of reversals and extended forward runs. We reasoned that KO/KDs in distal and proximal GluT might change the activity of GluRs at the membrane affecting the ASH-AVA circuitry. The average duration of forward runs for distal GluTs KO of glt-3 was significantly shorter than WT, in line with an increase in Glu activity in the ASH-AVA/D/E synapse (Fig. 11a). Surprisingly, the glt-7 mutant showed longer forward runs, which were not seen in distal GluT combination mutants. Currently, we do not have a satisfactory explanation to this observation, and it is not supported by the nose touch assay (another assay monitoring activity in these synapses). Therefore, it may possibly be a technical difficulty. We only saw a minor effect for either proximal GluT KO (in isolation (Fig. 11a), however, in combination we saw a major using RNAi (Fig. 11b)). Therefore, our data on
spontaneous mobility support a minor effect of proximal transporters and major effect of distal GluTs on the overall levels of GluR activity in the ASH-AVA/D/E circuit.

Figure 11. Spontaneous mobility: duration of spontaneous mobility of forward runs based on short reversals is increased in distal GluTs mutants and decreased in GluTs mutants. We examined forward runs in GluT mutants under conditions favoring forward movement (~30 min after transfer to a plate without food). Error bars represent ±SEM. n= 120 animals for each strain. a. We also examined a triple GluR mutant (nmr-1(ak4); glr-2(ak10); glr-1(ky176)) and compared the effect of Glu
understimulation in GluT KO mutations. For each strain ~30 animals were tested in 4 sessions. KO in distal GluTs deletions does not impair the nematodes forward runs. ***, P<0.001; **, P<0.01. b. Spontaneous mobility in response to RNAi against glt-1 gene in glt-4 mutants. For each bar ~30 animals were tested in 3-4 sessions. Control groups are animals from glt-1 and glt-4 feeding on L440 bacteria. A knock-down in proximal GluT deletions does not impair nematodes forward runs.

We further tested the physiology of aversive sensory stimuli mediated by glutamatergic synapses in the ASH-AVA circuit through GluRs by testing the mutant’s evasive reaction to nose touch\textsuperscript{37,49,54,57}. Again, we observe the major effect of glt-3, while the contribution of glt-6 and glt-7, remains unclear (Fig. 12a). Individual proximal mutant shows small effect; however, a combination by RNAi might be more effective (Fig. 12a and b). This further indicates that distal glt-3 plays a major role in the ASH-AVA circuitry whereas a proximal GluTs plays a more minor role.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{chart.png}
\caption{Bar chart showing sensitive animals percentage for different mutants.}
\end{figure}
**Figure 12:** Glu signaling is defective in both distal and proximal GluTs mutants. N=40-120 animals. We tested each worm twice. **a.** Nose touch in KO mutants ***, P<0.001; **, P<0.01; *, P<0.05. **b.** Nose touch in animals in response to RNAi against glt-1 gene in glt-4 mutants were highly significant ***, P<0.001. Control groups are animals from glt-1 and glt-4 feeding on L440 bacteria.

4) The effect of distal GluTs on the ASH-AVA/D/E synapse can be also seen by genetically encoded Ca$^{2+}$ imaging (Collaboration with Postdoc, Dr. KyungWha Lee)

To follow the changes in neuronal activity in GluT mutants, we examined the changes in activity of a postsynaptic interneuron by using G-CaMP imaging. We crossed our distal GluT mutant, glt-3;--6;--7, with G-CaMP (original version) under the control of nmr-1 promoter (CX7343) and generated Pnmr-1::G-CaMP x glt-3;--6;--7. We then recorded the calcium signal in AVA interneurons after sensory stimulation in the ASH using a standard repellent, high concentration of glycerol (Fig. 13). These results are preliminary because of the fast bleaching of G-CaMP1. To get an initial evaluation of neuronal responses, we numerically subtracted the bleaching-induced signal decline from the recording traces. Following this manipulation we
observed a sharp increase in Ca²⁺ signal intensity after the stimulation in the ASH in the triple distal GluT KO, while the response in WT was too small to detect (Fig. 13).

![Graph showing G-CaMP intensity of WT and distal GluT mutant in response to sensory stimuli in the ASH after subtracting the effect of G-CaMP bleaching (average of the two traces).](image)

**Figure 13:** G-CaMP intensity of WT and distal GluT mutant in response to sensory stimuli in the ASH after subtracting the effect of G-CaMP bleaching (average of the two traces).

5) The output of the volatile odorant sensitive neuron AWC is affected by both the distal GluT, GLT-3 and the proximal GluT, GLT-1

The Glu system homeostasis/balance was further examined by testing the nematodes’ response to volatile chemical stimuli sensed by AWC-AIB/Y circuit using the volatile chemical attractive stimuli, isoamylcohol (IAA)⁵⁰,⁵⁸. *C. elegans eat-4 (ky5) mutants have defective vGluTs, which disrupts the release of Glu, causing abnormal olfactory chemotaxis behavior which we used as a negative control⁴. The *glt*-3 mutation reduced response to IAA chemotaxis while the addition of other distal GluT KO was not consequential (Fig. 14a). There was a partial effect of proximal GluTs but more in combination in *glt*-1,-4 mutants (Fig. 14b). This suggests that both distal and proximal GluTs mutant seem to affect aversive circuitry through the AWC neuron.
Figure 14: Only distal GluT mutants are affected by chemotaxis to isoamyl alcohol. a. Average of 4-6 sessions. N= 20-150 worms each session *, statistical difference between WT N2 and glt-3,-6,-7 and glt-1 and glt-3 is p<0.01.

b. 3 sessions. N=20-100 worms each session. No statistical difference between RNAi glt-1 and glt-4 feeding on L440 bacteria and glt-4 feeding on glt-1 bacteria.

6) We could not detect a major effect of GluT KO on pharyngeal pumping

To better understand the role of GluTs in Glu clearance in inhibitory synapses, we observed another Glu-regulated behavior, pharyngeal pumping. There are three essential pharyngeal neurons in the nematodes that control feeding, M3, M4 and MC59. The M3 inhibitory motor neurons mediate their effect using inhibitory NMJ in the Glu synapses and thus control the timing of pharyngeal relaxation34,59. One of the function of distal GluTs, glt-3, is clear Glu from glutamatergic synapses in the pharynx42. I was unable to detect effects of GluT KOs on pharyngeal pumping rates (Fig. 15).
Figure 15: The pumping rate of WT and distal GluT deletion mutants. Lower pharyngeal pumping in distal GluTs KO due to elevated Glu signaling. n=18-42 *** p<0.001.

7) Glu spillover in the ASE-AIB circuit versus the ASH-AVA circuit through behavioral assays and neuro-imaging (Collaboration with Postdoc, KyungWha Lee)

One of the most important roles of GluTs is to regulate the functional separation of activity in adjacent synapses. The open architecture of C. elegans nervous system presents an interesting challenge to synaptic autonomy. Since mammalian neurons that use spillover as a mechanism of synaptic coordination depend on GluTs to regulate this activity, it is interesting to determine the role of GluT in inter-synaptic spillover in nematodes. In order to study the mechanisms involved in the balance of Glu neurotransmission, we will study Glu spillover and linkage between identified synapses by following animal behavior and neuro-imaging.

C. elegans has been shown to exhibit forward mobility due to chemoattraction to low NaCl concentrations mediated by the ASE sensory neuron36,39,60. The forward movement circuit
is known to be triggered by the activation of the AIB neuron\textsuperscript{39,60}. In contrast, avoidance of high NaCl concentration is sensed by the aversive circuit, where ASH stimulation causes a reversal movement mediated by the AVA neuron\textsuperscript{39,60}. Unlike a regular chemotaxis assay, the drop assay allows us to monitor the aversive response to precise concentrations of solutes. In wild-type, we see the nematode avoid NaCl with increasing concentration so that there is only minimal aversive response to low NaCl concentrations (Fig. 16a). Surprisingly, both proximal GluT single mutants (\textit{glt}-1 and \textit{glt}-4) and double mutant (\textit{glt}-1,-4) avoided very low NaCl concentration especially with 1mM of NaCl (Figs. 16b and c). While proximal GluT KOs showed an overall aversive response to any stimulation, \textit{glt}-1 and \textit{glt}-4 mutants were the only mutants that were able to separately detect either very low or very high concentrations of NaCl (Fig. 16a). \textit{glt}-1 mutants had a similar response to \textit{glt}-4 (Fig. 16a). No additional effect was observed with combination of proximal GluT KD in the nematodes (Fig. 16c). \textit{glt}-3,-6,-7 mutants were strongly defective in avoiding high NaCl concentrations (1000mM, Fig. 16a). The usual aversive response to 1mM of NaCl is partially observed also when combining deletions in all of the distal GluT mutants (Fig. 16b). The observation that low NaCl concentrations, which are normally sensed by chemoattractant sensory neuron, ASE, trigger aversive reactions in GluT KO strains (with emphasis on proximal GluT KOs) suggests that there may be Glu spilling over to the next synapse.
b)
Figure 16: GluT mutants are sensitive to low concentration of NaCl. Escape behavior assessed by drop test assay. Each animal was tested twice. The avoidance index indicates the fraction animals reversing following stimulus application; error bars indicate ±s.e.m. For each data point, N>30. a) Dose response curve for NaCl avoidance in GluT KO mutants. b) Effect of distal and proximal GluT mutations on avoidance (1mM NaCl). **, P<0.01 and ***, P<0.001. c) Dose response curve for NaCl avoidance in RNAi against glt-1.

Since we want to study this potential spillover effect more closely, we tried to envision possible scenarios where low NaCl concentrations cause GluT KO strains to show aversion. The aversive response to very low NaCl concentration can stem from either increased sensitivity of the ASH neuron to low NaCl concentration or from spillover of signals from low-NaCl-detecting ASE neurons to the avoidance circuit. To examine the other circuitry that might be affected by the Glu spillover in our GluTs mutant, we looked further at the ASH-AVA circuit to check for a possible increased sensitivity. We used CuCl₂ as the stimuli for our drop test assay separating ASE from ASH response because the ASH (but not ASE) neurons detect soluble repellants such
as the heavy metals, \( \text{Cu}^{2+} \) and \( \text{Cd}^{2+} \), while the drop assay can allow us to detect precise dependence of sensitivity of response on stimuli concentration\(^{38-39} \). Our preliminary results are hard to interpret. Current data show regular-sensitivity avoidance towards \( \text{CuCl}_2 \) in both distal and proximal GluTs mutants. However, we did not yet manage to see the very low threshold of this response (Fig. 17a and b). Analysis of ASE-specific stimulants such as cAMP and Biotin is underway (no data yet). Furthermore, as I am now finishing constructing strains harboring either distal or proximal GluT KO in combination with GCaMP3/5 expression in AVA neurons, we will perform the appropriate spill-over assays using the imaging system as well.

![Graph](attachment:image.png)
b) Figure 17: Glu Spillover does not occur in the ASH-AVA circuit in both distal and proximal GluTs mutants. Escape behavior assessed by drop test assay. Each animal was tested once. The avoidance index indicates the fraction animals reversing following stimulus application; error bars indicate s.e.m. For each data point, between 14 to 44 animals were tested. a) Effect of distal and proximal GluTs mutant on CuCl₂ avoidance, an ASH-dependent escape behavior. No significant data were detected across all concentrations. b) Effect of proximal GluTs mutant on CuCl₂ avoidance. No significant data were detected across all concentrations.

Discussion

*C. elegans* might have a unique mechanism of Glu clearance that relies heavily on shared responsibility of proximal and distal GluTs. After observing the behaviors in GluT mutants, we think that there are functional differences between proximal and distal GluTs, lending itself to efficient Glu clearance in the absence of an extensive barrier system.

*glt-1 shows heavy expression in hypodermis and probable expression in glia cells*

The construction of full-length *GLT-1::GFP* has allowed us to probe expression more precisely than before. Our analysis of *GLT-1::GFP* has shown similar expression patterns as
previously near the nerve ring, where the muscle arms extend from the cell soma and wraps around the nerve ring\cite{42}. We have reasons to believe that \textit{glt-1} may also be expressed in the CEPsh cells. This further supports that hypodermal and a glia cooperate to clear Glu near the synapses. However, further analysis is needed in order to fully determine this.

\textit{C. elegans distal GluTs have a preferential effect on the nematodes’ behavioral responses that involved in ASH-command interneuron aversive circuitry}

The mutations in GluTs were previously found to affect the length of time in forward runs. This suggests GluR activity in the command interneurons increased as the time of the forward movement decreases\cite{40,49,54,57}. Our data shows that single distal GluT, \textit{glt-3} affects both mobility and nose touch where as our triple distal GluT mutants, \textit{glt-3; glt-6; glt-7} affects glycerol response in imaging. Although these behaviors are triggered by different stimuli, they are all mediated by the same ASH neurons. This is suggesting that distal GluTs especially \textit{glt-3} regulate the ASH-AVA/D/E circuit function. Other Glu-dependent behaviors such as volatile attractive circuitry through the AWC neuron and the inhibitory NMJ through the M3 inhibitory motor neuron only show minor effect of distal and proximal GluTs. This further supports the prominent role of distal GluTs in the ASH-AVA/D/E circuitry.

Some of the results we obtained here do not correlate with what was previously found\cite{42}, an observation we might attribute to technical difficulties. Suprisingly we now find that mutations in the distal GluTs \textit{glt-3} and \textit{glt-7} have opposite effects, as seen in both forward mobility and in chemotaxis towards IAA (Fig. 11a and 12a). We do not have a complete explanation for these observations and to the fact that the GluT \textit{glt-7} mutants seem to have similar behaviors as those of GluR mutants. However, as a wild speculation, we could suggest that this may be due to the fact that \textit{glt-7} is found to be expressed very early in development.
whereas *glt-3* is expressed throughout later development\(^4^2\). It seems that mutations that manifest themselves later in development (as in *glt-3* KO) appear to produce an untamished, possibly full-scale effect. In contrast, it is possible that mutations that are expressed earlier in development might trigger some form of functional compensation. According to this (wholly unsubstantiated) speculation, as *glt-7* mutant matures, it was able to compensate for the excessive Glu signaling by a mechanism of developmental synaptic plasticity, for example, by down-regulating GluRs. Later in development, when other GluTs are in action, the reduced GluR activity remains the same. We are currently testing this hypothesis by directly determining GluR expression levels in *glt-7* using GLR::RFP. On an unrelated technical note, all of our RNAi results are also because we did not perform a wild-type N2 control with RNAi of empty vector L440.

A possible inter-synaptic Glu spillover from ASE-AIB to AVA command interneuron at low concentration of NaCl might cause abnormal chemorepulsion in proximal GluTs mutants

It is known that there are two signaling pathways mediating *C. elegans* response to NaCl: chemoattraction to low NaCl involving the ASE neuron and repulsion to high NaCl concentration involving the ASH neuron\(^4^1,6^0\). It appears that in GluT mutants there is an imbalance between attraction, resulting in avoidance of low concentrations of NaCl and suggesting a difference between distal and proximal GluT mutants. In wild-type nematodes’ attraction towards low concentration of NaCl causes the ASE-AIB circuitry to produce forward mobility (Fig. 16). However, the proximal GluT mutants have a strong repulsion to low concentration of NaCl suggesting that Glu is seeping into the next command interneuron affecting the AVA neuron causing backward mobility (Fig. 16).
ASH-command interneuron circuitry remains unaffected in mutants lacking proximal GluTs

The aversive response to very low NaCl concentration sensed by the ASE neuron is suggesting that Glu is spilling over to the avoidance circuit. In contrast to the proximal GluT mutation \textit{glt}-3, the mutation in either single or double proximal GluTs does not affect the nematodes’ spontaneous forward run suggesting that the ASH-AVA/D/B/E circuitry is less sensitive to proximal Glu clearance (Fig. 11). Unfortunately this could not be further confirmed in the CuCl$_2$ assay because we did not manage to go low enough in Cu$^{2+}$ concentration to see the area of the dose-response curve where differences might occur (Fig.17)$^{38-39}$. Possible changes in sensitivity of chemical detection by the ASE neuron and possible cross stimulation from the attractant ASE sensory neuron to the AVA/D/E aversive command interneuron will be followed in upcoming experiments.

Future/Ongoing Experiments

We are currently still testing the effects of distal and proximal GluTs mutations based on the following:

a) \textit{A detailed analysis of physiology of distal and proximal GluTs in vivo by swapping distal-(canal-) GluTs and proximal (ring-) GluTs expressions}  
To gain insight in the strategy of Glu clearance in \textit{C. elegans}, we would like to swap the expression of the distal GluTs (canal) and proximal GluTs (ring) \textit{in vivo} and compare the their function. We would like to test phenotypic rescue by ectopic expression by using the \textit{glt}-1 promoter to express \textit{glt}-3 ORF and vice versa. This will allow us to observe the functional differences between canal-GluTs and ring-GluTs and test the effects of point mutations and domain swapping. Furthermore, to determine possible expression of GLT-
1 in glia we are crossing our glt-1::gfp with a CEP sheath glia marker, nsIs143 [F16F9.3:dsRed].

b) **Behavioral assay: Biotin and cAMP Drop Assay**

The ASH-AVA circuit seems unaffected by proximal GluT mutations, arguing against its involvement in the aversive response to low NaCl concentration seen in these mutants. This suggests that Glu may be spilling over from the ASE-AIB synapse to ASH-AVA synapse when proximal GluTs mutants are exposed to low concentration of NaCl, causing a change in their behavior from chemoattraction to chemorepellent. To further confirm this, we are currently performing drop assay using biotin and Adenosine 3':5'-Cyclic Monophosphate (cAMP). Both biotin and cAMP are chemoattractants that are also sensed by the ASE neuron\(^4^1\).

c) **Direct visualization of inter-synaptic Glu spillover using Microfluidics/Ca\(^{2+}\) Imaging.**

(Collaboration with Post-Doctoral, KyungWha Lee)

To better understand the functional separation of activity in adjacent synapses for the underlying abnormal behaviors, we are currently using a better (more stable and effective) version of G-CaMP. We are using QW625 (zfls42) [prig-3::GCaMP3-SL2-mCherry;lin-15+], which is a strain where G-CaMP3 is expressed in the AVA interneuron. This strain allows us to monitor the Ca\(^{2+}\) activity while normalized to red signal in the same cell\(^6^1^\text{-}6^2\). We are currently crossing QW625 with our KO distal GluT strain and crossing QW625 with glt-4 and then using this strain to KD glt-1\(^2^7\). We will also be using another G-CaMP strain expressed in the AIB interneuron.
Conclusion

*C. elegans* might have a unique mechanism of Glu clearance that relies heavily on proximal and distal GluTs. We believe that there are functional differences between proximal and distal GluTs allowing efficient Glu clearance in the absence of an extensive barrier system. We are currently still testing the effects of distal vs. proximal GluTs through behavioral analysis and also using cutting-edge imaging techniques looking at neuronal activity in living animals. Using these techniques we can test the effects of proximal and distal GluTs at the detailed-level of cellular analysis yet still in the context of whole-animal physiology. We hope that understanding how proximal and distal GluT prevents Glu accumulation and spillover in the nematode might help us understand the role of similarly positioned GluTs in the mammalian brain. This knowledge would help us to better understand how the nematode uses Glu as their central excitatory transmitter and how this fits the overall arch of evolution of the nervous system. Furthermore, detailed understanding of the mechanisms involved in Glu clearance from afar could help us suggest new ways to affect inappropriate Glu clearance in medical scenarios that include either excitotoxicity or a breach of the BBB, such as seen in brain ischemia or hemorrhage. Since, GluTs expressed on the BBB are more easily manipulated than those beyond the BBB, these studies might provide new avenues for therapeutic interventions in stroke.
References


