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Screen for Suppressors and Enhancers of Excitotoxic Neurodegeneration

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Screen for Suppressors and Enhancers of Excitotoxic Neurodegeneration.

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Abstract

Excitotoxicity is an important and frequently observed neurodegenerative process. Excitotoxicity mediates brain damage in a range of diseases and conditions including stroke, and is triggered by excessive stimulation of glutamatergic synapses. In spite of extensive studies, the molecular mechanisms involved in excitotoxicity following the over-activation of postsynaptic glutamate receptors are not well understood, and clinical trials based on our partial understanding of the process ended with disappointment. Genetic screens in simple animal models offer a powerful alternative approach, since screens are unbiased, analysis is facilitated by strong research tools, and cellular mechanisms are highly conserved through evolution. We produced a reliable model for excitotoxicity in the nematode C. elegans (the ∆glt-3;nuIs5 strain) and we now use this model to screen for genes whose mutation can alter the extent of neurodegeneration. We are using two approaches to systematically knock-down/modify C. elegans genes and test their involvement in excitotoxic neurodegeneration: a) We are developing sensitive strains that will allow us to use an RNAi library to knock down each gene in the nematode’s genome. b) We are using EMS to introduce random mutations throughout the genome. In both cases we are monitoring the effect of genetic modification on the level of neurodegeneration. We have screened ~2,000 mutagenized genomes and identified 2 mutant strains that show enhanced excitotoxicity and 6 mutant strains that show suppression/decrease in the level of neurodegeneration. To identify the most interesting mutants, we are carrying out behavioral assays that can indicate if the mutation affects overall normal synaptic activity by changing presynaptic release or perisynaptic receptor activity. We are mostly interested in mutants where these functions are normal, suggesting that the modified levels of neurodegeneration come from a specific effect on postsynaptic cell-death pathways. Following the characterization of cell death pathway mutants, we will be using whole genome sequencing to identify enhancer or suppressor mutations and study their mechanism of action.
Introduction

Glutamate is an excitatory neurotransmitter with a key role in the synapse

Glutamate (Glu) is the major neurotransmitter that controls most excitatory signals in the brain \((1, 2)\). In addition to mediating basic neurophysiology, Glu plays a key role in long term potentiation (persistent increase in synaptic strength following stimulation of a chemical synapse) and it is important for learning and memory \((3)\). Glu is loaded in synaptic vesicles by a vesicular transporter \((4)\) and gets released from presynaptic cells by nerve impulses (stimulation). Once this is done, Glu binds to glutamate receptors (GluRs) on the opposing post synaptic neuron. There are two different classes of glutamate receptors: metabotropic GluR (mGluRs) and Ionotropic GluR (iGluR). mGluR’s act through G coupled protein signaling, with seven membrane-spanning domains that are linked to secondary messengers such as cyclic AMP (cAMP) or DAG (Di acyl Glycerol)/IP3 (Inositol-3-phosphate). Ionotropic GluR acts through a different mechanism and are divided into 3 different subtypes, each according to the specific Glu-derived synthetic substance that binds to it (as agonist): NMDA (N-methyl-D-aspartate), AMPA, (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and Kainate (KA-R) receptors \((5, 6, and 7)\). Each of these receptors have a docking site for Glu and change their conformation once Glu binds, thereby leading to the opening of an intrinsic channel domain and influx of \(Na^+\) and \(Ca^{2+}\). After Glu activates its receptors, it is removed from the synaptic cleft by specialized Glu transporters (GluTs) expressed on the cell membrane of cells surrounding the synapse \((8)\), to maintain homeostasis in the synapse (Figure 1).
This homeostasis must be maintained to ensure the precision of high-frequency synaptic activity and to prevent Glu spillover. The Glu molecules taken up by glia in the surrounding space are converted into glutamine (a synaptically inactive molecule), released from the glia, taken up by neurons, and reused once again in the presynaptic cells where they are converted back into glutamate and packaged into vesicles, in what is referred to as the Glutamate-Glutamine cycle [9-11]. GluTs use a secondary-active mechanism by the co-transport of Glu with 3 molecules of Na⁺ into the cell, and the counter transport of potassium (K⁺) along their electrochemical gradient. Using the gradient of ions established by the Na⁺/K⁺ ATPase allows the transporter to maintain extracellular concentration of Glu at the nanomolar range [12 and 13].
**Excessive Glu is Toxic to the brain, as seen during stroke**

In ischemia there might be an occlusion of a blood vessel, leading to a shortage of oxygen and Glucose. Once the supply of energy is depleted, the Na+/K+/ATPase cannot maintain ion gradients, and GluTs stops bringing Glu into the cell, resulting in the accumulation of Glu in the synapse as shown in (Figure 2).

![Figure 2](image-url)

Figure 2: During Ischemia there is a depletion of energy in the cell resulting in GluTs becoming slow or underpowered. This results in the accumulation of Glu in the synapse and over excitability of glutamate receptors, leading to toxic buildup of ions inside the cell, which ultimately lead to cell death.

The excess Glu over stimulates the post synaptic ionotropic Glu receptors (iGluRs), leading to excessive depolarization, a buildup of toxic intracellular Ca\(^{2+}\) and Na\(^{+}\) levels, cell swelling and necrotic cell death called excitotoxicity \(^{14}\).

**Stroke damage is caused by excitotoxicity**

Stroke is the third leading cause of death and the most important cause of long term disability and hospitalization in the United States \(^{15}\). Ischemia-triggered excitotoxicity is considered to be the main
cause of brain damage during stroke. Traumatic Brain Injury (TBI) also induces excitotoxicity, either due to the breach of the Blood-Brain Barrier and the penetration of Glu-rich serum into synapses, or by mechanically obstructing normal blood supply. Stroke and TBI related injuries also result in later damage to other brain areas, away from the initial site of injury. Patient admission to hospitals grants a narrow window of opportunity for therapies that might be able to reduce or slow down the gradual deterioration of other areas. However, our understanding of the cascade of events that lead to both acute and delayed neurodegeneration is very limited, hindering the effectiveness of current treatment. This tells us that we do not completely understand the events that occur during stroke; although current treatment provide some measure of relief for the patients, further understanding of the physiological processes that occur during stroke is still required.

**Stroke and TBI involve a range of forms of cell death mechanisms**

The stroke core, or center of the affected area in the brain is where a large number of cells undergo necrosis, a form of uncontrolled cell death that involves cells emptying their contents into the surrounding space, releasing large amount of cytoplasmic Glu and triggering inflammation. Waves of destruction propagating from the core to surrounding areas, called stroke Penumbra, resulting in delayed death of surrounding neurons. The cells in the penumbra are exposed to a weaker insult and undergo a different type of cell death called Apoptosis. This form of cell death is controlled and cells do not empty their contents into the surrounding space; thereby reducing the spread of neuronal death \{16, 17, 18 and 19\}. 
Figure 3: The red area depicted in the figure above is the center of the affected area which is called the core and cells in this area die by necrosis. This form of cell death results in the excretion of Glu into the surrounding space, and the propagation of death to surrounding cells. The cells in the outer area are better prepared for this shock because they sense the increase in Glu in the surrounding space and die by either Apoptosis or Necrosis (17 and 18).

Therefore, there are different forms of cell death that occur during stroke: while necrosis occurs at the core of the injury, cells in brain regions surrounding the core die by either apoptosis or necrosis, as determined by the spread of Glu and Ca$^{2+}$.

**Race is a factor in stroke risk, although the cause for this is not known**

Minority populations are the most vulnerable to stroke, most especially African Americans. The largest anomaly is seen within the age range of 35-54, with African Americans being four times more likely to suffer from stroke during this midlife period and twice as likely to die from stroke when compared to Caucasians (20). The precise explanation for racial differences in the occurrence of stroke is not yet known. Lower socioeconomic status, frequently associated with race in the US, has been linked with a number of stroke risk factors such as obesity, diabetes, lack of exercise and sharply limited access to both routine and acute (in the ER) medical care. However, this is not the full picture because
Hispanics live in similar conditions, but do not show high incidence of stroke. This tells us that other factors causing this higher occurrence of stroke amongst racial minorities are not yet known [21].

**We do not understand the process of neuronal damage in excitotoxicity**

Several clinical trials have tried to prevent excitotoxic neurodegeneration and ischemic cell death by using antagonists that prevent glutamate from binding to post synaptic receptors, but the results were highly disappointing. Although this method is feasible and likely to reduce the occurrence of Glu mediated over-excitation of GluR’s, a complete block of Glu signaling is apparently very detrimental to the cell. This may be due to the function of Glutamate as an excitatory neurotransmitter, makes it necessary for neuronal survival, and the viability of the cell depends on normal neuronal activity [22]. Therefore it was suggested that Glu plays a crucial role in both protecting the neurons from dying (i.e., Glu-mediated Neuro-protection and neuronal loss (i.e., Glu-mediated neurotoxicity) [23]. These clinical failures reveal that we need to go back to basic research and understand the next steps in the cell death pathway after the toxic buildup of ions in the cell, before we can create drugs that can improve patient health rather than dampen it.

**Previous techniques such as Proteomic mass spectroscopy and protein-protein interaction were good for identifying unknown components of a pathway but were subject to their flaws**

Despite the fact that the first few steps in the cascade of events that occur during excitotoxicity are known, the sequence of events that lead to neuronal loss and excitotoxicity remain a mystery [23]. Most of the data currently available on excitotoxicity were obtained by taking educated guesses, and were limited by the bias of targeted testing. In order to gain an unbiased and comprehensive understanding
of these mechanisms, molecular events must be studied at the cellular level by using techniques that are not limited to identifying only a particular set of candidates. For these purpose different techniques such as proteomic mass spectroscopy (24), genetic analysis and two hybrid screening (25) have been used to understand the cellular changes happening in excitotoxicity. Proteomic mass spectroscopy analyzes cellular processes by using protein-protein interactions. Since most proteins interact constantly in the cell and enzyme activities are regulated tightly by these interactions, it becomes easy to use one protein as an affinity reagent (bait) to isolate its binding partner. Once this interaction is observed, the two proteins are isolated and the structure of the unknown protein is deciphered via mass spectroscopy (24). Two hybrid screening uses a similar approach but can also be used to understand DNA-protein interaction in addition to protein-protein interaction. This method is used in yeast (Saccharomyces Cerevisiae) to identify unknown interactions that occur between proteins or between a proteins and DNA (25). Although both of these methods are very useful for identifying previously unsuspected interactions between proteins, they also have their draw backs. One major challenge with this technique is that if the physical interaction between the bait and the unknown protein is weak or short lived, or if one protein communicates a signal to the other via a soluble small molecule, it will be very difficult or impossible to detect this interaction. Another drawback is if the conditions for these two proteins to interact are only met at specific time points or conditions. This would hamper or slow down the progress of the research, Additionally, these methods are often employed in heterologous cellular system (tissue culture or yeast), while the physiological conditions inside the cells of the organism differ significantly from the dish or plates where these experiments will be done.

**Genetic screens and their unbiased approach**

Genetic analysis offers an unbiased evaluation of all cellular processes, regardless of prior knowledge, or strength of physical interactions between proteins or the brevity of the interaction.
Moreover, genetic analysis highlights the most important functional interactions, since the modifications (caused by the mutations) that result in the strongest effects will be easily identified, due to their strong phenotype. Mutations in genes along these cellular cascades can be arranged in logical flowcharts that correspond to the sequence of molecular events that lead to the observed phenotypes. Genetic screens were the cornerstone of great discoveries made in *Drosophila* {26}, *C. elegans* {27} and yeast {28}, resulting in improved understanding of key cellular processes. Once key steps that modulate excitotoxicity are discovered in nematodes they could potentially suggest neurodegeneration-blocking approaches in mammals.

**Unique features of *C. elegans* research make it the system of choice for genetic analysis of excitotoxicity:**

Several genetic studies in *C. elegans* including apoptosis {29}, synaptic functions {30}, Ras signal transduction {31} and RNAi {32}, have led to the discovery of conserved molecules and pathways significant to human biology and the subsequent development of therapeutic tactics {33-35}. *C. elegans* as a model for research analysis offers an especially strong combination of research tools. In addition to its short life cycle and ease of culturing, all cells have been identified, all neuronal connections have been intricately studied, and there is a fully sequenced genome, allowing large scale screens to be performed with ease and relatively quick {36, 37, and 38}. Unlike other invertebrates, Glu is the main excitatory neurotransmitter in *C. elegans*, and basic signaling machinery of glutamate synapses in *C. elegans* is very similar to their mammalian counterparts in terms of release mechanism, receptors, transporters and their interacting proteins {39- 51}. Our recent studies have shown that key processes that occur during excitotoxicity are conserved in nematodes, making our model a suitable candidate to study excitotoxicity {52}.
We created excitotoxic conditions in nematodes by knocking out transporters that remove Glu from synapses and combining this with a sensitized mutant background.

To study excitotoxicity in nematodes, we created *C. elegans* with excessive Glu in their synapse, by eliminating glutamate uptake using gene knockouts. Nematodes have a total of 6 glutamate transporters genes (*glt*-1, 3, 4, 5, 6, 7), and we were able to obtain deletion strains for each one of these genes by either creating the KO ourselves (*glt*-3, 4, and 5) or obtaining these strains from the knockout consortia (*glt*-1, 6, 7). Knockout of the genes that express these transporters were obtained via EMS mutagenesis and strains that had deletions in these genes were identified via doing PCR {53}. Amongst the knockouts obtained via EMS, only mutations in *glt*-3 led to excessive Glu buildup in the synapse that produced excitotoxicity (in the right background, figure 4).

Figure 4

Figure 4 is a representation of how many vacuole-like structures were observed in our sensitized background alone, and the combination of our *glt*-3 knockouts with our sensitized background (as seen on the right). The bar graph on the right hand corner shows the number of neurons that undergo necrosis in the head. These vacuole like structures appear more frequently in our combination strain than the single mutant which consisted of our sensitized background alone. To further show that the increase in necrotic cell death is as a result of excessive Glu, a knockout of all GluRs were made in the presence of our sensitized background as shown on the bar graph on the upper right corner of Fig. 4.
However, the knockout of the transporter alone was not enough to cause exacerbated cell death by necrosis. We combined our GluT knockout strain (Δglt-3) with a sensitized background known as nuls5 [53] (where GαS* is expressed under a GluR gene promoter), giving rise to our excitotoxicity strain Δglt-3; nuls5, where we identified more vacuole like necrotic structures in this transgenic mutant typical of neurons dying by necrosis. To ensure that the neuronal death observed in these worms were as a result of hyper-activation of post-synaptic receptors by Glu, we used the fact this strain combined the hyper active GαS background with a GFP reporter under the promoter of glr-1. A picture of this nematode strain is shown below

**Figure 5**

![Figure 5: The picture above shows a worm that has a GαS sensitized background mutation fused together with the promoter of glr-1 and a visual reporter (GFP). We can visualize neurons in the nerve ring that are stimulated by Glu and undergo Necrosis.](image-url)
We also verified that this excessive neurodegeneration is mediated by classical Glu toxicity by demonstrating that removing the GluRs from the postsynaptic neurons prevents this neurodegeneration even in the presence of excess Glu (in glt-3 KO animals, as shown in figure 4).

We now have a powerful system to study excitotoxic neurodegeneration in nematodes, and are well positioned to carry out a genetic screen to search for genes whose modification can reduce or enhance excitotoxic neurodegeneration.
Experimental approaches

Random Mutagenesis

Although genetic screens are invaluable and have proven to be useful in understanding cellular processes, they also face several challenges. One major impediment is that they are labor intensive especially at the stage of molecular identification of the phenotype-causing mutation by positional mapping. Long time periods are required to identify mutations that change the phenotype by examining crossovers with chromosomes that harbor known phenotype mutations e.g., *dumpy* or *roll*. Although advances in technology have introduced the technique of Single Nucleotide Polymorphism (SNP) mapping, which allows us to check for crossovers with multiple, easily detectable markers, it is still difficult to carry out positional cloning by crosses, and generation of recombinant animals remains challenging (54). Since neurodegeneration requires two genetic modifications (*Δglt3; nuIs5*), obtaining many recombinants while still maintaining these two modifications in the background makes genetic mapping tedious.

New technological advances, especially in whole-genome sequencing (55), now allow geneticist to circumvent these problems, and prompted us to take this approach to screen for suppressors or enhancers of excitotoxicity.

RNAi

RNA-interference is a method that is used to inhibit the translation of specific mRNAs by introducing an inhibitory double stranded RNA (silencing/small inhibitory RNA, siRNA) by feeding worms bacteria that produce RNAi targeting specific nematode genes (56). (figure 6).
Libraries of RNAi-inducing bacteria that consist of most ORF (open reading frames) identified in the *C. elegans* genome are readily available and have been very useful. The main advantage of this approach is that unlike classic mutagenesis where mutations are obtained by chance and their location needs to be identified, RNAi screens are more methodical (going through each gene one by one); once a phenotype is observed, the gene whose silencing is causing this effect will be easy to identify (since the bacteria expressing the specific sequence that is producing the RNAi effect is already known). However, until recently, a huge roadblock in the use of RNAi screen for neuroscience was that *C. elegans* neurons showed high resistance to effects of RNAi, making most attempts to block neuronal genes with dsRNA unsuccessful.

Fortunately, in recent years there were major breakthroughs in the use of RNAi for genetic screens in *C. elegans* neuroscience. Several mutations were found to distinctly increase the sensitivity of *C. elegans*
neurons to the effect of RNAi. These sensitizing mutant backgrounds were effectively used to perform whole genome RNAi-based screens for neuronal processes \{56, 57, 58 and 59\}. Several mutant backgrounds that increase RNAi sensitivity in neurons are now readily available at the \textit{C. elegans} Genetics Consortium. One such mutation is \textit{nre-1}, whose mechanism of action in unclear \{59\}. The other is \textit{sid-1}, a gene encoding part of the cellular RNAi import mechanism \{60\}. Since \textit{sid-1} is normally not expressed in neurons, ectopic expression of it (under the pan-neuronal \textit{unc-119} promoter) makes neurons sensitive to RNAi. I crossed these two enhancing background RNAi mutations (\textit{nre-1; lin-15B} and \textit{P unc-119:: sid-1}) with \textit{DglT3; nuls-5}, and I am in the process of analyzing these mutant combinations.

**EMS Mutagenesis**

Ethyl methyl sulfate (EMS) is a mutagenic compound that induces random mutations in genetic material by nucleotide substitution. These mutations typically fall in non-coding areas, primarily due to introns making up a large part of the genome. The mutagen causes a substitution or change of a single nucleotide base, resulting in a codon that produces a different amino acid. These modifications can have different effects on protein functions. Most point mutations can render proteins nonfunctional (\textit{loss-of-function (lf) mutation}), partially functional (\textit{reduction-of-function (rf) mutation}), or endow the protein with a novel function (\textit{gain-of-function (gf) mutation}). Other point mutations cause a nucleotide change that produces a codon for the same amino acid, and, therefore, these mutations do not change protein function at all. Alternatively the mutation might occur in areas that do not affect the protein’s configuration. However, genetically strong and important mutations will be identified because they show a strong change in phenotype. The technique of EMS mutagenesis has been very useful for genetic screens because of its effectiveness to generate different levels of genetic alternations in gene activity. Although this technique is very effective at generating genetic changes, it also has its classical drawbacks. One of the problems with this method is the high level of difficulty associated with
identifying phenotype-causing genetic variants. The second problem encountered with the use of EMS is the high level of mutagen efficiency; once the animal has been exposed to the mutagen, it most likely contains several EMS-induced background mutations in its genome [61 and 62].

**New method in sequencing makes EMS Mutagenesis more attractive**

Recent technological advances have made EMS mutagenesis screens an effective path to consider. This is due to the development of affordable services for whole genome sequencing (WGS), a technique that involves sequencing the whole genome and comparing it to a wild type or starting strain reference. In this approach, the use of positional mapping is no longer needed as the whole genome is sequenced, and the position of the mutation is identified with the aid of software [63 and 64]. The second problem of EMS can be resolved by doing several backcrosses of the mutant strain with the starting strain (Δglt-3; nuIs5) (as it may be different from the standard wild type, but does not contain the new phenotype producing mutations). This is done to get rid of background mutations that were randomly produced in the new strain but are not related to the phenotype of interest [65].
**Sorting interesting Mutants from the screen into different groups: Functional groups and Complementation groups**

Mutations can suppress or enhance neurodegeneration by a range of different mechanisms. However, some mutant effects are more important to us than others. For example, an overall reduction in Glu release is expected to reduce excitotoxicity, but given the evidence from clinical trials that overall suppression of Glu signaling is detrimental, reduction in Glu release is not high on our priority list. Instead, we want to focus on postsynaptic mechanisms that are specific to excitotoxic cell death. To that end, we would like to first sort the mutant strains into categories. The first step in identifying mutants with discernable changes in the level of neurodegeneration will be the visual identification necrotic-like structures present in the head of our mutant nematode strains and comparing it to our starting excitotoxicity strain. By doing this we can visually observe if the mutations have an effect on cell death i.e., increase the level of neurodegeneration (enhancer strains) or block/reduce neurodegeneration (suppressor strains). Since most mutations are loss-of-function, we expect most suppressor strains to carry loss-of-function mutations in death-promoting genes, and most enhancer strain to carry loss-of-function mutations in neuro-protective genes.

Once these mutants are identified via microscopy, the next step is to categorize these mutants into different complementation and functional groups. Categorizing these mutants into these groups gives us an understanding of how important these mutants are and where in the synaptic functional architecture these mutants are producing an effect. We divide our mutants according to their function: pre-synaptic (Type 1), peri-synaptic (Type 2), and post-synaptic (Type 3), mutations.
Type 1 mutants have alternations in the Presynaptic Cell

The first group of mutants falls into the category of presynaptic mutations; these mutations affect the overall packaging or release of neurotransmitters into the synaptic cleft. The overall mechanism of synaptic vesicle release is common to all small molecule neurotransmitter (e.g., Acetylcholine, Serotonin, and Dopamine): binding of the synaptic vesicle to the SNARE complex, and influx of calcium ions leads to vesicle fusion and neurotransmitter release (66). Since Glu is a compound found in all cells, the only type of enzyme that is necessary and sufficient to convert a neuron into a Glu-releasing one is the vesicular...
Glu transporters (vGluT) [67]. It should be noted that very large protein complexes are necessary for the overall process of synaptic vesicle release to take place, and a mutation in any one of these proteins could alter the overall release of neurotransmitters into the synaptic cleft. This category of mutations can manifest itself as either a suppressor mutation or an enhancer mutation. Suppressor mutations that affect the presynaptic cell can result in the reduction of neurotransmitter release, thereby limiting the overall release of glutamate into the synaptic cleft and activation of receptors. Enhancer mutations that affect the presynaptic cell might result in the increase in neurotransmitter release, these mutations can cause an increase in the level of neurodegeneration as the concentration of Glu in the synaptic cleft is increased. This increase in the level of neurodegeneration results in over activation of the receptors and increase in the level of excitotoxicity.

**Figure 8**

*Figure 8 A and B are a depiction of how suppressor and enhancer phenotypes can change the amount of readily release pool (RRP) of neurotransmitters in the presynaptic cell. Figure 6 A represents an enhancer mutation, while figure 6 B shows how a suppressor mutation would manifest itself in the synapse*
Using Genetic and pharmacological approaches to identify type-1 (presynaptic) mutants

Since the underlying mechanism for synaptic release is common to all neurotransmitters, we can quantify system wide changes in neurotransmitter release by monitoring acetylcholine (ACh) release at the neuromuscular junction (NMJ). In *C. elegans* this can be easily monitored by doing a drug assay. Normally when acetylcholine is released into the synaptic cleft, it binds to its receptor, and is rapidly degraded. Aldicarb is a drug that inhibits the enzyme acetylcholine esterase; thereby resulting in the buildup of ACh in the synaptic cleft and over activation of its receptors leading to paralysis in worms in a typical time frame. As shown in figure 9 mutants with enhance synaptic release will show faster rate of paralysis, while mutants with reduced synaptic release will show a slower rate of paralysis.
Figure 9a shows a representation of a wild type synapse and how the enzyme Acetylcholine esterase degrades ACh shortly after it is released from the pre-synaptic cell. 9b and b’) Wild-type animals are exposed to aldicarb and show paralysis after a certain time period. 9c and c’) mutant worms that show reduced neurotransmitter release are exposed to the drug and take longer time periods to paralyze when compared to a wild type strain. 9d and d’) mutant worms that show increased release of neurotransmitters are exposed to the drug and show reduced paralysis time when compared to Wild type.

With the large number of proteins involved in the unified synaptic release mechanism that is common to all NTs, we expect most type-1 mutations to affect ACh release as well. However, some mutations might specifically affect synaptic vesicle packing of Glu by vGluTs. Fortunately, a mutation in the vGluT eat-4 will affect more than the excitatory Glu synapses in the nerve ring. C. elegans also has Glu gated chloride channels (GluClβ and GluClα) [68], many of which are concentrated in the pharynx; Glu’s main function in the pharynx is to cause muscle relaxation in the pharynx muscle, and thus reduce the pharyngeal pumping rate. Glu is secreted from M3-neuronal cells in the pharynx onto these Glu gated chloride channels on the muscle. Once Glu binds to these receptors, it becomes active and chloride ions enter the cell. The reversal potential for chloride is -20mv, this acts as an inhibitory current to lower the activity of the cell. Mutations that specifically modify the release of Glu and change the concentration of
Glu in the synapses will also change the amount of Glu available to bind to these channels, resulting in a change of pharyngeal pumping rate. This phenotype can easily be detected by counting how many times the pharynx pumps within a specific period.

**Type 2 mutants: Perisynaptic**

The second group of mutations we call Peri-Synaptic mutations; these are mutations that affect the overall activity of Peri-synaptic proteins, such as changing the level of expression or activity of GluR on the post synaptic cells and GluT expressed close to Glu synapses. Mutations that affect the overall expression or activity of these GluTs can affect the overall concentration of glutamate in the synapse. Mutations that affect the expression, ER/Golgi maturation, trafficking to the cell surface or modulation (e.g., by phosphorylation) of receptors at the cell surface, can affect overall sum level of GluR activity. All these mutations can affect glutamate signaling in the synaptic cleft by altering either the number or activity level of GluRs or GluTs in the synapse, leading to a deviation from normal levels of Glu signaling. However, these changes will also affect normal Glu signaling, not only under excitotoxic conditions. The schematic below is a representation of this scenario.

Studies of GluR trafficking mutants demonstrate that even small changes in the level of activity of Glu synapses have clear effects on spontaneous and aversive behaviors mediated by the ASH-regulated circuit (69). Therefore, we can examine if mutants with reduced neurodegeneration achieved that by reducing the activity of Glu synapses. In that case the mutants will have modified levels of normal activity in Glu synapses, which we can test by monitoring behaviors known to be mediated by these cells, such as sensitivity to nose touch. This assay gives us an indication on the overall activity and expression of GluR in the synapse. The schematic below shows how we perform Glu signaling assay.
We can monitor overall activity of GluRs

Figure 10: The assay is performed by gluing an eye lash to a tooth pick and placing it in front of a moving worm (This assay is usually done on young adult worms, i.e., a little past the L4 stage), the worms senses the eyelash and a series of events which involve Glu already packaged into vesicles in the presynaptic cell, is released into the synaptic cleft. The binding of Glu to its receptors, ion influx and stimulation of command interneurons post-synaptically will tell the worm to go backward.

With this assay we can understand if our mutation affects Glu signaling by following the worm’s response to nose touch. An even more sensitive assay that detects small changes in GluR activity levels is spontaneous forward-backward mobility, again a behavior delicately regulated by the synapses that we study here. The level of nose touch response and spontaneous mobility is also affected by the mere survival and overall health of the command interneurons that are subjected to neurodegeneration in our study. This adds a potential complication in the interpretation of Glu activity assays. However, a mutation that suppresses neurodegeneration by a type-2 perisynaptic mechanism will enhance the survival of command interneurons while reducing Glu synaptic activity compared to the starting strain. In contrast, a post synaptic death suppressing effect (type-3) will enhance command interneuron
survival without reducing Glu synaptic effect (possibly even enhancing nose touch response and normal spontaneous mobility) compared to the starting strain. Therefore, a reduced neurodegeneration paired with reduced Glu activity suggest type-2 (perisynaptic) mutation, while reduced neurodegeneration paired with increased Glu activity suggests type-3 (postsynaptic) mutation. The second group of mutants (type 2 or peri-synaptic mutants) again will not be top priority for us as they suppress overall Glu signaling, while we want to focus on the postsynaptic death-specific signaling. Again, these mutations can definitely tell us important things about Glu signaling, and they do tell us that we are doing the screen correctly, but they will not be our first priority.

**Type 3 mutations are Post Synaptic**

Mutations in both the Presynaptic (Group 1) and Peri-Synaptic (Group2) categories do not give us an intricate insight into the underlying cause of cell death. The failure of Glu-antagonists during clinical trials demonstrated that an overall presynaptic or perisynaptic reduction in Glu signaling is not beneficial in the overall picture of excitotoxicity, urging us to focus on death-promoting signaling events that occur in the post-synaptic cell as a possible remedy for excitotoxicity. The next categories of mutations to which we will devote most of our effort are mutations that have their effect postsynaptically and affect the activity of the cell death pathway after ion influx. The mutants that fit into this category are very important to us as they affect cell death after large amount of ion influx, a situation that we observe during excitotoxicity. However, although mutations that occur post synaptically are important, it should be noted that not all of the mutations that fall into this category are of equal importance. One such example would be mutations that reduce the effect of the sensitizing background, where GalphaS* is expressed in the post synaptic neuron. While a loss of function (lf) mutation in GalphaS* is unlikely (since it is expressed from a multi copy array), a mutation in type-IX
adenylyl cyclase acy-1 (which is often identified in genetic screens as a suppressor of GαS*) would block all cell death in these animals, without being Glu-specific (70 and 71). We will monitor for the presence of these mutations by checking the sequence of acy-1. The figure below shows the cell death pathway that plays a crucial role in our mutant strain (Δglt-3;nuIs5).

**Figure 11**

![Figure 11](image)

Figure 11: The schematic above is a depiction of the cell death pathway that involves our sensitized background and is crucial for nematode excitotoxicity. Mutations that result in the loss of function (lf) of key proteins along this pathway will either block or reduce the level of excitotoxic cell death.

The mutants to which we will devote most of our attention will have normal Glu signaling (show no changes in response to nose touch or spontaneous mobility) and show normal paralysis time when tested for synaptic release. I will identify the genes that have these mutations by using whole-genome sequencing.
Materials and Methods

General Methods: *C. elegans* strains were grown and maintained on standard MYOB plates containing OP50 *Escherichia coli* bacteria at 20\(^\circ\) obtained from our lab. All strains used were obtained from the *C. elegans* Consortium and were available to us Δglt-3: Δglt3 (Δbz34)/nls (v), wild type (N2 var Bristol), ppk-1 (ok1411), glr-1 (ky176), *P*\(_{glr-1}\):: rfp transgene was obtained from Chris Rongo's lab, eat-4 (ky5).

Synchronization

20 ul of bleach solution was added onto an agar plate, using a 20-200 ul pipette without OP50. Gravid adult worms of our excitotoxicity strain (glt-3; nls5) were placed into the solution to release their eggs. Hatched worms were then transferred to standard MYOB plates with OP50 bacteria and incubated at 20 degrees for a 1 day period to arrest animals at L1 [72].

Mutagenesis

EMS is most effective at the time of generation of gametes, which occurs at the L4 stage of the parental *P*\(_0\). Once synchronized L4 worms were obtained, the plates were washed with 1ml of M9 solution into a 16mL conical tube and 10 ul of EMS was added to bring the final concentration to 50mM. The *P*\(_0\) animals were exposed to EMS by swirling the tube at room temperature for 3 hours. When this was finished M9 was used to wash the *P*\(_0\) animals 3 times, carefully removing the top solution. The *P*\(_0\) animals were then placed on 7 seeded plates with OP50 for 24 hours to allow them develop eggs carrying mutagenized chromosomes. The *P*\(_0\) animals were again bleached to obtain their F1 mutagenized eggs, eggs were
allowed to hatch, and F1 mutagenized animals were arrested on a plate without food. These F1 animals were allowed to hatch over a 2 day period before they were transferred on to individual plates with food {62}. These F1 single animals self-fertilized and generated F2 animals that were put on individual plates and screened for the presence of a subpopulation of animals with reduced or enhanced neurodegeneration (as would be expected of a recessive mutation).

**RNAi**

This is being done as a side project, and will therefore not be described in detail here. RNAi protocol was followed as described by the Ewald et al. {72}. I am currently crossing our excitotoxic strain *glt-3; nul5* with worms that have an RNAi sensitive background *P*unc-119::SID-1*{60} and *nre-1;lin-15B*{59}.

**Behavior Analysis**

Nematode locomotion and worm paralysis by aldicarb were performed blindly, using standard protocol for behavior analysis. For Glu regulated forward and backward mobility we followed the protocol from V. Maricq’s lab (U. of Utah). For aldicarb assay we followed Caldwell’s lab protocol (U. of Alabama) {73}. Standard MYOB plates were soaked with aldicarb and allowed to dry, bringing the final concentration to 0.5mM. After the plates were dry, a spot of OP50 was placed in the center making it easier to score the animals as they become paralyzed. 25 freshly picked young adult worms from each strain were placed onto each plate. The percentage of paralyzed worms was scored every 10 minutes for a total of 1 hour. Each experiment was repeated 4 times.
Analysis of Neurodegeneration

We observed the extent of necrotic cell death in strains that express Gαs* by observing free-moving animals with an inverted scope under Nomaski Differential Interference Contrast (DIC). These animals were observed with no anesthetics on agar chunks with fresh nematode culture flipped over on the side. We scanned through the animals on the agar chunk, and in each animal we identified its developmental stage and counted degenerating neurons. Swollen cells in the nerve ring area were counted as head necrotic figures, representative of neurodegeneration [52]. In some cases we verified the identity of these swollen cells as glr-1expressing command interneurons by viewing Green Fluorescence Protein (GFP) expression (indicative that nuls is functioning in these neurons since Gαs* and GFP are both expressed from the glr-1 promoter). ~50 animals for each developmental stage (L1-adult) were scored for the level of neurodegeneration. Statistical significance between control groups and experimental groups at L3 (typically the stage with highest levels of neurodegeneration) were analyzed using t-test score.

Fluorescence Microscopy

To identify possible changes in the level of expression of the key GluR subunit GLR-1, mutant strains were crossed with animals carrying an integrated construct of P_{glr-1}GLR-1::RFP [74]. From the F2 progeny of this cross we re-isolated glt-3; nuls5 homozygous animals, and continued sib-selection to identify animals that show consistently reduced neurodegeneration (indicating they are also homozygous to the unidentified suppressor of neurodegeneration). Mutant animals that have P_{glr-1}:: rfp transgene were mounted on a coverslip that had a drop of sodium azide (a compound that paralyses worm after a few seconds of exposure), and observed on Nomaski Differential Interference Contrast (DIC) with
epifluorescence  optics. Wild-type worms that expressed the \textit{rfp} transgene were scored as 100 percent fluorescence, and \textit{rfp} in other worms with smaller expression as less than 100 percent.

**Pharyngeal Pumping analysis**

I performed this assay as follows and used the protocol from previous studies \textsuperscript{50}

Adult worms are placed on seeded plates and allowed to lay eggs and shortly after, the adult worms are removed from the plate. The plates are then incubated at 20 degree Celsius for 3 days to synchronize all the animals. Adult worms are transferred onto freshly seeded plates and were allowed to move around the plate for 1 hour. After 1 hour the numbers of pulses are counted every 30 seconds under a dissecting microscope. The counts are repeated for 7 sessions with a total of 10 animals per session.
**Results**

Classic EMS mutagenesis protocols (62) generate 1 strong loss-of-function (lf) mutation every 2,000-10,000 genes. With ~20,000 genes/genome, and 2 genomes/animal, we expect ~5-20 mutations in each F1 mutagenized animal. Random distribution of mutations suggests that 99% of the genes will have at least one mutation if there is an average of 4.6 mutations/gene. With ~20,000 genes in the genome, this requires us to obtain ~100,000 mutations to saturate the genome. With 5-20 strong mutations per animal, we will need to screen 5,000-20,000 F1 mutant animals to saturate the genome with at least 1 mutation in every gene in a P=0.99. At this stage I therefore performed only a pilot screen (using 1,000 animals, statistically covering at most ~50% of the genes). I used our excitotoxicity strain *glt-3; nul5* as my starting strain. After screening 1000 mutagenized F1 worms with our excitotoxicity background (*glt-3; nul5*), we obtained several mutants with different phenotypes. The primary way through which we select mutants that are important to us is to visibly observe the changes in the level of Neurodegeneration and compare them to our starting strain (*glt-3; nul5*). I observed all the mutant strains that I obtained from doing EMS, and noted mutants that showed no change or increased or decreased levels of neurodegeneration when compared to our starting strain. Mutants that showed increased levels of excitotoxicity levels were not our immediate highest priority. As knockout mutations in genes that induce neuro-protection and are activated by Glu (e.g., DAF-16 and CREB) can result in exacerbated levels of neurodegeneration. Enhancer mutations are very important, but they are less likely to represent a direct trigger of excitotoxicity, and are, therefore, not as high priority to us as suppressor mutations.
The graph below shows mutants strains that were obtained from our EMS screen.

Figure 12

![Bar chart showing mutant strains in comparison to starting strain](image)

Figure 12: The bar chart above shows the level of neurodegeneration of important strains obtained from EMS in comparison with our Excitotoxic model (\(\Delta glt\text{-}3;\ nuls5\)) labelled as the starting strain.
The horizontal categories show the developmental stages of the worms, while the vertical axis shows the average number of neurons dying per head of the animal. A key note: we mainly care about neurodegeneration at L3 during development because at this stage all the glutamatergic synapses mature and neuronal Glu signaling is prominent. The signaling peaks at L3 and remains the same throughout the remaining stages of development (L4 and Adult). Although we observe a decrease in the number of vacuole like structures later on in development, this is primarily because the necrotic corpses are being cleared faster than they are being produced. As can be seen in the figure above, we have isolated 5 suppressor strains and 2 Enhancer strains.

8 EMS strains that show interesting phenotypes have been isolated based on their change in neurodegeneration

I obtained a total of 8 excitotoxicity mutants; 5 of them showed suppressor phenotypes, one of them shows no neurodegeneration at all and 2 of them show an enhancer phenotype. All the strains were tested for normal Glu signaling and aldicarb sensitivity.
The strongest enhancer strain that showed up on our screen is IMN8 (cny-8; glt-3; nuls5) as it shows significant increase in neurodegeneration at all stages. Figure 13 is a comparison of neurodegeneration levels between our mutant strain IMN8 and our starting strain Δglt-3; nuls5.

Figure 13: The bar graph in red depicts our enhancer mutant strain obtained from EMS (IMN8), while our starting strain (Δglt-3; nuls5) highlighted in purple. At L3 both strains only show a significant difference in neurodegeneration level.
Figure 14: Aldicarb analysis of our Enhancer mutant shows a defect in synaptic release (Quicker paralysis time); indicating that the phenotype observed may be as a result of a type 1 mutation.

Fig. 14 presents the Aldicarb assay for this mutant, showing the IMN8 is defective on Synaptic release, pointing to increased aldicarb sensitivity (short time to paralysis), suggesting an increased level of neurotransmitter being released into the synaptic cleft.
Fig. 15 presents preliminary data on nose touch assays. It also shows no nose touch sensitivity in IMN8 animals, suggesting that Glu signaling is defective in this strain, as mutant worms do not respond to this assay. The nose touch assay suggests that Glu receptor activity/expression may be affected by the cny-8 mutation.

Therefore, IMN8 exhibits increased neurodegeneration, increased synaptic release, and decreased function of Glu synapses. This combination is somewhat surprising; however, it is reminiscent of the phenotypes observed for glt-3 KO itself, where excess synaptic Glu causes increased neurodegeneration (compared to nuls5), but reduced nose touch sensitivity due to the lingering effect of synaptic Glu. To test this idea we will perform additional tests such as mobility assays. At the moment, the significance of this strain remains unclear.

Figure 15: The graph above shows that IMN8 is not responsive to nose touch when compared to our starting strain and mutants that are defective for GluR expression. This indicates that there is a mutation in Glu mediated behavioral circuitry (i.e a class 2 mutation).
IMN5: cny-5; glt-3; nuls5

Figure 16: Comparison between our starting strain (Δglt3; nuls5) and our mutant strain IMN5 show similar levels of neurodegeneration at L3. Although the difference is not statistically significant it was still treated as an enhancer mutation.

Fig. 16 demonstrates that IMN5 shows somewhat increased levels of neurodegeneration during L3 larval stage. We next tested aldicarb-sensitive release and Glu signaling in this strain.
Figure 17: The plot above depicts our enhancer strain IMN5 when exposed to aldicarb. As seen above IMN5 paralyzes relatively quicker than our sensitive control, indicating an increase in synaptic release. This tells us that our enhancer mutation may be as a result of an increase in synaptic release.

Fig. 17 presents the aldicarb data for IMN5, and shows that our mutant has increased rate of paralysis, suggesting excessive synaptic release.

Aldicarb data for IMN5 show that our mutant has excessive synaptic release (similar to IMN8) and points to a type 1 mutation being the result of the slight increase in neurodegeneration levels.
Figure 18: Spontaneous mobility was performed for IMN5 and the results show that Glu signaling is affected in this mutant, indicating a mutation in behavioral circuitry, and possibly a type 2 mutation.

The spontaneous mobility assay presented in Fig. 18 shows longer forward runs in IMN5, which is in line with reduced Glu signaling. This is somewhat surprising in light of the (slightly) increased levels of neurodegeneration and increase in synaptic release in this mutant. Although these are different assays, it is interesting to compare Glu activity in IMN8 and IMN5. Both strains have increased neurodegeneration, increased synaptic release, and reduced Glu signaling. However, in IMN8 Glu signaling is lower than that of glr-1 mutants (fig 15), while Glu signaling in IMN5 is midway between WT and glr-1 mutants.
Suppressor Phenotypes

**IMN7:cny-7;glt-3;nuIs5**

Figure 19: The graph above is comparing the overall level of neurodegeneration of our suppressor strain (IMN7) with our starting strain ($\Delta$glt-3; nuIs5). We see a significant decrease in the level of neurodegeneration at L3.
Fig. 19 shows significant decrease in the level of neurodegeneration in IMN7 at all stages including L3. This strain has the lowest levels of neurodegeneration amongst all suppressor strains isolated via EMS. Unfortunately, it had a viability problem and could not be maintained and studied further. This strain had a developmental defect that caused 70% percent of the worms to not hatch and reach adulthood. Eventually the strain was not viable and the data are preliminary as only 30 worms could be screened for neurodegeneration.

**IMN6:** \textit{cny-6;glt-3;nuIs5}

Fig. 20 shows significant decrease in the level of neurodegeneration in IMN6 at L3.

**Figure 20**

![Graph](image)

Figure 20: The graph above compared our suppressor strain (IMN6) in comparison with the starting strain (\textit{Δglt-3; nuIs5}). Significant reduction of neurodegeneration was observed at L3, with the starting strain showing on average about 3.7 necrotic vacuoles per head and the suppressor strain showing on average 1.7 vacuoles per head.
Figure 21: Aldicarb assay on IMN6 shows a defect in synaptic release. IMN6 appears resistant to Aldicarb when compared with our sensitive Aldicarb strain \textit{ppk-1}, indicating synaptic release is decreased in this mutant strain, hinting at a type 1 mutation.

As seen in Fig. 21, Aldicarb data for IMN6 shows that these animals are relatively insensitive to paralysis, suggesting that synaptic release is reduced in this mutant.
As seen in fig 22, the nose touch sensitivity of IMN6 is decreased suggesting a decreased activity of Glu synapses (although the survival of command interneurons is probably increased).

Put together, the phenotype of IMN6 includes reduced neurodegeneration, reduced synaptic release, and reduced activity of glutamatergic synapses. This combination of phenotypes can be explained by reduced synaptic release leading to reduced synaptic Glu activity and reduced excitotoxicity (type 1).
**IMN4:cny-4;glt-3;nuIs5**

The fourth suppressor mutant that I identified during my screen is IMN4, another mutant that showed significant reduction in neurodegeneration at L3 (Fig. 23).

**Figure 23**

Figure 23 shows the neurodegeneration levels of the suppressor strain IMN4; this mutant shows significant reduction in neurodegeneration at L3 with the mutant strain showing on average 1.7 neurons dying per head of the animal and 3.8 neurons dying per head in our starting strain (glt-3; nuIs5).

Since this mutant showed an interesting phenotype at L3 the next step was to perform Synaptic release and Glu signaling assays to categorize where this mutation fell in our outline (Synaptic, pre-synaptic and post-synaptic). Preliminary data from aldicarb assays was not conclusive but showed a
change in the level of synaptic release, indicating that our mutation affects neurotransmitter release and Glu receptor activity.

**Figure 24**

![Glu signaling results for IMN4](image)

Figure 24 shows the comparison between IMN4 and our starting strain (\textit{glt-3;nuIs5}) and strain that are mutant for Glu signaling. It can be seen from the graph that IMN4 is mutant for Glu signaling, and this could be the cause of the reduction of neurodegeneration phenotype.

Fig. 24 shows that nose touch assay for IMN4, indicating a reduced sensitivity to nose touch and suggesting a reduced Glu synaptic activity. The data obtained from Glu signaling assay shows that our mutant phenotype affects Glu signaling, and may be a type 2 mutation that affects the overall activity/expression of Glu receptors on the post synaptic cell. Although we see a significant decrease in the level of neurodegeneration at L3, this phenotype can be attributed to the change in the reduced concentration or activity of Glu receptors in these mutants. Preliminary data from aldicarb assays also
show a change in the level of synaptic release, indicating that our mutation affects neurotransmitter release and Glu receptor activity.

**IMN3: cny-3; glt-3; nuls5**

As seen in Fig. 25, another suppressor mutant is IMN3, as it shows significant decrease in the level of neurodegeneration throughout development (L1 - adult). Although this mutant looks interesting, it would have to be normal when tested for Glu signaling and synaptic release.

Figure 25

![Figure 25](image)

Figure 25, shows another suppressor that was isolated from my EMS screen and it shows significant reduction in the level of neurodegeneration at L3 with 1.2 neurons dying per head of the animal in our mutant strain (IMN3) and 3.7 neurons dying per head of the animal in our starting strain (glt-3; nuls5).
I next exposed these worms to aldicarb, to see if the synaptic release was affected. The result of this assay is shown below.

**Figure 26**

![Aldicarb assay for EMS mutant strains](image)

Figure 26 depicts the aldicarb data for my EMS mutant IMN3, the graph tells us that IM3 has a mutation in synaptic release due to its similarity to our control (*ppk-1*), which is very sensitive to Aldicarb and paralyzes relatively quickly when its exposed to aldicarb.

Fig. 26 indicates that IMN3 has an increased rate of paralysis in the aldicarb assay, suggesting that IMN3 may have a mutation that results in increased neurotransmitter release. We next performed Nose touch assay to see if this mutant is defective in Glu signaling.
Fig. 27 shows that the nose touch sensitivity of IMN3 is reduced, suggesting a reduced activity of Glu synapses. The combination of phenotypes for IMN3 is therefore surprising: reduced neurodegeneration, increased synaptic release, and reduced Glu responses. We therefore decided to test this strain further to ensure that we were performing Glu signaling assays correctly, and the results obtained were an indication of changes in either expression/activity of GluRs. I made a cross between my mutant and another strain that had an extra chromosomal reporter fused with the promoter of Glr-1 \((P_{glr-1}:: rfp)\) \(^{74}\). This reporter labels Glu receptor expression along the pharynx, allowing visual identification of changes in expression of these reporters. Fig. 28 shows the reporter expression in wild type and our mutant IMN3.
Figure 28 shows the wild type strain with the transgene $P_{glr-1}::rfp$ on the left and our mutant strain IMN3; $P_{glr-1}::rfp$ on the right.

Figure 28 shows the overall expression of GLR-1::RFP in the wild type worm and our mutant EMS strain. Our EMS mutant strain shows 50% decrease in the overall expression of GLR-1 in the nerve ring. We therefore conclude that the reduced neurodegeneration in IMN3 is likely due to reduced Glu receptor expression, while the reason for the increased synaptic release remains obscure. Nonetheless we classify IMN3 as a class 2 mutation.
**IMN2: cny-2; glt-3; nuls5**

As seen in fig 29, IMN2 shows steep decline in the levels of excitotoxicity. In combination with other assays (see below), we suggest that this mutant is a leading candidate with top priority because it shows reduced neurodegeneration throughout development in comparison to the starting strain while not reducing it below the level of neurodegeneration observed in *nuls5* (where *acy-1* mediates Glu-independent necrosis).

Figure 29

Figure 29 shows another suppressor strain that shows significant levels of neurodegeneration.
Fig. 30 presents the aldicarb assay for this mutant. These mutants show interesting results as they show similar paralysis time to our starting strain ($\Delta glt$-3; $nuls5$). Since this was the first mutant to show similar paralysis time to our starting strain, I used spontaneous mobility to test for Glu signaling. Since this assay is a more sensitive, I decided to use it and see if the mutant will show any changes in Glu signaling.
Fig. 31 shows that the spontaneous mobility of IMN2 animals is somewhat tilted towards forward mobility, suggesting reduced Glu activity. This mutant might therefore correspond to a type 2 mutation (peri-synaptic).

**Experimental Concerns**

Although IMN2 is a very good candidate for whole genome sequencing, one major issue with our assay had to be dealt with. Although our aldicarb assay has shown no change in overall mechanism of synaptic release, it does not tell us if there is a specific defect in Glu release (e.g., due to a mutation in eat-4). To address this concern, we know from previous findings that Glu is used to control pharyngeal pumping in *C. elegans*. Pharyngeal muscle contraction is regulated by glutamate gated chloride channels, and mutations in eat-4, were shown to affect muscle contraction in the anterior pharynx (39). In *C. elegans*,

![Spontaneous Mobility for IMN2](image)
**eat-4** is responsible for the packaging of Glu into vesicles via a proton exchange mechanism, and is important for regulating a variety of processes in the synapse. M3 cells in the pharynx use Glu as their neurotransmitter, and these receptors mediate pharyngeal pumping by using Chloride channels expressed by AVR-15[75]. Once the receptor is activated by Glutamate, chloride ions move into the cell and causes hyperpolarization. We used this study to our advantage, and tested if our IMN2 mutation showed a phenotype as a result of an **eat-4** mutation. Protocol for pharyngeal pumping is described in the methods section.

**Figure 32**

![Pharyngeal Pumping assay for IMN2](image)

Figure 32: Pharyngeal pumping appears normal in IMN2 mutant worms, indicating **eat-4** function is normal.
We can conclude that the reduction in Neurodegeneration phenotype is not as a result of a mutation in *eat-4*, as the pumping rate of our mutant is not different statistically from the starting strain. This makes IMN2 a very important candidate for sequencing, and an impressive mutant.

**IMN1:cny-1;glt-3;nuIs5**

This strain does not show any necrotic vacuole-like structures in the nerve ring, indicating that the mutation is affecting the cell death pathway dramatically. However, the extensive effect also suppresses our background neurodegeneration observed in *nuIs5* which is independent of Glu. Therefore this mutation may not be our top priority.

Aldicarb and Glu signaling assay results are shown below.

Figure 33

![Aldicarb assay for EMS mutant strains](image)

Figure 33: IMN1 shows similar paralysis time to our starting strain, indicating synaptic release is normal
As shown in Fig. 33 Aldicarb results show that synaptic release is normal in terms of synaptic release.

Figure 34

**Glu signaling results for IMN1**

![Glu signaling assay for IMN1](image)

Figure 34: Glu signaling assay for IMN1 show similar results to our starting strain, indicating Glu signaling is normal.

Figure 34 shows Glu signaling assay for IMN1. However, the complete lack of neurodegeneration is similar to that seen in acy-1 mutants. Therefore, although this mutant shows some interesting results, a follow up of possible mutations in the cell death pathway is required before further conclusions can be made.
A tabular representation of all assays performed on mutant candidates is shown below.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Excitotoxic Level when compared to starting ($\Delta$glt-3;nuIs5)</th>
<th>Synaptic release (Aldicarb sensitivity)</th>
<th>Glutamate Signaling (Nose touch and forward backward mobility)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMN1</td>
<td>None</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>IMN2</td>
<td>Reduced</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>IMN3</td>
<td>Reduced</td>
<td>Increased</td>
<td>Reduced</td>
</tr>
<tr>
<td>IMN4</td>
<td>Reduced</td>
<td>Increased</td>
<td>Reduced</td>
</tr>
<tr>
<td>IMN5</td>
<td>Increased</td>
<td>Increased</td>
<td>Normal</td>
</tr>
<tr>
<td>IMN6</td>
<td>Reduced</td>
<td>Reduced</td>
<td>Reduced</td>
</tr>
<tr>
<td>IMN7</td>
<td>Reduced</td>
<td>Could not be performed</td>
<td>Could not be performed</td>
</tr>
<tr>
<td>IMN8</td>
<td>Increased</td>
<td>Increased</td>
<td>Reduced</td>
</tr>
</tbody>
</table>
Discussion

Genetic screens are the cornerstone of unbiased research, and have been used to understand different pathways that regulate a variety of processes in *Drosophila*, yeast and *C. elegans*. To study the process of excitotoxicity, we created a nematode model that has a knockout of *glt-3* in combination with a sensitive background GalphaS (GαS*) and performed a genetic screen on this strain. The main goal for using a genetic screen was to obtain mutants that showed an overall decrease in neurodegeneration, and were having an effect in the post-synaptic cell. We used mutagenesis to induce random mutations in the genome of our construct, and screen 1000 worms for phenotypes that show a change in the levels of excitotoxicity.

We have identified 8 mutants; 6 of them show suppressor phenotypes, and 2 show an Enhancer phenotype. Once the mutants were identified; they were classified into different functional groups to have an understanding of where in the synapse our mutations were having an effect. We tested this by performing synaptic release assays using the drug aldicarb, and mutants that showed a defective phenotype were grouped according to their corresponding class of mutations e.g. Class 1. Mutants were also tested for Glu signaling using nose touch and forward and backward mobility tests, to see if mutants were defective in Glu receptor expression. We also identified a mutant that showed no neurodegeneration, but have an idea that this mutation is affecting the cell death pathway post synaptically independent of Glu and is not important to us. We did identify 2 mutants that showed interesting phenotypes; IMN8 and IMN2 are interesting candidates. IMN8 is defective in synaptic release and Glu signaling, but still shows increased levels of neurodegeneration. This mutation could result in phenotypes that could compensate each other, and may seem like type and type 2 mutations but may
not be the case. Mutations that result in increased Glu release could over stimulate the GluRs and result in reduced Glu signaling because these receptors become desensitized and unavailable to new stimulation. The other possibility that could occur would be, the increase in Glu release from the pre-synaptic cell could be a compensatory mechanism for the lack of Glu receptors on the post synaptic cell. These behaviors have been observed in synapses, where receptor expression were reduced due to mutations or developmental defects, hinting at this also occurring in our mutant strains that showed increased neurotransmitter release and reduced response to nose touch. We will pinpoint the molecular basis of this mutation by genetic mapping and then doing whole genome sequencing.

IMN2 is our suppressor mutant that is normal for synaptic release, and Glu signaling while showing a decrease in neurodegeneration. This mutant is very important to us as we have a mutant strain that shows reduced neurodegeneration and is normal for all assays.

Although we have two mutants that show interesting phenotypes, the other mutants we screened are not useless. They were very informative and tell us that we are doing the screen correctly because we expect mutations in certain areas to affect synaptic release and Glu receptor expression, and we see these mutations in some mutants. Furthermore we could collaborate with research groups that specialize in the study of neurotransmitter release or GluR expression.
**Future Experiments**

Since we have two mutants that show interesting phenotype from my screen, the next steps for EMS mutants will be:

a) Backcrossing these mutants with our starting strain ($\Delta$glt-3; nuIs5) and re-isolating our mutation to remove any other mutations in the background.

b) Sending both strains to sequencing once they are backcrossed

c) Positional mapping with SNP can also be done on these mutants by crossing IMN2 and IMN8 to a Hawaiian background strain that has our excitotoxicity setup

d) RNAi to knockdown specific genes in our sensitized background strain and observe which gene has a pronounced effect on neurodegeneration.

e) Identifying if the mutation in IMN-1 is caused by a knockout of AC-9 by PCR and sequencing.

f) Using Differential Interference Contrast with epiflourescence optics to observe surviving neurons in the RIG area of our mutant strain IMN2.

g) Classifying mutants with similar phenotypes into complementation groups. E.g., IMN8 and IMN5 show similar phenotypes, and may be allelic to each other. Crossing these two strains together and seeing if they complement or not will tell us if these mutations are present on the same or different genes.
Conclusion

Using a genetic screen to understand key steps in a pathway grants us an unbiased approach to understanding central mediators that are involved in these pathways. Although this method has its drawbacks, its insight into key processes cannot be undermined. We used genetic screens to induce random mutations in the nematode worms that have Excitotoxic-like conditions in the genome, and have identified several strains that have a change in their levels of excitotoxicity. These mutants were tested for changes in neurotransmitter release by using the drug aldicarb (An acetyl cholinesterase inhibitor) and Glu signaling by doing Nose touch and Spontaneous mobility. Mutants that showed defects in these areas were classified into functional groups based on the phenotypes that were observed from the assays. Only mutations that did not affect the presynaptic architecture and still showed reduced levels of excitotoxicity were given top priority. Future understanding of these mutants will be very important for understanding key processes in nematode excitotoxicity, and provide insight into similar processes happening in mammals.
References


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