

# Seizing the Role of Vesicle Targeting in Epilepsy Via CarpeDB and Roundworms

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## Abstract

Genes throughout development govern synaptic transmission and, thus, neuronal excitability in both roundworms and humans. Notably, malfunction of synaptic vesicle targeting through the absence of essential regulatory proteins often leads to neurological dysfunction. Experimental results from our laboratory demonstrate that mutations or RNA interference (RNAi) against synaptic vesicle targeting proteins DHC-1 (dynein heavy chain), LIS-1, SNB-1 (synaptobrevin), and UNC-104 (kinesin) greatly increase a susceptibility to epileptic-like convulsions in the roundworm, *C. elegans*, by disrupting neurotransmission of GABA, the principal inhibitory transmitter of the central nervous system. Using bioinformation from our comprehensive database on epilepsy genetics, CarpeDB, and RNAi-mediated convulsion analysis with an antagonist of GABAergic reception, pentylenetetrazole (PTZ), we have accelerated the characterization of *C. elegans* synaptic vesicle targeting proteins that share significant human homologies (e.g. cytoskeletal motors, rabs, and SNAREs). These results help establish *C. elegans* as a model system for discovering new genes with implications for epilepsy.

## Introduction

Epilepsy ranks as the third most common neurological disorder, affecting fifty million people worldwide, and is characterized by paroxysmal electrical disturbances of the brain that are manifested as seizures. Epilepsy is often etiologically classified into two major categories: symptomatic and idiopathic. Symptomatic epilepsy is of known cause and is often typified by damage to the central nervous system. Conversely, idiopathic epilepsy lacks an identifiable cause and is believed to result from both environmental and, most notably, genetic factors (1).

Thus far, much research into the molecular basis of epilepsy, as well as closely related

disorders like Alzheimer's disease, dystonia, lissencephaly, and tuberous sclerosis, has led to the identification of numerous genes implicated in this disorder. For example, gene products such as ion channels, molecular chaperones, synaptic vesicle components, and transcription factors have been shown to influence seizure susceptibility. However, despite countless hours of research, scores of "epilepsy genes" have not yet been isolated (2, 3).

Much of the difficulty surrounding the identification and characterization of epilepsy genes is intimately associated with prevalent experimental methods. That is, most epilepsy researchers have chosen to limit their studies to mammalian organisms, such as *Mus musculus* (the mouse) and *Rattus norvegicus* (the rat), which exemplify nervous system complexities quite similar to those of humans. Unfortunately, this complexity substantially hinders screening for epilepsy genes, as it necessitates costly and inefficient procedures (3).

Quite possibly, an absence of sufficient data compilation has also impeded the search for novel genetic factors influencing susceptibility to epilepsy. Although multiple Web-based resources, such as NCBI, the Gene Ontology Consortium, the Human Gene Mutation Database (HGMD), Pfam, Uniprot, FlyBase, Mouse Genome Informatics, Rat Genome Database, and Wormbase offer valuable information that may be applied toward epilepsy genetics, those sites frequently truncate their information and disallow routine submission of nascent data from users. Furthermore, none of the aforementioned resources is comprehensive with each resource containing only fragments of pertinent information on specific genes and their epileptic roles. Consequently, these resources are recalcitrant to the specificity and autonomy for data submission or retrieval ideally desired for the acceleration of epilepsy research.

Accordingly, we have utilized the aforementioned resources to establish a publicly accessible, dynamic Web site on epilepsy genetics.

CarpeDB comprises all known genes, both in humans and animal model systems, which have been directly implicated in seizure susceptibility. Moreover, this resource provides researchers with abstracts and hyperlinks to accompanying full-text articles, thus validating the incorporation of specific genes into the database. Presently, CarpeDB serves as the only available Web-based resource designed specifically for epilepsy genetics (2).

It is our hope that CarpeDB will soon include many new updates linking specific genes to epilepsy, because we have established a simple animal model, a roundworm, for studying epilepsy's molecular basis. The utilization of this model system, classified as *Caenorhabditis elegans*, is based on our past identification and functional analysis of a *C. elegans* homologue of a human disease gene, *LISI*. The gene product of *LISI* is important in regulating microtubule-associated targeting of synaptic vesicles, which contain neurotransmitters, (e.g. GABA, the principal inhibitory neurotransmitter of the central nervous system) and has been linked to classical lissencephaly. The chief *LISI*-associated form of classical lissencephaly is Miller-Dieker syndrome (MDS), a neurological disorder characterized by profound mental retardation and, most notably, aggressive epileptic convulsions leading to short lifespan. Similarly, the roundworm *LISI* homologue, termed *lis-1*, also seems to mediate synaptic vesicle targeting of the inhibitory neurotransmitter GABA. More specifically, we showed that a mutant strain of *lis-1*, named *pnm-1*, exhibits presynaptic bunching of synaptic vesicles containing GABA. Other *C. elegans* homologues of human genes with roles in the same cellular pathways as *LISI* have analogously been shown to disrupt synaptic vesicle targeting when mutated or silenced by RNA interference (RNAi). Such silenced or mutated homologues, like *dhc-1* (dynein heavy chain), *snb-1* (synaptobrevin), and *unc-104* (kinesin), yield presynaptic bunching of GABA-containing synaptic vesicles in *C. elegans*, as well. Although these data have many implications, our most exciting discovery could be that presynaptic bunching of inhibitory synaptic vesicles via aberrant *LIS-1* pathway function may contribute to the generation of epileptic-like convulsions, which mimic those convulsions of MDS patients (3).

We believe that our model system, *C. elegans*, will prove to be an exceptionally advantageous tool for the epilepsy research community. This roundworm is microscopic, transparent, and genetically tractable. In addition, *C. elegans* has a short generation time of only three days and a life expectancy of fourteen to seventeen days, making it relatively efficient for experimentation. The roundworm also shares about 50% of its genome with humans and has only 302 neurons (compared to humans with  $10^{11}$  neurons), which have essentially all of the fundamental nervous system components (i.e. ion channels, neurotransmitters, neurotransmitter receptors, transporters, and axon guidance cues) that would be critical for translational epilepsy research with model organisms. Another notable application of the roundworm to epilepsy research is its responsiveness to RNAi feeding. RNAi feeding is a gene silencing technique in which bacteria containing double-stranded RNA molecules with the capacity to block translation of complementary mRNAs to proteins are ingested by *C. elegans*. As a result, the biological function of a possible epilepsy gene, obtained through RNAi-mediated genetic screening, can be determined in a matter of days without much cost (3). Finally, *C. elegans* is unique when compared to other animal models of human disease, because elaborate bioinformatics resources are specific to it. For example, the expression profile for every gene in the *C. elegans* genome has been constructed and made freely accessible online (4). Likewise, thousands of protein-protein interactions in the *C. elegans* proteome have been elucidated and are also available free-of-charge through the Internet (5). These facts, coupled with the array of complimentary *C. elegans* mutant strains assembled over forty years of research and obtainable from the Caenorhabditis Genetics Center, as well as the union of our previous experimental results, should facilitate the development of therapeutics. Perhaps, our research makes a cure for epilepsy even more attainable, giving hope to the millions confined by this disorder.

Indeed, we hold that our research will assist in the understanding of the various roles that genes play in regulating neuronal excitability and, thus, epilepsy. CarpeDB now serves as an impetus for

our research by indirectly providing us with a collection of potential epilepsy gene targets. Because some synaptic vesicle targeting dysfunction in *C. elegans* has been associated with susceptibility to epileptic-like convulsions (3), we decided to search CarpeDB for other synaptic vesicle targeting genes that might alter neuronal excitability in the roundworm. For representative synaptic vesicle targeting genes, which are important for movement, docking, tethering, or fusion of synaptic vesicles, we chose to examine cytoskeletal interactors (*i.e.* putative *LIS-1* pathway members), rabs, SNAREs, and synaptic vesicle recycling components. Upon ascertaining that convulsions mimicking those of human epilepsy patients do in fact result from mutations or RNAi knockdown of roundworm homologues of such synaptic vesicle targeting genes found in CarpeDB, we are even more convinced that our experimental methods represent a valid approach to epilepsy gene discovery.

## Results

### Convulsions in Synaptic Vesicle Targeting Mutants

Despite the many notable functions of synaptic vesicle targeting genes in *C. elegans*, the viability of this model organism does not require wildtype function of each of the synaptic vesicle targeting genes selected for convulsion analysis. That is, the contribution of each synaptic vesicle targeting gene to the regulation of neuronal synchrony can often be thought of as threshold-based, meaning several mutations or environmental factors may be needed in sum for an observable change in behavior. With this concept in mind, we first assessed whether various mutant roundworm strains with synaptic vesicle targeting defects could demonstrate spontaneous convulsions in the absence of neural stimulants. After witnessing no epileptic-like phenotypes in our mutant strains, we employed a chemical antagonist of the inhibitory neurotransmitter, GABA. This GABA antagonist, called pentylenetetrazole (PTZ), is a common seizure inducer in animal models of epilepsy, such as mice and rats. PTZ was also used in previous epilepsy-associated research with *C. elegans*, in which *pnm-1* homozygotes exhibited rapid anterior contractions not unlike those shown by roundworm mutants of GABA synthesis and transport (3).

Because PTZ-mediated inhibition of GABAergic receptors should hypothetically lower a threshold for neuronal overexcitation, adding a predisposition to epileptic-like convulsions via synaptic vesicle targeting gene mutations seemed to be a logical step. Similar to the reported behavior of *pnm-1* homozygotes when exposed to PTZ, the selected synaptic vesicle targeting mutant strains displayed rapid anterior convulsions at all tested PTZ concentrations.

Using digital video imaging, we captured the phenotypic effects of chemically inducing convulsions in a variety of *C. elegans* synaptic vesicle targeting mutant backgrounds. The convulsion analysis results are depicted in Table 1. As previously reported, wild-type N2 roundworms (0/300) do not exhibit PTZ-induced convulsions at concentrations of up to 20 mg/mL (3). The extraordinary number of convulsions seen in many of our mutant strains, however, allowed us to cease convulsion analysis at 10 mg/mL PTZ (*i.e.*, PTZ 10). Moreover, the percentage of synaptic vesicle targeting mutants with epileptic-like convulsions directly correlated with the concentration of PTZ.

### Convulsions in Synaptic Vesicle Targeting RNAi Escapers

Following our discovery that mutations in synaptic vesicle targeting genes can greatly enhance susceptibility to PTZ-induced convulsions, we hoped to characterize the contributions of supplementary, putative *LIS-1* pathway members to neuronal synchrony. Ruefully, mutant strains of such genes are rarely available, as the *lis-1* pathway serves a vital role in life through regulating the cell cycle, and impeding this pathway's function can yield embryonic lethality. Nonetheless, we hypothesized that RNAi feeding might disrupt neuronal synchrony, while permitting the cell cycle to function normally, by reducing the number of functional *LIS-1* pathway members. Our RNAi feeding in an N2 background of *nud-1* and *nud-2*, computationally-identified homologues of human *LIS1* pathway members with roles in the cytoskeletal control of neurotransmission (*i.e.* *NUDC* and *NDE1*, respectively), was neither adequately severe to cause embryonic lethality, nor sufficient to promote spontaneous seizure-like activity. Yet, the contribution of each gene to

mitigating neuronal excitability became apparent after the use of PTZ to lower the convulsion threshold.

Although *nud-1* (RNAi) and *nud-2* (RNAi) animals in the presence of PTZ demonstrated increased susceptibilities to seizure-like activity, they were not quite like the behavioral phenotypes of synaptic vesicle targeting mutant strains (Table 1). These RNAi “escapers” did not have clear head-bobbing convulsions, but were rigid and immobile. We speculate that such phenotypic variation, which we call “tonic,” is not necessarily a result of different underlying mechanisms, though. Instead, the observed differences in seizure-like activity of these synaptic vesicle targeting defective roundworms could result from gene expression disparity between mutations and RNAi. No matter the case, these data indicate that RNAi feeding, coupled with the use of sensitized backgrounds and neural stimulants, could assist in the isolation of novel genes implicated in epilepsy. Furthermore, the prospective contribution of other bioinformatic sources, such as Internet databases (*e.g.* CarpeDB), need not be disregarded.

## Materials and Methods

### CarpeDB Development

MySQL functions as the relational database management system (RDMS) for CarpeDB. Additionally, the user interface for CarpeDB was developed using ColdFusion, HTML, CSS, and JavaScript. CarpeDB is hosted by The University of Alabama at <http://carpedb.ua.edu>.

### Behavioral Assays

Convulsion analysis was performed by adding 2.5-10 mg/mL PTZ (Sigma) to normal growth media (NGM) plates. Drug plates were then seeded with a concentrated stock of OP50 bacteria. Roundworms were placed on the drug plates and observed for a period of 30 minutes. Roundworms were scored positive for convulsions if they showed repetitive bodily contractions (“tonic-clonic” convulsions). Animals in this broader class of contractions were subdivided into head-bobbing only, characterized by anterior contractions with posterior immobilization, or as full body contractions, wherein simultaneous contraction of both the anterior and posterior occurred. If animals

appeared overtly rigid, but still viable (following observation of pharyngeal pumping activity) and did not respond to touch with a platinum wire, they were deemed tonic. In total, 50 roundworms of each strain were analyzed at each concentration of PTZ.

### Digital Imaging of Convulsions

Roundworms were exposed to 2.5-10 mg/mL PTZ for fifteen minutes and then examined under a Zeiss M<sup>2</sup> Bio-Quad stereomicroscope. Convulsions were recorded for twenty seconds using a Q Imaging Retiga Exi digital video camera at 25 frames per second. Using Northern Eclipse software (Empix Imaging, Mississauga, Ontario), we saved the images onto an Intel Pentium RAID. Subsequently, streaming videos were converted to QuickTime format at 25 frames per second.

### RNA Interference

RNA interference by bacterial feeding was performed using standard procedures with minor modifications (6). HT115 (DE3) cells transformed with the L4440 vector containing the sequence of our gene of interest were inoculated overnight in LB + 100 mg/mL ampicillin. Overnight cultures were plated onto NGM plates containing 100 mg/mL of ampicillin and 0.5%  $\beta$ -lactose. The following day, dauer larvae were placed onto the plates for developmental synchronization. Young adult offspring of these wild-type (Bristol N2) animals were analyzed for convulsions using PTZ.

## Conclusion

Extensive research should still be a prerequisite for determining the roles of synaptic vesicle targeting components in mediating neuronal excitability. Countless genes and gene products involved in establishing a convulsion threshold via alteration of synaptic vesicle targeting pathways almost certainly remain unidentified. Several obstacles in the journey to elucidate such genetic roles in epileptogenesis exist. However, we are confident that our methods for identification and characterization of the genetic players in epilepsy should aid numerous individuals in the battle against this disorder.

Our comprehensive epilepsy genetics database, CarpeDB, will help to fuel the selection of *C. elegans* genotypes for convulsion analysis.

CarpeDB should not only make accessible summaries of all known genes implicated in epilepsy, but also it should provide crucial genomic and proteomic data for computationally-identified roundworm homologues of the epilepsy genes found in other model organisms and humans. Proper application of CarpeDB should, most notably, afford researchers the ability to expand the known catalog of epilepsy genes.

Increasing the number of genes implicated in epilepsy will likely be an arduous task, but use of bioinformatics and laboratory techniques for altering gene expression should lessen the ultimate demands. As a prime example, our computational identification of *C. elegans* homologues of synaptic vesicle targeting genes through CarpeDB, and subsequent convulsion analysis with corresponding mutant strains and RNAi escapers, has given us a reference point for promptly finding novel epilepsy genes. Our objective is to now begin testing other *C. elegans* genes interacting with or coexpressed with our synaptic vesicle targeting genes of interest to determine their roles in regulating neuronal excitability. The relatively simple, yet fundamentally comparable, nervous system of *C. elegans* may be the key to abolishing epilepsy in our society.

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**Table 1: Synaptic vesicle targeting mutants or RNAi escapers are predisposed to PTZ-induced epileptic-like convulsions**

Worm strain	Functional class	PTZ 2.5 (%)	PTZ 5 (%)	PTZ 10 (%)
Wildtype Bristol N2	N/A	0	0	0
<i>nud-1</i> (RNAi) in N2	cytoskeletal	6	34	44
<i>nud-2</i> (RNAi) in N2	cytoskeletal	26	40	46
<i>aex-3</i> (n2166)	rabs (GTPases)	2	8	44
<i>aex-3</i> (sa5)	rabs (GTPases)	0	2	4
<i>rab-3</i> (y250)	rabs (GTPases)	0	0	36
<i>rab-3</i> (js49)	rabs (GTPases)	4	8	38
<i>unc-10</i> (e102)	rabs (GTPases)	24	42	72
<i>unc-10</i> (md1117)	rabs (GTPases)	28	56	66
<i>ric-4</i> (md1088)	SNAREs	8	14	86
<i>snt-1</i> (ad596)	SNAREs	2	22	40
<i>snt-1</i> (md290)	SNAREs	90	90	96
<i>snt-1</i> (n2665)	SNAREs	94	96	96
<i>unc-13</i> (n2813)	SNAREs	0	54	58
<i>unc-18</i> (e81)	SNAREs	72	82	100
<i>unc-64</i> (e246)	SNAREs	12	24	98
<i>unc-64</i> (md130)	SNAREs	74	92	94
<i>unc-11</i> (e47)	recycling	56	64	70
<i>unc-26</i> (e1196)	recycling	96	94	98
<i>unc-26</i> (e205)	recycling	94	92	100
<i>unc-26</i> (m2)	recycling	96	96	100
<i>unc-57</i> (e1190)	recycling	88	100	98
<i>unc-57</i> (e406)	recycling	92	100	100
<i>unc-57</i> (ok310)	recycling	76	90	100