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**Research Report**
**Genetic interactions among cortical malformation genes that influence susceptibility to convulsions in *C. elegans***
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**ABSTRACT**

Epilepsy is estimated to affect 1–2% of the world population, yet remains poorly understood at a molecular level. We have previously established the roundworm *Caenorhabditis elegans* as a model for investigating genetic susceptibilities to seizure-like convulsions in vivo. Here we investigate the behavioral consequences of decreasing the activity of nematode gene homologs within the LIS1 pathway that are associated with a human cortical malformation termed lissencephaly. Bioinformatic analysis revealed the *nud-2* gene, encoding the worm homolog of mammalian effectors of LIS1, termed NDE1 and NDEL1. Phenotypic analysis of animals targeted by RNA interference (RNAi) was performed using a pentylentetrazole (PTZ) exposure paradigm to induce convulsions. Worms depleted for LIS1 pathway components (NUD-1, NUD-2, DHC-1, CDK-5, and CDKA-1) exhibited significant convulsions following PTZ and RNAi treatment. Strains harboring fluorescent markers for GABAergic neuronal architecture and synaptic vesicle trafficking were employed to discern putative mechanisms accounting for observed convulsion behaviors. We found that depletion of LIS1 pathway components resulted in defective GABA synaptic vesicle trafficking. We also utilized combinations of specific genetic backgrounds to create a sensitized state for convulsion susceptibility and discovered that convulsion effects were significantly enhanced when LIS-1 and other pathway components were compromised within the same animals. Thus, interactions among gene products with LIS-1 may mediate intrinsic thresholds of neuronal synchrony.

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**1. Introduction**

Epilepsy affects over fifty million people worldwide and it is estimated that genetic factors account for the origins of ~40% of epilepsies (Noebels, 2003). Genetic epilepsies are often complex disorders in which only about 1% of patients

show simple inheritance (Robinson and Gardiner, 2004). Lissencephaly is a developmental abnormality associated with a failure in the final migration event of neurons within the periventricular zone to the cortical plate; the majority of cases are caused by mutations or deletions of the *LIS1* gene. Patients often display intractable epilepsy that generally

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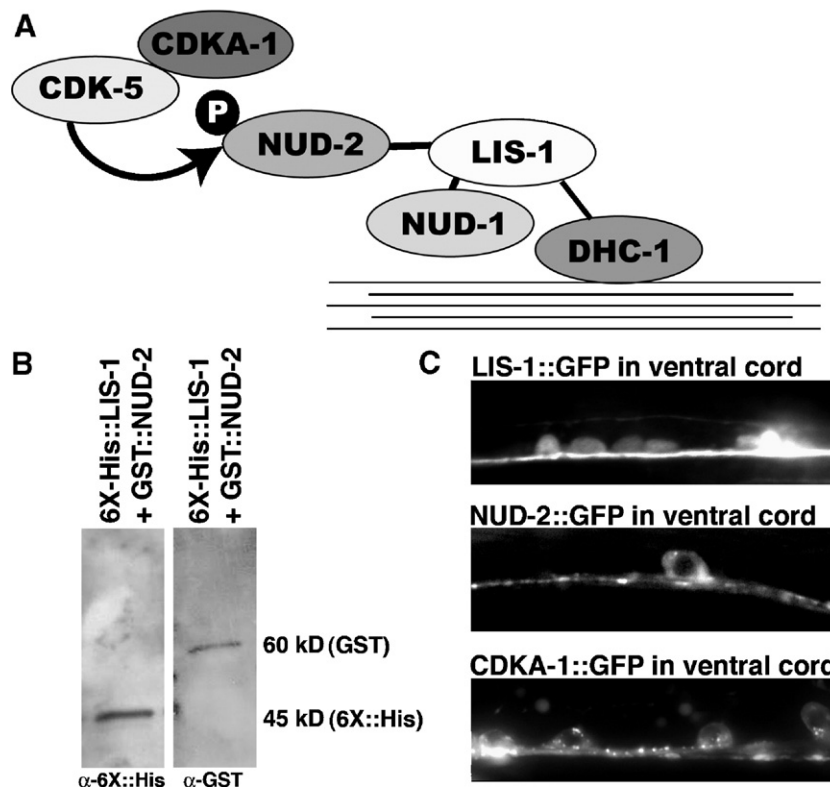
begins late in the first year of life (Dobyns, 1989; Miller, 1963).

Insights into the cellular functions of LIS1 have been elucidated through investigations of this protein within model organisms. Originally identified in *Aspergillus nidulans*, a LIS1 homolog termed NUDF was shown to have an essential role in nuclear distribution within hyphae of this filamentous fungus (Xiang et al., 1995). This mutation was termed *nud* for nuclear distribution defective. There are also well-characterized *nud*/LIS1 pathway members in both higher and lower eukaryotic model organisms (Morris et al., 1998; Vallee et al., 2000). These gene products are highly conserved and function as a biochemical complex in multiple cell types, including migrating neurons and asymmetrically dividing cells.

The LIS1 protein is a member of the WD-40 repeat protein family (Neer et al., 1994), molecules characteristically involved in protein–protein interactions. Therefore, it is not surprising that many proteins have been identified as interacting partners of LIS1 (Emes and Ponting, 2001; Morris, 2000; Reiner, 2000). In multiple systems, LIS1 has been shown to interact with dynein, NUDC, and NUDE (Efimov and Morris, 2000; Faulkner et al., 2000; Gonczy et al., 1999a; Miyajima et al., 1995; Morris et al., 1998; Reiner, 2000; Smith et al., 2000; Vallee et al., 2000; Xiang et al., 1995). Dynein is a well-characterized motor protein while NUDC associates with the dynein motor complex and is a highly conserved protein of unknown function with

roles in cell division and neuronal movement (Aumais et al., 2001, 2003). There are two mammalian homologs of NUDE, termed NDE1 and NDEL1. The NDEL1 protein associates with dynein heavy chain (Liang et al., 2004; Niethammer et al., 2000; Sasaki et al., 2000) and is also phosphorylated by Cdk5, a unique member of the cyclin dependent kinase family that is activated by p35 in neurons (Gilmore et al., 1998; Lew et al., 1994; Smith et al., 2000; Tsai et al., 1994). Loss of *Ndel1* gene activity in mice results in neuronal migration defects that are exacerbated by *Lis1* mutation (Sasaki et al., 2000). Mice lacking Cdk5 or p35 exhibit neuronal migration defects in which the normal inside-out neurogenic gradient of the cerebral cortex is reversed (Chae et al., 1997; Liu and Kipreos, 2000). Furthermore, p35 homozygous knockout mice display an increased number of lethal seizures compared to wild type siblings (Liu and Kipreos, 2000).

Despite evidence that the LIS1 pathway is involved in neuronal development, little is known about the relative importance of these cytoskeletal factors in the epileptic process. As the neuronal cytoskeleton interacts with synaptic trafficking components, neurotransmitter receptors and ion channels, disruption of these components can dramatically alter normal neuron function (Whatley and Harris, 1996). Given the complexity of the human brain and central nervous system, we contend that genetic analysis in a simple model organism may provide a more rapid means to discern the



**Fig. 1** – LIS-1 pathway components in *C. elegans*. (A) Simplified interaction pathway of LIS-1 and related proteins. Phosphorylation of NDEL1 (NUD-2 in *C. elegans*) by the p35/Cdk5 (*C. elegans* CDKA-1/CDK-5) complex facilitates its interaction with LIS-1, NudC (NUD-1) and subsequent regulation of dynein (DHC-1)-mediated microtubule processes. (B) Western blot showing *in vitro* interaction between 6X-HIS::LIS-1 and GST::NUD-2 following pulldown of these proteins using nickel sepharose. (C) *In vivo* expression of GFP translational fusions with LIS-1 (top), NUD-2 (middle) or CDKA-1 (bottom) in separate transgenic *C. elegans* lines. Expression of all three proteins is evident within the ventral nerve cord.

contribution of LIS1 and associated proteins to development of the epileptic seizures associated with cortical malformations. The nematode *Caenorhabditis elegans* is ideal in this regard, as a wealth of traditional mutant analyses have been performed in this animal. Likewise, this animal is amenable to rapid phenotypic screening via RNA-mediated interference (RNAi), there are a plethora of GFP markers that highlight neuronal processes and a wide variety of straightforward assays for neuronal function (Bargmann, 1998; Hamilton et al., 2005; Kamath et al., 2003; Simmer et al., 2003; Sonnichsen et al., 2005).

We have previously established *C. elegans* as a simple model for studying seizure-like convulsions where animals genetically compromised for LIS-1 were responsive to the common seizure inducer pentylentetrazole (PTZ) and displayed a distinct convulsive phenotype (Williams et al., 2004). We further combined our phenotypic analysis of convulsions with an examination of specific changes in neuronal cell division, architecture, and intracellular neurotransmitter trafficking. These analyses revealed that LIS-1-dependent convulsions could be uncoupled from gross anatomical changes in neuron number and migration, and were instead coincident with intrinsic deficits in synaptic vesicle transport.

Here we extend our initial efforts toward a more expansive investigation of LIS-1-associated gene products to determine which of these factors influence the susceptibility to epileptic-like convulsions in *C. elegans*. We employed RNAi to systematically inactivate several established LIS-1 pathway components (Fig. 1A) in three distinct genetic backgrounds, two of which reduce the function of either LIS-1 or GABA. This strategy facilitates the investigation of complex genetic interactions within the context of sensitized states, enabling evaluation of the relative contribution of specific proteins to the regulation of LIS-1 and susceptibility to convulsions. In this regard, we determined that a combined reduction of LIS-1 and any of the LIS-1 pathway members results in significantly enhanced levels of convulsions.

## 2. Results

### 2.1. Bioinformatic identification of *C. elegans* NUD-2 as a LIS-1 interacting protein

Before initiating our expanded analysis of the convulsive phenotype, we identified the worm homolog of the gene encoding the mammalian protein, NDE1. *C. elegans* proteins are often unusually divergent from other metazoan proteins, and *A. nidulans*, in which NUDE was first discovered, is phylogenetically distant from the metazoa (King, 2004). We thus identified NUDE orthologs in *C. elegans* and other organisms by position-specific iterated (psi)-Blast and profile searches (Altschul et al., 1997; Schaffer et al., 2001), as well as by TblastN searches of ESTs. This identified open-reading frame R11A5.2 in *C. elegans*, named NUD-2, along with 27 other orthologs from metazoa and fungi (Supplemental Table 1). The orthology of NUD-2/R11A5.2 to mammalian NDE1 has been independently observed in the NCBI and Inparanoid groups of eukaryotic protein orthologs (O'Brien et al., 2005; Tatusov et al., 2003).

Comparison to metazoan and fungal orthologs reveals two highly conserved regions in NUD-2/R11A5.2 (Supplemental Figs. 1 and 2). These two regions correspond precisely with predicted coiled-coil domains of NUD-2/R11A5.2 (Lupas, 1996). They also correspond with subsequences found to be necessary and sufficient for binding NUDF/LIS1 proteins in *A. nidulans* and vertebrate orthologs of NUD-2/R11A5.2 (Efimov and Morris, 2000; Feng and Walsh, 2000; Sasaki et al., 2000).

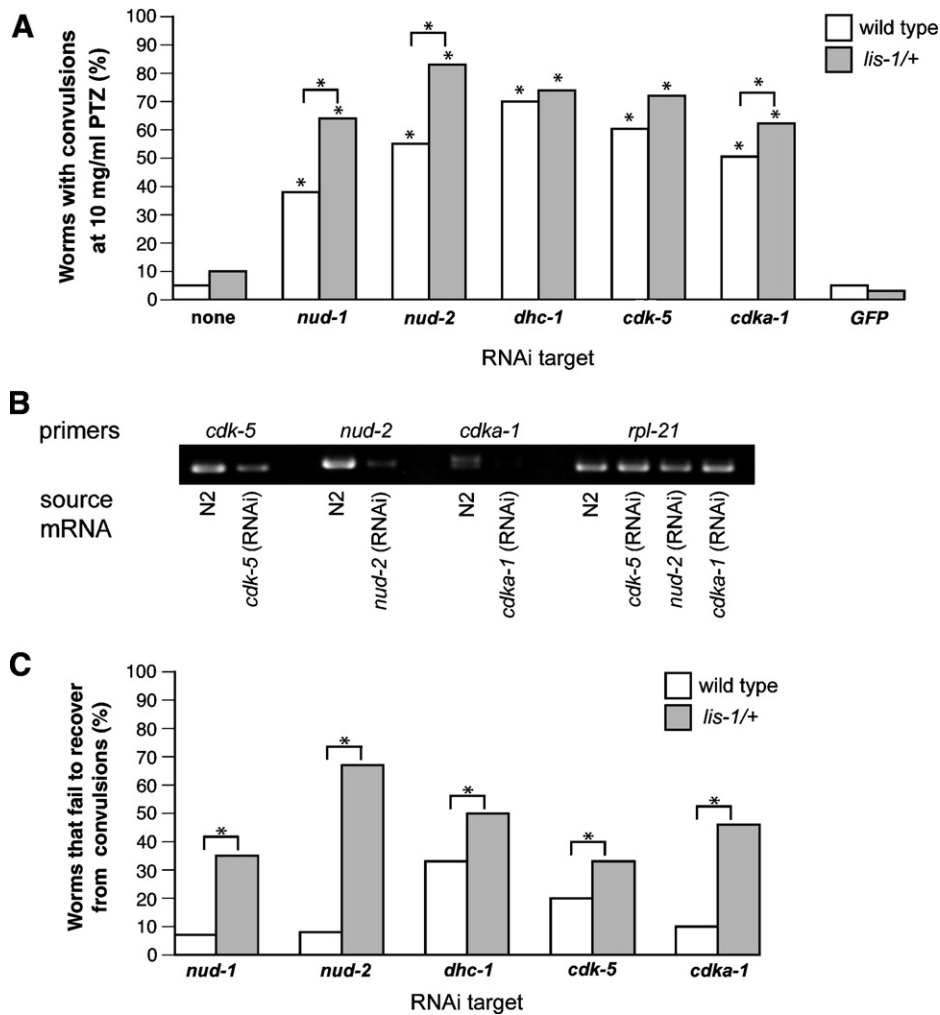
Therefore, to functionally corroborate these bioinformatics data, we demonstrated that NUD-2 directly interacts with LIS-1 in vitro. Specifically, the *nud-2* cDNA was fused to a glutathione *S*-transferase (GST) tag while the *lis-1* cDNA was fused to a 6X-histidine tag. Both constructs were generated using N-terminal tag fusions. Following a coupled transcription/translation reaction, full-length GST::NUD-2 and 6X-HIS::LIS-1 proteins were synthesized. These proteins were combined together to promote binding and attached to appropriate columns containing glutathione or nickel sepharose, respectively, and washed. The binding between NUD-2 and LIS-1 was assessed by SDS-PAGE of the bound samples. We found that NUD-2 and LIS-1 are co-precipitated following incubation on either column, indicating that they were able to bind to each other directly in vitro (Fig. 1B shows data after binding on nickel sepharose). As controls, we incubated NUD-2 protein (without LIS-1) in a nickel sepharose column, and vice versa, to ensure that precipitation did not occur without both proteins present (data not shown). Purified GST protein was eluted from a glutathione column as a positive control (data not shown).

LIS-1 is expressed in all classes of neurons (GABA, cholinergic, glutamatergic) within the ventral cord of *C. elegans* (Dawe et al., 2001) (Fig. 1C). Therefore, we sought to determine the in vivo gene expression pattern of *C. elegans nud-2*. We constructed transgenic animals expressing a translational fusion ( $P_{nud-2}$ ::NUD-2::GFP) between the complete *nud-2* open reading frame and the green fluorescent protein (GFP) driven by a promoter sequence upstream of *nud-2*. The NUD-2 fusion protein was also expressed within neurons of the ventral cord (Fig. 1C) as well as the pharynx, seam cells of the hypodermis, and in the vulva muscle cells (Supplemental Fig. 3).

### 2.2. Induction of convulsions in *nud-2* (RNAi) animals

We have previously demonstrated that PTZ, a GABA antagonist frequently utilized to induce seizures in mammalian systems, is capable of inducing seizure-like convulsions in *C. elegans* (Williams et al., 2004). It is generally thought that PTZ exposure reduces an intrinsic level of GABA-related neurotransmission in neurons. In this regard, PTZ reveals sensitized neuronal conditions that may contribute, either genetically or physiologically, to seizure susceptibility. We used this assay to determine the functional consequences of NUD-2 knockdown on the propensity of worms to exhibit convulsions in the presence of PTZ treatment.

To deplete the levels of NUD-2 within *C. elegans*, the technique of RNAi was employed via ingestion of dsRNA producing bacteria (RNAi feeding) (Timmons et al., 2001). When *nud-2* is knocked down in wild type worms (N2), convulsions are observed in 55% (83/150) of the worms exposed to 10 mg/ml PTZ (Fig. 2A). Convulsions are only



**Fig. 2** – Response to PTZ treatment following targeted knockdown of LIS-1 pathway proteins in different genetic backgrounds. **(A)** Comparison of convulsion incidence in response to *nud-1*, *nud-2*, *dhc-1*, *cdk-5*, *cdka-1*, and GFP RNAi knockdown following exposure to 10 mg/ml PTZ. RNAi knockdown significantly increased convulsions ( $*p < 0.05$ ) compared to controls (no RNAi or GFP RNAi). Worms exhibited convulsions in both *lis-1/+* (grey bars) and the wild type N2 background (white bars), with *nud-1*, *nud-2*, and *cdka-1* showing significant enhancement in convulsions in *lis-1/+* when compared with the N2 background ( $*p < 0.05$ ; denoted with a bracket). **(B)** Semi-quantitative RT-PCR demonstrating target-specific RNAi knockdown for *cdk-5*, *nud-2*, and *cdka-1*, as well as control ribosomal subunit transcript, *rpl-21*. **(C)** RNAi-treated N2 and *lis-1/+* worms recover differentially from PTZ exposure. Animals demonstrating convulsions were assessed following 1 h of recovery from PTZ treatment. In all cases, recovery is reduced in the *lis-1/+* genetic background ( $*p < 0.05$ ).

observed in 5% (10/200) of N2 worms at this concentration of PTZ and in only 5% (8/150) of worms exposed to a negative control (dsRNA vs. GFP) (Fig. 2A). In all cases, *nud-2* (RNAi) worms presented with total loss of wild type locomotion and instead had an overall stiffened appearance. It is tempting to speculate that this might be similar to mammalian tonic seizures, which manifest with rigid posture and limb position. These worms are not dead, as they resume normal movement after removal from PTZ (see below; Fig. 2C). Tonic convulsions are also displayed when worms are depleted of *lis-1* via RNAi (Williams et al., 2004).

It is likely that complex genetic factors impact the development of convulsions. Thus, we proceeded to analyze the effect of knocking down NUD-2 activity in a *lis-1* mutant strain. The strain used, GE2730, encodes a nonsense allele of *lis-1* (Cockell et al., 2004; Williams et al., 2004). This truncated

protein contains the LisH and coiled-coil domains but not the WD40 interaction domain (Emes and Ponting, 2001; Williams et al., 2004). Despite an embryonic requirement for LIS-1, approximately 30% of *lis-1* homozygotes (*lis-1/lis-1*) are able to survive to adulthood (Williams et al., 2004). We have previously demonstrated that these homozygous *lis-1* animals display 96% convulsions following exposure to 10 mg/ml PTZ (Williams et al., 2004). In contrast, *lis-1/+* heterozygous animals, which carry one mutant and one wild type copy of *lis-1*, display 10% (15/150) convulsions following 10 mg/ml PTZ exposure (Fig. 2A). Human lissencephaly is caused by a haploinsufficiency and is more accurately reflected by *lis-1* heterozygous animals (*lis-1/+*). Therefore, we sought to examine the effect of knocking down NUD-2 levels in *lis-1/+* animals. In combination, the knockdown of *nud-2* in *lis-1/+* worms resulted in 83% (124/150) of the worms demonstrating

convulsions in the presence of 10 mg/ml PTZ (Fig. 2A). Notably, the percentage of *nud-2* (RNAi) animals displaying convulsions increased by 51% in the *lis-1/+* sensitized background compared with the N2 background ( $p < 0.05$ ).

### 2.3. Knockdown of additional LIS-1 interacting proteins induces convulsions

To determine if the significantly enhanced convulsion effect observed was specific to the interaction between NUD-2 and LIS-1, we examined two additional characterized LIS-1 interactors, NudC and cytoplasmic dynein heavy chain, for their potential influence on convulsion levels. The *C. elegans* homologs of these two proteins, NUD-1 and DHC-1, were knocked down via RNAi feeding in both wild type and *lis-1/+* heterozygous worms. Following RNAi, both N2 and *lis-1/+* worms displayed convulsions that appeared tonic and rigid and were statistically greater than untreated controls (Fig. 2A). Additionally, the *lis-1/+* genetic background significantly enhanced convulsion susceptibility ( $p < 0.05$ ) when compared to the N2 background for NUD-1 depletion at 10 mg/ml PTZ where 38% (57/150) and 64% (32/50) of N2 and *lis-1/+* worms, respectively, had convulsions when exposed to *nud-1* (RNAi) (Fig. 2A). However, this significant enhancement for convulsion susceptibility in the *lis-1/+* background was not observed for *dhc-1* (RNAi) at 10 mg/ml PTZ. This is most likely because the convulsion levels were already high, or at saturation, in the N2 background, as 70% (35/50) of N2 animals had convulsions when exposed to *dhc-1* (RNAi), compared with 74% (37/50) of *lis-1/+* worms (Fig. 2A). Since the combination of *dhc-1* RNAi and 10 mg/ml PTZ did not provide sufficient phenotypic separation to distinguish potential neuronal excitability differences between the N2 and *lis-1/+* genetic backgrounds, convulsions were investigated in the presence of 2.5 mg/ml PTZ. The lower concentration of PTZ did not resolve a potential genetic interaction because there was still a high level of convulsions observed in the N2 background with 48% of N2 worms (24/50) displaying convulsions when treated with *dhc-1*(RNAi); the level of convulsions for *lis-1/+* worms was 70% (35/50) following depletion of DHC-1 ( $p = 0.07$ ; data not shown).

### 2.4. Convulsion induction in LIS-1 pathway members

These studies indicate that NUD-1, NUD-2, and DHC-1 may function in maintaining a threshold for normal neuronal activity within the *lis-1* heterozygous background (*lis-1/+*). Therefore, we examined the *C. elegans* homologs of other genes in the LIS-1 pathway for convulsion susceptibility. The genes we examined were *Cdk5* and *p35*, which hereafter are referred to as *cdk-5* and *cdka-1* (for cyclin-dependent kinase activator) using standard worm nomenclature. LIS-1 and CDK-5 are expressed in all classes of neurons within the ventral cord of *C. elegans* (12; Gian Garriga, personal communication) (Fig. 1C). To determine the expression pattern of *cdka-1*, we made a *cdka-1* translational fusion with GFP ( $P_{cdka-1}::CDKA-1::GFP$ ). This construct showed that CDKA-1 is also localized within all neuronal classes in the ventral cord (Fig. 1C).

When *cdk-5* and *cdka-1* are depleted via RNAi in N2 worms, animals exposed to 10 mg/ml PTZ exhibit statistically significant numbers of convulsions when compared to con-

trols (Fig. 2A). A similar trend is observed when *lis-1/+* worms are exposed to *cdk-5* and *cdka-1* (RNAi) where a significant percentage of the population exhibited convulsions [74% (74/100) and 59% (59/100), respectively] compared to 10% (15/150) of *lis-1/+* control worms exposed to PTZ. Notably, the percentage of convulsions was significantly different when induced in *lis-1/+* worms compared with the wild type N2 background at 10 mg/ml PTZ when CDKA-1 is depleted with 59% (59/100) and 32% (48/150), respectively. However, this genotypic specificity is not observed when CDK-5 is depleted as we observed 60% convulsions (93/150) in the N2 background vs. 74% convulsions (74/100) in *lis-1/+* worms (Fig. 2A).

One of the advantages of the RNAi feeding technique is the ability to titrate the RNAi effect by changing the concentration of inducing chemical (IPTG or  $\beta$ -lactose). Modulating the amount of inducer can result in a differential RNAi effect, allowing for the equivalent of an allelic series to be examined (Timmons et al., 2001). We have previously shown that a low level of RNAi knockdown of essential genes such as *lis-1* can be used to elicit post-embryonic neuronal phenotypes, specifically PTZ-induced convulsions resulting from intraneuronal deficits in presynaptic GABA vesicle trafficking (Williams et al., 2004). While all RNAi induction conditions discussed thus far were elicited with 0.5%  $\beta$ -lactose, we decided to investigate the consequences of using 0.25%  $\beta$ -lactose for knockdown of *dhc-1* and *cdk-5*, as these two targets exhibited the greatest level of convulsions in the N2 background. We therefore reasoned that a lower level of convulsions in N2 worms might unmask a greater convulsion propensity in *lis-1/+* worms. As a control, we also induced dsRNA vs. *nud-1* and assayed for convulsions. Notably, for all mRNA targets tested, the RNAi effect was stronger at 0.25%  $\beta$ -lactose. It has been previously reported that RNAi knockdown of various targets in *C. elegans* is not necessarily correlated to higher inducer concentrations; lower quantities of inducer can yield enhanced knockdown in a gene-specific manner (Kamath et al., 2001; personal communication, Eric Lambie). In fact, this concentration of  $\beta$ -lactose resulted in almost complete lethality for *dhc-1*, precluding the analysis of convulsions. However, RNAi induction of *cdk-5* and *nud-1* at 0.25%  $\beta$ -lactose revealed notable observations at 10 mg/ml PTZ. Specifically, at 0.25%  $\beta$ -lactose, worms depleted of CDK-5 displayed convulsions in 51% (76/150) of N2 worms compared with 81% (122/150) in *lis-1/+* worms (Supplemental Fig. 4A). Thus, a genetic interaction between CDK-5 and LIS-1 was uncovered ( $p < 0.05$ ). The lower concentration of  $\beta$ -lactose was also correlated with enhanced knockdown of *cdk-5*, as demonstrated by RT-PCR with *cdk-5* specific primers (Supplemental Fig. 4B). Likewise, while *nud-1* (RNAi) displayed a genetic interaction with *lis-1/+* at 0.5%  $\beta$ -lactose induction, the convulsions were significantly greater when 0.25%  $\beta$ -lactose was utilized ( $p < 0.05$ ). The level of convulsions for knockdown of *nud-1* changed from 38% (57/150) to 63% (94/150) in the N2 background and from 64% (32/50) to 96% (144/150) in *lis-1/+* worms for  $\beta$ -lactose concentrations of 0.5% and 0.25%, respectively (Supplemental Fig. 4A).

While knocking down LIS-1 pathway components results in convulsions in combination with PTZ, we did not observe any convulsions when depleted worms were not exposed to this GABA antagonist (0/50 for each target). To address the

specificity of the convulsions, we knocked down GFP in both N2 and *lis-1/+* backgrounds. We found 5% (8/150) and 3% (5/150) convulsions in N2 and *lis-1/+* worms following exposure to 10 mg/ml PTZ (Fig. 2A). These convulsion levels are not significantly different from the convulsion levels observed in the absence of RNAi treatment (Fig. 2A). Additionally, several other genes unrelated to GABA or LIS-1 processes were analyzed following RNAi in the N2 wild type background to obtain background levels of convulsions. These targets included *cat-1*, a synaptic vesicular monoamine transporter required for the presence of dopamine and serotonin in nerve terminals (Duerr et al., 1999) as well as *rrf-3* and *eri-1*, genes that provide enhanced sensitivity to RNAi effects (Kim et al., 2005; Simmer et al., 2002). Knockdown of *cat-1*, *rrf-3*, and *eri-1* in combination with 10 mg/ml PTZ produced 0% (0/50), 6% (3/50), and 8% (4/50) convulsions, respectively. To ensure the gene targets were knocked down, we performed semi-quantitative RT-PCR using target specific primers on N2 worms depleted for *nud-2*, *cdk-5*, and *cdka-1*. This analysis was not performed on *nud-1* or *dhc-1* because RNAi treatment for these targets is predominantly lethal, precluding sufficient quantities of worms for RNA extraction. Regardless, the results of the analysis on *nud-2*, *cdk-5*, and *cdka-1* mRNA depletion are shown in Fig. 2B. In all cases, the amount of targeted transcript is substantially reduced when compared to the control RT-PCR reactions with *rpl-21*, a ribosomal protein large subunit.

## 2.5. Convulsion recovery

The ability of worms to recover from convulsions was monitored following exposure to *nud-1*, *nud-2*, *dhc-1*, *cdk-5*, or *cdka-1* RNAi. Both N2 and *lis-1/+* worms were assayed. Following a 1-h exposure to 10 mg/ml PTZ, animals displaying a stiffened posture (worm tonic convulsion) were transferred to a fresh plate containing no drug. Animals were then observed for their ability to resume normal behavior, including foraging (head movement from side to side) and wild type sinusoidal locomotion. The majority of N2 worms depleted of NUD-1, NUD-2, DHC-1, CDK-5, or CDKA-1 recovered from convulsions within an hour after removal from PTZ (Fig. 2C). In all cases, the *lis-1/+* worms were less likely to recover from convulsions than wild type N2 worms ( $p < 0.05$ ). Although fewer *lis-1/+* worms recovered in general, a striking difference was evident among the targets analyzed. Specifically, when *nud-1*, *dhc-1*, *cdk-5*, or *cdka-1* were depleted, there was ~15–30% reduction in *lis-1/+* worm recovery compared to N2 worms. However, when *nud-2* was depleted, there were ~50% fewer recovered *lis-1/+* animals compared to N2 wild type animals (Fig. 2C).

## 2.6. The consequences of altering LIS-1 pathway proteins on neuronal migration

We proceeded to investigate the underlying cellular mechanisms associated with the observed tonic convulsions in these animals. One of the many advantages of the *C. elegans* field is the wealth of available transgenic lines carrying fluorescent protein fusions that illuminate a particular cell type in these transparent animals. Specifically for this work, we utilized a strain carrying a fusion of the *unc-47* promoter to GFP ( $P_{unc-47}::GFP$ ), enabling visualization of the overall

architecture of GABAergic neurons within *C. elegans*, since the convulsions were induced with PTZ, a GABA antagonist. The *unc-47* gene encodes a transmembrane vesicular transporter of GABA and is required in all GABAergic neurons for neurotransmission. There are precisely 26 GABAergic neurons within the worm, including 20 easily visualized neurons located within the ventral nerve cord of the animal (McIntire et al., 1993). Worms were fed dsRNA-containing bacteria against our chosen targets and examined for gross changes in the architecture of the GABAergic neuronal processes.

We did not find discernable expression pattern differences among most of the RNAi targets (Table 1). This was not entirely surprising as we previously performed a similar experiment with *lis-1/lis-1* homozygous mutant animals and did not find obvious differences (Williams et al., 2004). This does not, however, preclude the possibility that electron microscopy analysis would reveal more subtle subcellular neuronal defects in microtubule structure. As summarized in Table 1, the only exception was *nud-1* (RNAi) where 12% (24/200) of the animals had noticeable GABA architecture defects where some of the axons exhibited small branches (Figs. 3A, B). As a control, *unc-47* (RNAi) was tested to ensure that the  $P_{unc-47}::GFP$  animals were being exposed to the dsRNA targets. In this case, 41% (41/100) of the *unc-47* (RNAi) worms demonstrated alterations in the normal *unc-47*:GFP pattern (Table 1).

## 2.7. LIS-1 pathway proteins contribute to defects in GABA vesicle transmission

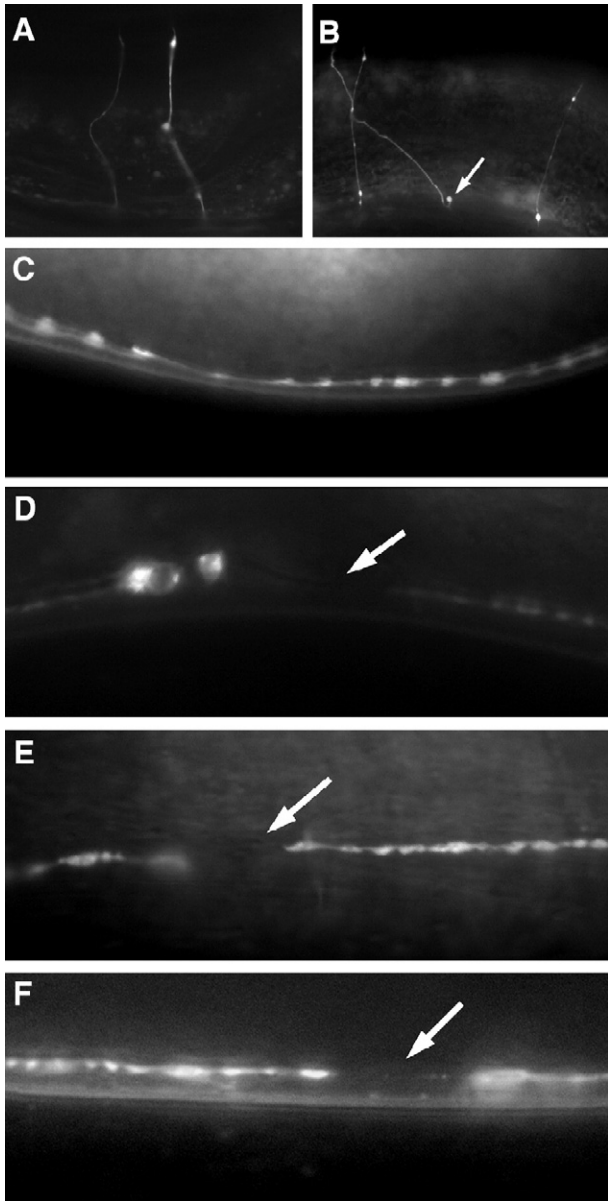
We previously reported a high percentage of defects in GABAergic vesicle transmission following alterations in cytoskeletal elements (Williams et al., 2004). The *unc-25* gene serves as a ubiquitous marker for the GABAergic system (Nonet, 1999). A transgenic strain carrying a fusion of GFP to the synaptic vesicle-associated membrane protein synaptobrevin (SNB-1) driven by the *unc-25* promoter allows for specific visualization of the GABAergic vesicle system ( $P_{unc-25}::SNB-1::GFP$ ). In this work, RNAi-treated  $P_{unc-25}::SNB-1::GFP$  animals were examined at the level of the compound microscope for the

**Table 1 – Depletion of most LIS-1 pathway proteins does not cause changes in GABAergic architecture in  $P_{unc-47}::GFP$  worms<sup>a</sup>**

RNAi target	Wild type expression (%)	Axon extension defects (%)	Branched commissures (%)	Significant difference <sup>b</sup>
None	96	0	4	n/a
<i>lis-1</i>	94	6	0	none
<i>nud-1</i>	88	12	0	$p \leq 0.01$
<i>nud-2</i>	96	4	0	none
<i>dhc-1</i>	97	3	0	none
<i>cdk-5</i>	96	4	0	none
<i>cdka-1</i>	95	4	1	none
<i>unc-47</i>	59	41	0	$p \leq 0.001$

<sup>a</sup> For the condition of no RNAi and *unc-47* mutant worms,  $n = 100$  worms; for all other targets,  $n = 200$  worms.

<sup>b</sup> Significant difference was calculated using the Fisher Exact Test by comparing  $P_{unc-47}::GFP$  worms with worms exposed to a dsRNA for a specific target, as specified.



**Fig. 3 – Knockdown of LIS-1 pathway targets by RNAi results in alterations in GABAergic architecture and/or synaptic vesicle distribution. (A) Wild type GABAergic architecture as demonstrated by expression of  $P_{unc-49}::GFP$ . (B) Axon extension defect observed following  $nud-1(RNAi)$  (arrow). (C–F) GABAergic vesicle localization was illuminated in the *C. elegans* ventral cord using the  $P_{unc-25}::SNB-1::GFP$  construct, as illustrated in (C) wild type, (D)  $lis-1(RNAi)$ , (E)  $nud-2(RNAi)$ , and (F)  $cdk-5(RNAi)$ . Puncta represent the accumulation of vesicles at a synapse. Arrows highlight areas absent of puncta, illustrating apparent defects in normal vesicle trafficking.**

accumulation of vesicles within synaptic termini. All LIS-1 pathway targets showed a significant percentage of defects within the GABAergic vesicle system (Table 2). The most commonly observed defects for all tested targets were readily visible gaps within the GFP pattern along the ventral cord (Figs. 3C–F). Notably, all pathway components displayed statistically

significant gaps in the ventral cord at 0.5%  $\beta$ -lactose induction, except  $nud-1$  (RNAi) where significance was uncovered at 0.25%  $\beta$ -lactose (Table 2).

## 2.8. LIS-1 pathway proteins function presynaptically to alter GABA transmission

We sought to determine if the vesicle transmission defects were pre- or post-synaptic by exposing RNAi-treated animals to the GABA agonist muscimol. Animals exposed to muscimol will display body muscle paralysis if a defect occurs pre-synaptically, due to an inhibition of GABA post-synaptic function (McIntire et al., 1993). An animal with a post-synaptic defect in GABA reception will display no response to muscimol exposure. In the case of our RNAi treatment regimen, 100% of animals depleted for  $nud-1$ ,  $nud-2$ ,  $dhc-1$ ,  $cdk-5$ , and  $cdka-1$  responded to muscimol exposure with overall body paralysis (20/20 for each target). As a positive control, we also fed animals bacteria producing dsRNA against  $unc-49$ , the predominant GABA<sub>A</sub> receptor in *C. elegans* (McIntire et al., 1997). Exposure to muscimol following  $unc-49$  (RNAi) treatment resulted in only 5% paralysis (1/20).

## 2.9. Increased sensitivity to convulsions is associated with decreased GABA threshold

Our data suggest that the occurrence of convulsions in animals depleted for LIS1 pathway members is associated with an apparent defect in GABAergic vesicle transmission (Williams et al., 2004). Therefore, we sought to determine if convulsions would be enhanced by knockdown of the LIS-1 associated targets in a sensitized GABA mutant background.

**Table 2 – Reduction of LIS-1 pathway proteins results in a change in GABAergic synaptic vesicle localization in  $P_{unc-25}::SNB-1::GFP$  worms<sup>a</sup>**

RNAi target	Wild type expression <sup>b</sup> (%)	Synaptic vesicle gaps <sup>c</sup> (%)	Unevenly distributed vesicles (%)	Significant difference <sup>d</sup>
None	96	0	4	n/a
$lis-1$	69	31	0	$p \leq 0.001$
$nud-1$ (0.5%) <sup>e</sup>	85	15	0	none
$nud-1$ (0.25%) <sup>e</sup>	42	58	0	$p \leq 0.001$
$nud-2$	80	17	3	$p \leq 0.01$
$dhc-1$	73	27	0	$p \leq 0.001$
$cdk-5$	64	36	0	$p \leq 0.001$
$cdka-1$	78	20	2	$p \leq 0.01$
GFP	2	51	47	$p \leq 0.001$

<sup>a</sup> For each condition  $n = 100$  worms except for  $nud-1$  at 0.25% where  $n = 50$  worms.

<sup>b</sup> See Fig. 3C where the ventral nerve cord displays evenly spaced synaptic vesicles.

<sup>c</sup> See Fig. 3D–F where arrows point to gaps within the ventral nerve cord.

<sup>d</sup> Significant difference was calculated using the Fisher Exact Test by comparing  $P_{unc-25}::SNB-1::GFP$  worms with worms exposed to a dsRNA for a specific target, as specified.

<sup>e</sup> dsRNA induction of  $nud-1$  at either 0.5% or 0.25%  $\beta$ -lactose.

For this study, we chose a mutant strain, *unc-25(e156)*, encoding an allele of the GABA synthesis gene glutamic acid decarboxylase. The *unc-25* homozygous mutant strain shows defects in all GABA-mediated behavioral functions (McIntire et al., 1993). This strong mutation is, however, sensitive to PTZ with dose-specific effects. At 2.5 mg/ml PTZ 24% of *unc-25* mutant worms had convulsions, while at 10 mg/ml 84% of the worms exhibited convulsions (Williams et al., 2004). We took advantage of this dose-specific response and exposed RNAi-treated *unc-25* worms to 2.5 mg/ml PTZ to determine if the onset of convulsions was influenced by depletion of LIS-1 pathway components. Indeed, when LIS-1 pathway members were depleted, they show statistically significant levels of convulsions at 0.5%  $\beta$ -lactose induction (*nud-1*, *nud-2*, or *dhc-1*) or 0.25%  $\beta$ -lactose induction (*cdk-5* and *cdka-1*) ( $p < 0.05$ ) (Supplemental Figs. 5A, B). As an additional control, *lis-1* (RNAi) was also tested in the *unc-25* background, and it enhanced convulsions to 70% (35/50) compared with 26% (13/50) convulsions resulting from *lis-1* depletion in the N2 wild type background (N2 data not shown).

### 3. Discussion

Here we present data that gene products known to function in neuronal migration (*lis-1*, *cdk-5*, *cdka-1*) and their interacting proteins (*nud-1*, *nud-2*, *dhc-1*) contribute to the development of a convulsion-prone neuronal state. While knocking down these target genes alone does not yield spontaneous convulsions in *C. elegans*, further alterations in the neural environment through the application of PTZ serve to pass a critical threshold within these animals. Recovery from PTZ-induced convulsions is commensurate with removal of animals from exposure, where they return to typical sinusoidal locomotion. Moreover, in the case of most genes targeted, the extent of recovery was inversely proportional to the severity of convulsions observed with a given gene. Taken together, these data imply that a threshold of neuronal activity was exceeded and restored in a manner that is at least partially dependent upon genetic constitution.

By exploiting the availability of stable genetic variants in *C. elegans*, we observed an increase in the manifestation of convulsive phenotypes that was further revealed by combining target gene knockdown with genetically compromised or sensitized backgrounds. Depletion of all targets enhanced susceptibility to convulsions following exposure to PTZ in both N2 and *lis-1/+* worms. Additionally, most of these targets also showed a statistically significant increase in convulsions in the *lis-1/+* animals when compared with the N2 wild type background. These data thus demonstrate that there is a potential genetic interaction among these pathway members in *C. elegans*. It is therefore possible that these genes may be susceptibility markers for epilepsy in the context of *lis-1* associated polymorphisms.

GABA, an inhibitory neurotransmitter, has been previously linked with seizure occurrence and is understandably associated with perturbations that could directly generate hyperexcitability (Cossette et al., 2002; DeLorey et al., 1998). The specific *unc-25* allele (*e156*) utilized in these studies is a strong mutation in GABA synthesis that does not exhibit sponta-

neous convulsions. Thus, either a sufficient level of GABA production or an uncharacterized compensatory mechanism for GABA synthesis or secretion may be present in these animals. It is also intriguing to speculate that PTZ may potentially provide an additive effect on disinhibition via interaction with a nematode-specific set of inhibitory glutamate receptors that appear evolutionarily-related to GABA<sub>A</sub> receptors (Bargmann, 1998; Schuske et al., 2004). Regardless, it seems likely that disruption of proteins that function with LIS1 contributes to the development of a convulsive phenotype, as a minimum, through a reduction of GABA transmission. Moreover, the behavioral changes observed corresponded to an enhanced level of defects associated with either GABA synaptic vesicles or GABA architecture.

While defects in neuronal architecture were only observed for NUD-1, it is important to note that such evidence does not supplant or preclude a role for any of these gene products in neuronal migration during mammalian cortical development. In contrast to mammals, nematode neurons are exceedingly small and the migration distances encountered during the course of development are equally minuscule. The scale of the animal represents the greatest drawback of this system, as electrophysiological studies are greatly limited in scope as well. Physical differences aside, the concurrence of vesicular trafficking defects with genetic perturbation and altered behavior clearly points to an intrinsic cellular deficit as contributory to the observed convulsive phenotype. Furthermore, since none of the targets examined generated spontaneous convulsions following knockdown and required further perturbation of the neuronal environment through PTZ exposure, we contend that LIS-1 related proteins may contribute to manifestations of complex epilepsies, but are not likely themselves 'epilepsy' genes. This distinction is arguably arbitrary, but serves to distinguish between contributory vs. purely causative factors to epileptogenesis.

The unknown root causes of the idiopathic epilepsies that afflict the vast majority of patients necessitate the formulation of novel hypotheses and strategies toward gene discovery (Noebels, 2003). While the existing cellular focal point for epilepsy researchers has rightly been on ion channels, receptors, and their accessory proteins, we contend that proteins regulating the cytoskeleton, like LIS-1 and associated proteins, represent a large and understudied group of molecules whose impact on neuronal synchrony remains to be fully discerned. The application of simple animal models toward epilepsy research may serve to define the variety of cellular factors that contribute to epileptogenesis and more rapidly inform subsequent target validation studies in mammalian systems.

### 4. Experimental procedures

#### 4.1. Identification and sequence analysis of NUDE homologs

Position-specific iterated (psi)-Blast (Altschul et al., 1997) with composition-based statistics (Schaffer et al., 2001) was used to search the NCBI non-redundant (NR) protein database (Schuler et al., 2001) with human NDE1 as the query sequence (NDE1). psi-BLAST was run at a highly stringent threshold of



1e-13, to exclude spurious matches to coiled-coil proteins (Lupas, 1996) and allow the search to converge. This analysis identified various metazoan and fungal sequences as putative NUDE homologs, including R11A5.2; their sequences were obtained from NCBI in Genpept format by batch Entrez (Baxevanis, 2003) and reformatted to FASTA with Perl (Schwartz and Phoenix, 2001).

#### 4.2. Reporter gene fusions and microinjection

GenBank accession numbers for the *C. elegans* cosmid R11A5 containing *nud-2* and cosmid T23F11 containing *cdka-1* are Z83122 and Z46343, respectively. There are no predicted ORFs upstream of *nud-2* for more than 3 kb or upstream of *cdka-1* for more than 7 kb. Genomic DNA was used with Primers 1: aac-tgc-agt-acc-tga-att-att-gtt-att-caa-agc-gc and 2: cgg-gat-cca-gcc-cgt-gtc-gtt-gta-aga-tgt-ctg-g to generate a translational fusion containing 3.3 kb upstream plus coding sequence without the final stop codon of the *nud-2* gene. The PCR product was cloned into *C. elegans* GFP expression vector pPD95.77 (a gift from A. Fire, Stanford University) to create  $P_{nud-2}::NUD-2::GFP$ . Likewise, genomic DNA was used with primers 1: acg-cgt-cga-ccg-cca-act-gtc-gaa-acg-ttg-c and 2: cgg-gat-cct-tcg-gaa-ctt-gaa-caa-tgc-ttg-c to generate a translational fusion containing 3.4 kb upstream plus coding sequence without the final stop codon of the *cdka-1* gene, prior to cloning into the pPD95.77 GFP expression vector to create  $P_{cdka-1}::CDKA-1::GFP$ . These reporter constructs and a dominant marker DNA (*rol-6*) were co-microinjected into wild type N2 young adults and 2 stable lines of each construct were propagated at 20 °C. One representative line was chosen for analysis in this work. The strains were named UA40 for the strain carrying the  $P_{nud-2}::NUD-2::GFP$  fusion and UA41 for the strain carrying the  $P_{cdka-1}::CDKA-1::GFP$  fusion.

#### 4.3. Protein–protein interaction studies

To isolate cDNAs encoding *lis-1* and *nud-2*, total RNA was extracted from a mixed population of *C. elegans* using Tri Reagent (Molecular Research Center); mRNA was then isolated using the PolyATract mRNA Isolation System III (Promega). RT-PCR methods employing the SuperScript II system (Invitrogen) were utilized to isolate the target cDNAs. Isolated cDNA for *lis-1* was then cloned into the pCR8/GW/TOPO vector (Invitrogen) to create a Gateway entry clone. Isolated cDNA for *nud-2* was cloned directly into pDONR221 (Invitrogen) to create an entry vector. The primers utilized for *lis-1* cDNA isolation were Primer 1: 5' ggt-acc-atg-agt-ttg-tcg-gag-agg-c 3' and Primer 2: 5' gaa-ttc-tca-acg-gcg-att-ccc-aaa-ctt-tgc 3'. The primers used for *nud-2* cDNA isolation were Primer 1: 5' ggg-gac-aag-ttt-gta-caa-aaa-agc-agg-ctc-cat-gga-ttt-gtc-tga-gg 3' and Primer 2: 5' ggg-gac-cac-ttt-gta-caa-gaa-agc-tgg-gtt-taa-gcc-cgt-gtc-gtt-g 3'. Sequence verification was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer. The cDNAs in entry clones were then introduced into pDESTGEX-5x-3 (*lis-1* for an N-terminal histidine fusion) or pGWB8 (*nud-2* for an N-terminal glutathione S-transferase fusion) using Gateway technology as previously described (Cao et al., 2005). Fusion

proteins were then expressed in *E. coli* BL21 cells and isolated using batch isolation with either Ni-Sepharose High Performance (for isolation of 6X-Histidine fused proteins) or Glutathione Fast 4B (for isolation of GST fused proteins) (Amersham Biosciences). Protein gel electrophoresis and Western blotting were performed as previously outlined (Caldwell et al., 2003). For 6X-histidine fused protein detection, a 1:2000 dilution of mouse anti-histidine (Sigma) primary antibody and 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Amersham Biosciences) were used. For GST fusion protein detection, a 1:4000 dilution of rabbit anti-GST (Sigma) primary antibody and 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Amersham Biosciences) were used.

#### 4.4. Worm strains and maintenance

*C. elegans* growth and maintenance were performed via standard procedures (Brenner, 1974). Worm strains used in this study include strain GE2730 [*unc-32(e189) pnm-1(t1550)/qC1 dpy-19(e1259) glp-1(q339) III; him-3(e1147)*] (Gonczy et al., 1999b), N2 Bristol, *lin-15(n765ts) X juIs1* (an integrated *unc-25::SNB::GFP* strain) (Simmer et al., 2002), *lin-15(n756) oxIs12 X* (an integrated *unc-47::GFP* strain) (McIntire et al., 1993), and strain CB156 [*unc-25(e156) III*].

#### 4.5. RNA interference

RNA interference (RNAi) by bacterial feeding was performed using standard procedures with minor modifications (Timmons et al., 2001). Briefly, for all targets except *dhc-1*, HT115 (DE3) cells transformed with the L4440 dsRNA production vector containing the appropriate target sequence were inoculated overnight in LB +100 mg/ml ampicillin. Overnight cultures were plated onto M9 minimal media plates containing 100 mg/ml ampicillin and either 0.5% or 0.25%  $\beta$ -lactose to induce dsRNA production. The following day, dauer stage larvae were placed onto the plates. Several days later, young adult offspring of these animals were analyzed for convulsions, muscimol response, ability to recover from PTZ exposure, or GFP expression. Production of dsRNA for *dhc-1* was performed using a previously described method (Williams et al., 2004). As noted below, the following target DNA sequences were cloned into the L4440 vector utilizing traditional restriction enzyme cloning methods (*lis-1*, *nud-1*, *nud-2*, *cdk-5*, *cdka-1*) with the following primers.

<i>lis-1</i>	Primer 1: 5' aac-gcg-tcg-acc-atg-agt-ttg-tcg-gag-agg-c 3' Primer 2: 5' aac-tgc-agt-caa-cgg-cat-tcc-caa-ac 3'
<i>nud-1</i>	Primer 1: 5' aac-tgc-agt-tat-cca-att-tta-gca-ttg-ctg 3' Primer 2: 5' aac-gcg-tcg-acc-atg-tct-caa-tat-gag-cga-ttc 3'
<i>nud-2</i>	Primer 1: 5' ggg-gta-ccg-act-acc-gca-cca-tga-gct-tct-tg 3' Primer 2: 5' aac-tgc-agt-gtc-atc-gat-gcg-cac-ttc-gtc 3'
<i>cdk-5</i>	Primer 1: 5' ggg-gta-ccg-gtg-ttc-caa-gct-ctg-ctc-tcc-gtg-aaa-ttt-g 3' Primer 2: 5' aac-tgc-agg-tgc-aaa-aat-aca-ccc-ggc-aga-cca-cat-atc 3'
<i>cdka-1</i>	Primer 1: 5' gac-tag-tac-ccc-gat-cac-tac-ttc-ggg-

(continued on next page)

acg-aca-atg-c 3' Primer 2: 5' aac-tgc-aga-tta-cca-att-aaa-aac-ggc-ttc- aac-gga-tag-g 3'
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As a positive control, GFP was cloned in a Gateway-converted L4440 plasmid using Gateway recombinational cloning (see above). The following primers were used.

GFP Primer 1: 5' ggg-aca-agt-ttg-tac-aaa-aaa-gca-ggc-tcc-atg-agt- aaa-gga-gaa-gaa-c 3' Primer 2: 5' ggg-gac-cac-ttt-gta-caa-gaa-agc-tgg-gtc-tca-tcc- atg-cca-tgt-gta-atc-cc 3'
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Two of the L4440 dsRNA production vectors, for *dhc-1* and *unc-47*, were obtained from a *C. elegans* RNAi library (Molecular Research Council, England) (Kamath et al., 2003). These targets were originally amplified with the following primers.

<i>dhc-1</i>	Primer 1: 5' aag-gaa-gga-gct-caa-cga-ca 3' Primer 2: 5' cct-ttc-ctt-cct-ggg-tct-tc 3'
<i>unc-47</i>	Primer 1: 5' gaa-tag-tgc-cgg-cca-gat-aa 3' Primer 2: 5' aac-gct-tga-tcc-aag-gaa-tg 3'

#### 4.6. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated using TRI Reagent (Molecular Research Center) from mixed age populations of worms fed dsRNAs specific for *cdk-5*, *nud-2*, or *cdka-1* at either 0.25% or 0.5%  $\beta$ -lactose induction. Total RNA was treated with amplification grade RNase-free DNase I (Invitrogen) to minimize genomic DNA contamination. SuperScript III RT (Invitrogen) and oligo dT primers were used to create cDNA. PCR was then performed using Platinum Pfx DNA polymerase (Invitrogen). PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. The following primers were designed to select for cDNA derived from mRNA:

<i>cdk-5</i>	Primer 1: 5' ggg-gat-gat-gag-ggt-gtt-cca-agc 3' Primer 2: 5' ggc-gac-cgg-cat-tg-aga-tct-ctg-c 3'
<i>nud-2</i>	Primer 1: 5' ggg-gaa-caa-gga-tcg-aca-gga-cg 3' Primer 2: 5' ccg-gta-gat-act-cgt-tgg-ctt-ttt-tc 3'
<i>cdka-1</i>	Primer 1: 5' ccg-ggt-cat-cgt-cag-aag-cca-cgt-c 3' Primer 2: 5' gac-gtt-cgt-ctg-gta-aca-cgt-cac-g 3'
<i>rpl-21</i>	Primer 1: 5' atg-act-aac-tcc-aag-ggt-c 3' Primer 2: 5' tca-cgc-aac-aat-ctc-gaa-ac 3'

#### 4.7. Fluorescence microscopy

Identification of neuronal cell types expressing  $P_{nud-2}::NUD-2::GFP$  or  $P_{cdka-1}::CDKA-1::GFP$  was performed by comparing GFP expression patterns to well-characterized control lines displaying GFP fluorescence within cholinergic, GABAergic, or dopaminergic neurons (data not shown). To observe possible alterations in GABA neuronal architecture, *lin-15(n765ts)* X *juIs1* animals were fed HT115(DE3) cells carrying DNA specific to the target gene cloned into vector L4440 as previously described (Williams et al., 2004). Briefly, RNAi-treated animals were examined for proper extension of axonal processes and branching of commissures; additionally, the number of GABAergic cell bodies within the ventral nerve cord was

scored. To be considered wild type, the number of commissures scored in each animal was 14–16. Likewise, the number of GABAergic cell bodies considered wild type within the ventral cord was 20. To score GABA vesicle distribution defects, *lin15(n765)* *oxIs12* X animals were exposed to dsRNA and mounted in 3 mM levamisole on 2% agarose pads for observation using a Nikon Eclipse E800 epifluorescence microscope equipped with DIC optics and Endow GFP HYQ and UV-2E/C DAPI filter cubes (Chroma, Inc.). Analysis was performed at 600–1000 $\times$  magnification.

#### 4.8. Behavioral assays

Convulsion assays were performed as previously described by adding either 2.5 or 10 mg/ml pentylenetetrazole [(PTZ) Sigma] to NGM plates (Williams et al., 2004). Worms were placed onto the drug plates and observed for a period of 30 min. Animals were scored positive for convulsions if they demonstrated repetitive body contractions (“tonic-clonic” convulsions) or a stiffened body posture (“tonic” convulsions). If worms appeared overtly rigid but still viable and did not respond to touch stimulus following gentle prodding with a platinum wire, they were deemed “tonic”. Fifty  $F_1$  offspring of animals fed bacteria producing dsRNA against the target genes were observed at each concentration of PTZ, unless noted otherwise.

Muscimol paralysis assays were performed as previously described (McIntire et al., 1993) with the following modifications: worms were observed for pharyngeal pumping immediately following placement upon NGM plates seeded with concentrated *E. coli* OP50 containing muscimol (Sigma) at a concentration of 3 mM. If the pharynx continued to pump for 1 h, worms were scored as unresponsive to muscimol; worms responding within the first hour (i.e., no pharyngeal pumping) were scored as responsive to muscimol. A total of 20  $F_1$  offspring of animals fed bacteria producing dsRNA against the target genes for each strain were observed.

Recovery assays were performed by placing animals on NGM plates supplemented with PTZ as noted above. After 1 h, animals displaying the tonic convulsive phenotype were removed from the PTZ drug plates and placed on NGM plates seeded with *E. coli* OP50 containing no drugs. Animals were observed after 60 min on these PTZ-free NGM plates. The number of animals resuming normal wild type movement was scored. Normal movement refers to sinusoidal crawling as well as normal foraging behavior, where the head moves side to side. A total of 100 animals displaying the tonic phenotype were observed for each target gene. Both N2 and *lis-1/+* animals were examined in the recovery assays, but only for those targets and strains determined to display a significant level of response in the original convulsion assays.

#### 4.9. Statistical analysis

Statistical analysis of all data sets was performed using the online program for the Fisher Exact Test found at <http://home.clara.net/sisa/fisher.htm>. Results given are two-sided *p* values found by comparing two data sets as appropriate for specified comparisons. Statistical analyses were performed

comparing convulsion results for target gene knockdown to the genotypic background not fed dsRNA, convulsion results between different genotypic backgrounds for a given target, rate of recovery between genotypic backgrounds, and results of GFP assays following RNAi knockdown of target genes as compared to the genotypic background not treated with dsRNA.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at, [doi:10.1016/j.brainres.2006.08.067](https://doi.org/10.1016/j.brainres.2006.08.067).

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