The C2H2 zinc-finger protein SYD-9 is a putative posttranscriptional regulator for synaptic transmission

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Communication between neurons is largely achieved through chemical synapses, where neurotransmitters are released from synaptic vesicles at presynaptic terminals to activate postsynaptic cells. Exo- and endocytosis are coordinated to replenish the synaptic vesicle pool for sustained neuronal activity. We identified *syd-9* (syd, synapse defective), a gene that encodes multiple C2H2 zinc-finger domain-containing proteins specifically required for synaptic function in *Caenorhabditis elegans*. *syd-9* loss-of-function mutants exhibit locomotory defects, a diffuse distribution of synaptic proteins, and decreased synaptic transmission with unaffected neurodevelopment. *syd-9* mutants share phenotypic and ultrastructural characteristics with mutants that lack synaptic proteins that are required for endocytosis. *syd-9* mutants also display genetic interactions with these endocytotic mutants, suggesting that SYD-9 regulates endocytosis. SYD-9 proteins are enriched in the nuclei of both neuron and muscle cells, but their neuronal expression plays a major role in locomotion. SYD-9 isoforms display a speckle-like expression pattern that is typical of RNA-binding proteins that regulate premRNA splicing. Furthermore, *syd-9* functions in parallel with *unc-75* (unc, uncoordinated), the *C. elegans* homologue of the CELF/BrunoL family protein that regulates mRNA alternative splicing and processing, and is also required specifically for synaptic transmission. We propose that neuronal SYD-9 proteins are previously uncharacterized and specific posttranscriptional regulators of synaptic vesicle endocytosis.

BrunoL/UNC-75 | *Caenorhabditis elegans* | endocytosis | synaptic function

Chemical synapses mediate neuronal communication through the regulated release of neurotransmitters from synaptic vesicles. Extensive studies have focused on the molecular machinery that mediates and regulates exocytosis, the process in which membrane fusion between synaptic vesicles and the plasma membrane leads to neurotransmitter release and endocytosis, the process that recovers synaptic vesicles from the plasma membrane (1, 2).

The release of neurotransmitters is stimulated by the influx of Ca^{2+} at the presynaptic active zones. Protein components of the vesicle membranes (synaptobrevin, SNB) and plasma membranes [syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa)] form a SNARE complex that functions as a minimal fusion machine to drive vesicle fusion (3–5). The formation and conformational change of the SNARE complex requires modulation by other proteins (6–9). Synaptotagmin (SNT) is a vesicle membrane protein that binds to Ca^{2+} and forms complexes with phospholipids and SNARE complexes. It is a proposed Ca^{2+} sensor for Ca^{2+}-dependent exocytosis (10–12). SNT-1 knockout mice display selective loss of Ca^{2+}-triggered fast exocytosis, and loss of SNT function in *Caenorhabditis elegans* and *Drosophila* leads to a large reduction of exocytosis (13, 14). Disrupting the function of UNC-13/mUNC13 and UNC-18/mUNC18 (UNC, uncoordinated) also leads to a reduction in exocytosis and an accumulation of unreleased synaptic vesicles (15, 16). After exocytosis, the recycling of synaptic vesicles serves as a major mechanism to replenish synaptic vesicle pools (2, 17, 18). The best-characterized mechanism is clathrin-mediated endocytosis, in which the adaptor proteins AP2 and AP180 recruit a clathrin matrix at the sites of endocytosis, generally considered to be at plasma membranes flanking the active zones, to initiate the membrane invagination and budding of vesicle precursors. The budded vesicles are cleaved from the plasma membrane and stripped of clathrin, and they mature into synaptic vesicles. Synaptojanin, a phosphoinositol phosphatase that forms a protein complex with AP2, is required for the budding and uncoating of vesicle precursors and the maturation of synaptic vesicles from the resulting endosomes (19, 20). Endophilin binds both synaptojanin and dynamin and is required for multiple steps of endocytosis through localizing synaptojanin at the synapse (21, 22). In *Drosophila* and *C. elegans*, synaptojanin and endophilin mutants show severe depletion of synaptic vesicles, and synaptic vesicle precursors are arrested at various endocytotic stages (21–23). SNT also regulates endocytosis through its interaction with AP2 (24). *Drosophila* and *C. elegans* SNT mutants display depletion of vesicles at synapses (13, 25). Furthermore, SNT is required during endocytosis for the replenishment of vesicles at *Drosophila* neuromuscular junctions (NMJs) (26).

Recent studies suggest that specific regulation or fine-tuning of synaptic activity can also be achieved at posttranscriptional levels (27). Rapid protein synthesis is associated with changes in synaptic activity (28–31). Alternative splicing of ion channels and other synaptic proteins plays a key role in modulating the activity of synapses (27, 32). Identification of a vertebrate splicing factor, Nova, which is not only nervous system-specific but also exhibits a profound preference for RNAs encoding synaptic proteins (33), suggests that transcriptional regulatory machinery dedicated to synaptic genes is present. Two neuronal nuclei-restricted RNA-binding proteins, UNC-75 and EXC-7, are required for synaptic transmission without affecting neuronal development or synaptogenesis in *C. elegans* (34). UNC-75 is the *C. elegans* ortholog of the CELF/BrunoL family of proteins that control pre-mRNA splicing in mammals (35, 36) and mediate translation repression in *Drosophila* oocytes (37, 38). EXC-7 is the *C. elegans* ELAV (embryonic lethal abnormal vision)/Hu family protein, a neuron-specific alternative splicing factor in *Drosophila* (39–42) and neuron-specific RNA-processing regulator in mammals (43–45). We identified a previously uncharacterized nuclear protein, *syd-9* (syd, synapse defective), in *C. elegans* that specifically regulates synaptic transmission: in particular, endocytosis. *syd-9* mutants are synthetically lethal with *unc-75* mutations. We propose that *syd-9* may regulate RNA splicing events that are specific for synaptic transmission.

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Abbreviations: AP, adaptor protein; NMJ, neuromuscular junction; SNB, synaptobrevin; SNT, synaptotagmin; SNG, synaptogyrin; RNAi, RNA interference.

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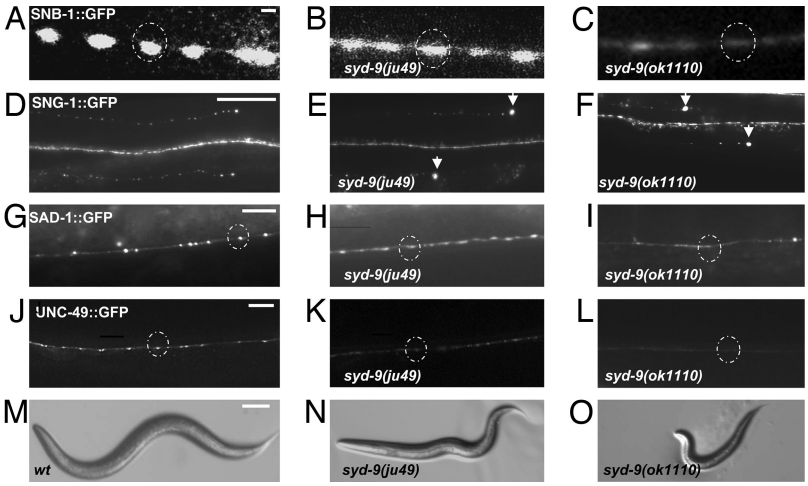

Fig. 1. Synaptic defects in *syd-9* mutant animals. (*A–C*) The SNB-1::GFP vesicle marker in GABAergic neurons appears diffuse and weaker in *syd-9* animals (*B* and *C*) than in WT adults (*A*). (*D–F*) another vesicle marker, SNG-1::GFP, is dimmer and smaller in *syd-9* animals (*E* and *F*) compared with WT animals (*D*) and accumulates in some neuronal cell bodies (arrows). (*G–I*) The presynaptic marker SAD-1::GFP is diffuse and dim in *ju49* (*H*) and *ok1110* (*I*) animals compared with WT animals (*G*). (*J–L*) The postsynaptic marker UNC-49::GFP GABA receptor appears moderately or severely diffuse and dim in *ju49* (*K*) and *ok1110* (*L*) animals. (*M*) WT young adult animal. (*N*) *ju49* young adult. (*O*) *ok1110* young adult. Individual fluorescent puncta were circled in WT and mutants for comparison of their shapes. (Scale bars: *A–L*, 5 μm; *M–O*, 80 μm.)

Results

Identification and Behavioral Phenotypes of *syd-9* Mutants. *juIs1* is a synaptic vesicle marker that expresses the GFP-labeled vesicle protein SNB (SNB-1::GFP) in GABAergic motoneurons of *C. elegans* (46). We identified a mutant named *syd-9(ju49)* that displays moderate diffusion and reduction in the brightness of the individual SNB-1::GFP punctum at presynaptic termini (Fig. 1 *A–C*). A second *syd-9* allele, *ok1110*, was later generated by the *C. elegans* Gene Knockout Consortium (Oklahoma City, OK). Both alleles of *syd-9* mutants display a drastic decrease in locomotion and a significant delay in development. Wild-type (WT) *C. elegans* displays a continuous and smooth sinusoidal movement that does not vary during development. By contrast, *syd-9* mutant animals are sluggish, with very slow locomotory activity and no obvious sinusoidal wave (Fig. 1 *M–O*). Although the locomotion defects are obvious upon hatching for *ok1110* animals, *ju49* animals start to display obvious defects from the late second-larval stage. Both alleles of *syd-9* mutants are also slow to develop, exhibiting a thinner and smaller body size and producing very few progeny, with *ok1110* being slower than *ju49* animals (Fig. 1 *M–O*). All *syd-9* animals display a starved appearance characterized by enlarged gut lumen as well as weak and sporadic pharyngeal pumping and enteric activities. *syd-9* mutants also retain eggs. Severity of the phenotypes and complementation tests by deficiencies suggest that *ju49* represents a severe loss-of-function allele, whereas *ok1110* represents a close to null allele of the *syd-9* gene. Because *ok1110* was obtained toward the end of the study, some studies were carried out only in the *ju49* allele.

***syd-9* Regulates Synapse Morphology and Presynaptic Protein Distribution.** *C. elegans* locomotion, pharyngeal pumping, and egg laying are controlled by defined neural circuits that innervate specific muscle cells. These phenotypes suggest a decrease in general neuronal or muscle activities in *syd-9* animals. The number, position, and morphology of neurons and their axonal projections, as visualized by a panneural GFP marker, *edIs20*, and a GABAergic-specific GFP marker, *juIs76*, were normal in both alleles of *syd-9* mutants (Fig. 7, which is published as supporting information on the PNAS web site). The ventral and dorsal nerve cords appeared slightly defasciculated, with some extra branching in *ok1110* animals, which is likely a secondary phenotype caused by synapse defects (see below). We did not detect gross abnormality in body wall, uterine, and vulva muscles by phalloidin staining.

The generally normal morphology of the nervous system and musculature led us to examine whether the behavioral defects were caused by defects at synapses. In addition to the SNB-1::GFP marker, abnormalities were observed in the distribution of other synaptic proteins, including the vesicle protein synaptogyrin SNG-1 (47), the presynaptic Ser/Thr kinase SAD-1 (48, 49), and the postsynaptic GABA receptor UNC-49. In *ju49* mutants, SNB-1::GFP, SNG-1::GFP, SAD-1::GFP, and UNC-49::GFP puncta were moderately dimmer and diffuse along the axons (Fig. 1 *B*, *E*, *H*, and *K*). These markers were severely diffuse and reduced in brightness in *ok1110* animals (Fig. 1 *C*, *F*, *I*, and *L*). There was also abnormal accumulation of SNG-1::GFP in some neuronal cell bodies (Fig. 1 *D* and *E*).

***syd-9* Specifically Regulates Synaptic Transmission.** The localized defects in synapse morphology in *syd-9* mutants suggest that SYD-9 regulates synaptic activity specifically. We examined the spontaneous and evoked neurotransmitter release at NMJs in *syd-9(ju49)* animals (50, 51). The evoked response was measured by recording the postsynaptic current in voltage-clamped muscles resulting from stimulation of the ventral nerve cord (Fig. 2*A*). *syd-9* mutants showed a 60% decrease in the amplitude of the evoked response,

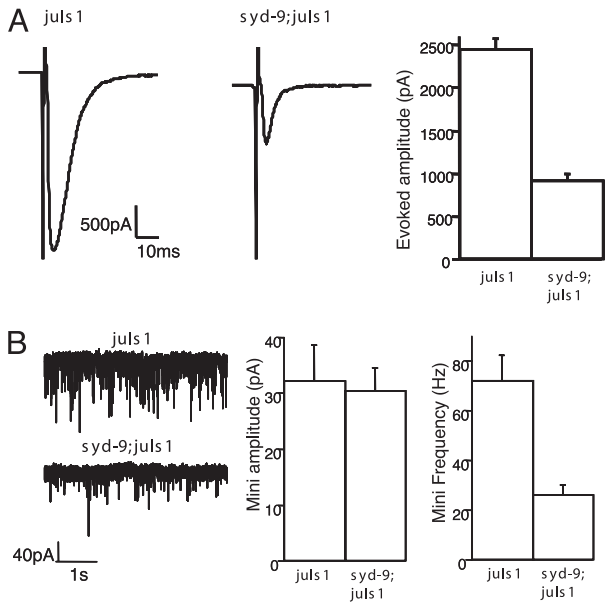

Fig. 2. Neurotransmitter release defects in *syd-9* animals. (*A*) The evoked response of voltage-clamped body wall muscles to ventral nerve cord stimulation was reduced in *ju49* mutants, as shown in sample traces and mean response amplitudes. (*B*) The endogenous synaptic event frequency was reduced in *ju49* mutants, but the remaining event amplitudes were indistinguishable from WT.

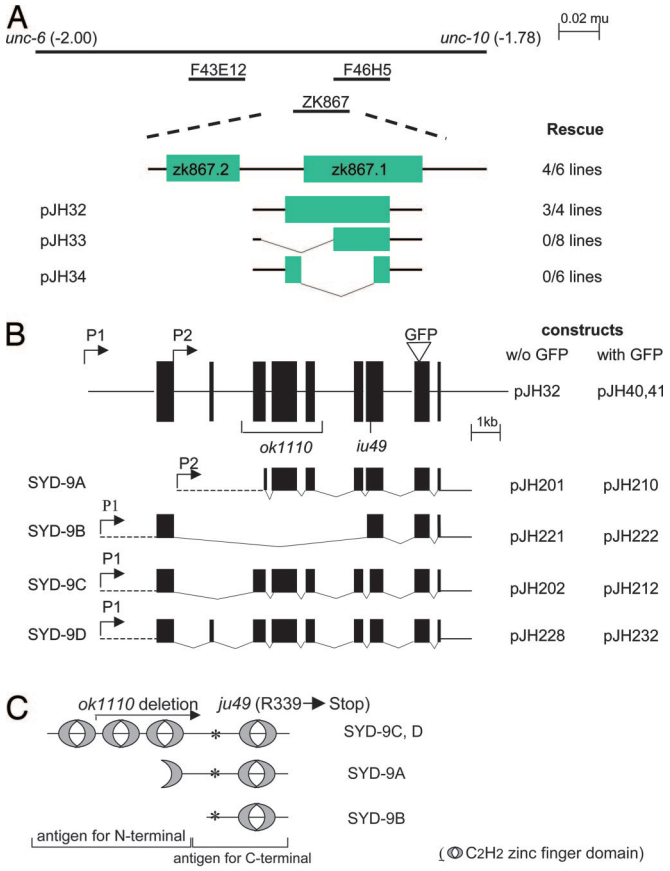

Fig. 3. Cloning of the *syd-9* gene. (*A*) *syd-9* was mapped to the X chromosome between *unc-6* and *unc-10* and rescued by a genomic fragment that contains the ORF ZK867.1. Partial deletion of ZK867.1 abolishes the rescuing activity. (*B*) Genomic organization of the four isoforms of *syd-9* resulting from alternative transcription and splicing of ZK867.1. P1 and P2 are presumptive promoters for respective isoforms. Black bars, individual exons; solid lines, introns. (*C*) Protein structure of four SYD-9 isoforms.

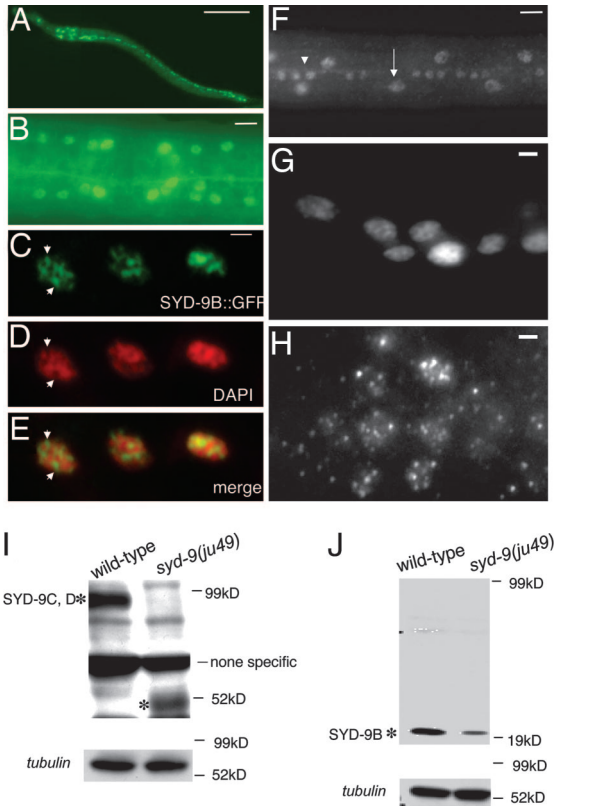

Fig. 4. SYD-9 proteins are predominantly expressed in nuclei of neuron and muscle cells. (*A* and *B*) *C. elegans* carrying a functional SYD-9::GFP construct stained with antibodies against GFP. (*A*) Larvae showing strong GFP expression in neurons of the ventral nerve cord, pharynx, and tail ganglia. Weak muscle expression is out of the focusing plane. (*B*) A ventral view of adults showing GFP in vulval and uterinal muscle nuclei. (*C–E*) Double staining with GFP and DAPI in *hpIs6* animals carrying only the SYD-9B::GFP isoform. SYD-9B::GFP signal was predominantly localized at the interchromatin region (arrows). (*F–H*) Staining of WT animals with SYD-9 antibody N (*F* and *G*) and SYD-9 antibody C (*H*). SYD-9 is expressed in the nuclei of body wall muscles (arrow) and ventral cord motoneurons (arrowhead). At higher magnification, SYD-9 expression shows a distinct subnuclear pattern similar to that of SYD-9::GFP; in particular, antibody C shows a speckle-like pattern (*G* and *H*). Antibody N staining in the retrovesicular ganglion neurons (*G*) and antibody C staining in the nerve ring ganglion neurons (*H*) are shown. (*I* and *J*) Western blot analysis of mixed-stage *C. elegans* lysates by using antibody N (*I*) and antibody C (*J*). Tubulin is the loading control for lysate. (Scale bars: *A*, 20 μm; *B* and *F*, 3 μm; *C–E*, *G*, and *H*, 1 μm.)

indicating a dramatic decrease in regulated release. Compared with WT animals, the frequency of endogenous synaptic events at *syd-9* mutant NMJs was reduced by 70%, whereas the amplitudes of the remaining release events remained similar (Fig. 2*B*). Thus, the reduced synaptic transmission in *syd-9* mutants likely results mainly from a decrease in presynaptic vesicle fusion rather than from alterations in postsynaptic response to neurotransmitters. Exposure to aldicarb, an inhibitor for acetylcholine esterase, causes hypercontraction and lethality in *C. elegans*. Response to aldicarb has been used as an indirect measure for synapse activity in cholinergic synapses (52, 53). *ju49* adults displayed sensitivity to aldicarb comparable to that of WT animals, whereas *ok1110* mutants were acutely resistant to aldicarb (not shown), suggesting a strong reduction in cholinergic synapse activity in *ok1110* mutants.

***syd-9* Gene Encodes Multiple Isoforms of C2H2 Zinc-Finger Proteins.** We identified a single ORF ZK867.1 that restored active sinusoidal locomotion and *juIs1* marker morphology in *syd-9* animals (Fig. 3*A*). It also improved the brood size in *syd-9* animals. The predicted *syd-9* gene encodes four protein isoforms resulting from alternative splicing. All predicted SYD-9 proteins contain C2H2 zinc-finger motifs (Fig. 3*C*) that can potentially mediate interactions with DNA, RNA, and protein domains (22). These motifs are most homologous to zinc fingers in human proteins ZNF574, Glis2 (54, 55), and ZNF74 (Fig. 8, which is published as supporting information on the PNAS web site).

Two antibodies were generated against SYD-9 proteins to analyze their expression (Fig. 4 *F–H*). One antibody, which was developed against the N-terminal region of the large isoforms, recognized SYD-9C and -9D (Fig. 4*I*). The other antibody, generated against the C-terminal portion, recognized only the smallest isoform, SYD-9B (Fig. 4*J*). We were unable to detect SYD-9A. In *ok1110* animals, a deletion from exon 2 results in a frameshift for SYD-9A, -9C, and -9D transcripts that abolishes their expression. Whole-mount staining using the antibody against SYD-9B also showed a drastic reduction in *ok1110* animals (not shown). In the *ju49* allele, a point mutation converts Arg-339 (as in SYD-9D) into a premature stop codon immediately before the last C2H2 domain that is shared by all SYD-9 isoforms (Fig. 3 *B* and *C*). This mutation led to a truncation of SYD-9C and SYD-9D and a severe reduction in the level of SYD-9B (Fig. 4 *I* and *J*).

To examine whether all SYD-9 isoforms contribute to the *syd-9(ju49)* phenotype, we generated minigenes that express each isoform with their predicted endogenous promoter regions (Fig. 3*B*; see also Table 1, which is published as supporting information

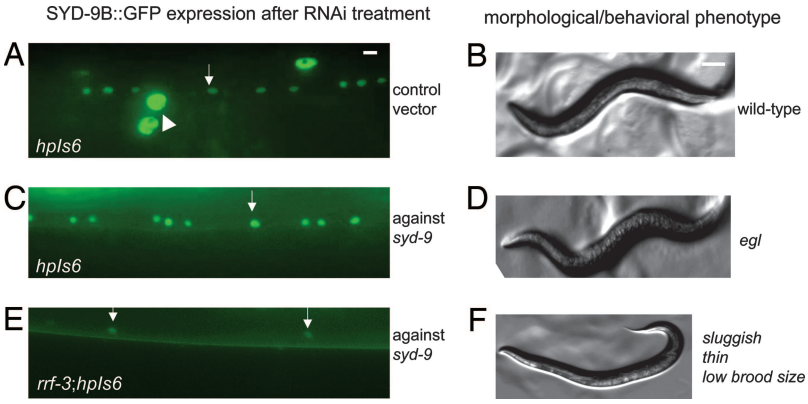

Fig. 5. SYD-9 plays a major role in neurons. *hpIs6* animals expressing SYD-9B::GFP in the nuclei of muscles (arrowheads) and neurons (arrows) were used to monitor the efficiency of RNAi-induced down-regulation of endogenous SYD-9. (*A* and *B*) *hpIs6* animals treated with empty RNAi vector display normal expression of SYD-9::GFP and no behavioral defects. (*C* and *D*) *hpIs6* animals treated with pJH147, an RNAi construct against all SYD-9 isoforms. Muscle GFP disappeared, whereas neuronal GFP remained bright (arrows). These animals were *egl* (retaining eggs) but otherwise normal. (*E* and *F*) *rrf-3;hpIs6* animals treated with pJH147 showed a reduction of GFP in neurons in addition to the absence of GFP in muscle. These animals were sluggish and developmentally delayed. (Scale bars: *A*, *C*, and *E*, 1 μm; *B*, *D*, and *F*, 80 μm.)

on the PNAS web site). The robust rescue of *syd-9(ju49)* phenotypes could only be achieved by cotransforming plasmids expressing the small isoforms with either one of the large isoforms, suggesting that both large and small isoforms are required for *syd-9* function.

SYD-9 Protein Is Predominantly Expressed in the Nuclei of Neurons and Muscles. Whole-mount staining with SYD-9 antibodies showed specific staining in the nuclei of neurons and muscles in WT animals (Fig. 4 *F–H*), whereas in *ju49* and *ok1110* animals, the staining either disappeared completely or was drastically reduced. Both antibodies revealed staining patterns enriched at specific subdomains within the nuclei (Fig. 4 *G* and *H*). The C-terminal antibody detected a speckle-like pattern exclusively in the nuclei (Fig. 4*H*). The N-terminal antibody stained a broader region but also included the speckle-like pattern in the nuclei (Fig. 4*G*), suggesting that SYD-9 isoforms may have slightly different subcellular distribution.

SYD-9::GFP expression is consistent with the expression pattern of endogenous SYD-9 proteins. A functional SYD-9::GFP construct was generated by inserting GFP after the C-terminal zinc-finger domain so that it tags all SYD-9 isoforms (Fig. 3*B*). GFP expression was observed throughout the nervous system and muscle cells (Fig. 4 *A* and *B*). SYD-9::GFP also accumulates in speckle-like patterns. To further define the subnuclear pattern, we counterstained for DNA in *hpIs6*, a strain that expresses the smallest isoform, SYD-9B::GFP. SYD-9B::GFP preferentially accumulates at interchromatin regions (Fig. 4 *C–E*). Speckle-like patterns at the interchromatin regions are hallmarks for many RNA-binding proteins and proteins regulating pre-mRNA processing (56), suggesting that SYD-9 is likely involved in pre-mRNA processing.

SYD-9 Is Predominantly Required in Neurons to Rescue Synaptic and Behavioral Defects. The expression of SYD-9 in both muscles and neurons raises the question of where SYD-9 function is required. We performed mosaic analysis using *syd-9(ju49);hpEx[sur-5::GFP;pJH32]* in which *syd-9* mutants were rescued by an extrachromosomal array that carried the rescuing genomic fragment and a marker that expressed GFP in cell nuclei that inherit the array (57). We generated five *syd-9* mosaic animals that had lost the array in the lineage that generates all neurons but retained it in most muscle cells. All these animals were reminiscent of *syd-9* mutants, with severe locomotion defects, slow development, thin bodies, and low brood sizes. By contrast, 10 of 20 mosaic animals that maintained the rescuing array in the neuronal lineage, but not the muscle lineage, displayed normal sinusoid locomotion and healthy body morphology in larvae. As they approached adulthood, the rescue was less apparent and brood size was still low, suggesting that rescue was partial. The mosaic analysis suggests that neuronal SYD-9 plays a more dominant role in locomotion.

We next used RNA interference (RNAi) to determine the functional requirement for SYD-9. A tissue-specific down-regulation of SYD-9 expression is possible because the *C. elegans* nervous system is generally resistant, whereas muscles are susceptible, to RNAi effects (58). We used the *hpIs6* line (Fig. 5*A*) to monitor the effectiveness of the RNAi on endogenous SYD-9 proteins. After treating *hpIs6* animals with dsRNA against all *syd-9* isoforms, GFP completely disappeared from the nuclei of muscles, whereas the level of GFP in neurons was unchanged (Fig. 5*C*). These animals displayed sinusoidal and active locomotion and healthy body morphology, suggesting that the muscle SYD-9 is largely dispensable (Fig. 5*D*; see also Movies 1 and 2, which are published as supporting information on the PNAS web site). Mutation in the *rrf-3* gene leads to an increase in RNAi sensitivity in all tissues, including neurons (59). Consistently, >90% of *rrf-3;hpIs6* animals treated with the same RNAi construct displayed a significant decrease of neuronal GFP intensity and absence of GFP in muscles (Fig. 5*E*). Sluggish locomotion was observed in these animals, with some displaying starved-looking body morphology and low brood size (Fig. 6*F*; see also Movie 3, which is published as supporting information on the PNAS web site), similar to *syd-9*

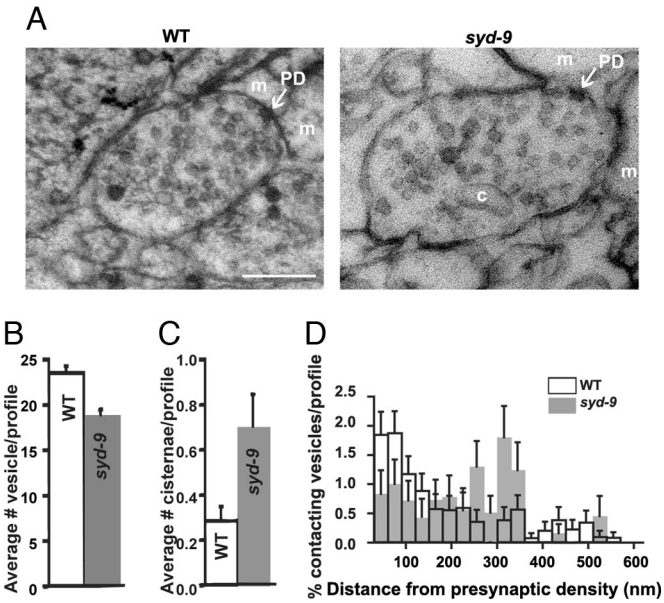

Fig. 6. EM analysis of *syd-9(ju49)* mutants suggests that vesicle recycling is impaired. (*A*) Sections from WT and *ju49* ventral nerve cord showing a NMJ. n, neuron; m, muscle; PD, presynaptic density; c, cisternae. (*B*) The average number of vesicles per synaptic profile is reduced in *ju49* animals. (*C*) The number of large membrane-bound organelles (cisternae) is increased in *ju49* profiles. (*D*) The distribution of plasma membrane-contacting vesicles in *ju49* mutants is redistributed away from the presynaptic specialization toward a region involved in vesicle endocytosis. Error bars show SEM.

mutants. These results also indicate that neuronal SYD-9 plays a more important role in regulating locomotion.

Last, we generated constructs in which a panneuronal promoter drives the expression of individual isoforms. Coinjection of these constructs significantly improved the locomotion and body morphology defects of *ju49* animals in larvae. It also restored the accumulation of GABA receptors (*oxIs22*) at the NMJs in *ok1110* animals (Fig. 9, which is published as supporting information on the PNAS web site), suggesting that the postsynaptic receptor clustering depends on neuronal SYD-9 proteins. These constructs did not rescue morphology in adults and the low brood size of *syd-9* mutants. By contrast, expressing SYD-9 in muscles did not rescue locomotion or restore GABA receptors at NMJs in *ok1110* animals (Fig. 9). In these animals, GABA receptors were increased in abundance but failed to concentrate at synapses (Fig. 9). This result supports the conclusion that SYD-9 is required in neurons and also suggests a participating role of muscle SYD-9 in regulating GABA receptors. Taken together, we demonstrated that *syd-9* functions predominantly in neurons to regulate synaptic function.

***syd-9* Mutants Show Genetic Interactions with Endocytotic Mutants.** The predominantly neuronal requirement of SYD-9 and specific defects for synaptic function in *syd-9* mutants suggest that *syd-9* may regulate synaptic transmission through known synaptic vesicle recycling pathways. We examined the genetic interactions between *ju49* and known regulators of synaptic function, including SNT-1, synaptojanin (UNC-26), endophilin (UNC-57), AP180 (UNC-11), syntaxin (UNC-64), SNAP-25 (RIC-4), UNC-13, RAB-3, SYD-2, RPM-1, and SAD-1. *syd-9(ju49)* mutants showed no specific genetic interactions with mutations in UNC-64, RIC-4, UNC-13, RAB-3, SYD-2, RPM-1, and SAD-1 because the double mutants displayed the locomotion and developmental characteristics of both single mutants. The double mutants were "sicker" than the single mutant alone, as expected from an additive effect; they proceeded in development and propagated.

syd-9(ju49) mutants displayed specific genetic interactions with *snt-1*, *unc-26*, *unc-57*, and *unc-11*, all genes that participate in endocytosis (13, 14, 19, 21, 60). In fact, the morphological defects of *syd-9* (specifically, the thin body and lack of sinusoidal wave) are reminiscent of these mutants. SNT is required for both exocytosis and endocytosis in *C. elegans* (13, 14). *snt-1;syd-9* double mutants were 100% lethal upon hatching. Although *snt-1(md290)*$^{/+}$*;syd-9(ju49)*$^{/+}$ heterozygous animals appeared WT, they did not segregate viable *snt-1;syd-9* progeny, and *snt-1;syd-9*$^{/+}$ or *snt-1*$^{/+}$*;syd-9* animals passed on so few progeny that the strain could not be maintained. Because neither the *snt-1* nor *syd-9* gene is haploid-insufficient in the WT background, this genetic interaction suggests that SYD-9 functions either in the same pathway or in parallel to SNT-1 to regulate synaptic transmission. A slightly different genetic interaction between *syd-9* and *unc-26*, *unc-57*, and *unc-11* was observed. *syd-9(ju49);unc-26(m2)*, *syd-9(ju49);unc-57(ok310)*, and *syd-9(ju49);unc-11(e47)* animals could be recovered from *syd-9;unc-26*$^{/+}$, *syd-9;unc-57*$^{/+}$, and *syd-9;unc-11*$^{/+}$ parents. They were initially viable and behaviorally identical to *unc-26*, *unc-57*, and *unc-11* null animals but became arrested and passed on few progeny. The arrest displayed by these double mutants is different from the additive "sickness" of the single mutants. These genetic interactions support the hypothesis that *syd-9* regulates synaptic transmission, particularly endocytosis.

***syd-9(ju49)* Mutants Display Endocytosis Defects.** We performed electron microscopy analysis to examine whether *syd-9* mutants display ultrastructural features consistent with a defect in endocytosis. Several characteristic ultrastructural defects have been observed in *C. elegans* endocytosis mutants (13, 14, 19, 21, 60). These defects include a reduction in the total synapse vesicle number, reflecting the defective replenishment of synaptic vesicles, and an altered distribution pattern of synaptic vesicles, with more membrane-contacting vesicles present away from active zones. Vesicle accumulation in these membrane regions is thought to reflect a disruption or delay in the maturation and mobilization of endocytosed vesicles. Endocytosis mutants also display an increased number of vesicles with large sizes and abnormal shape or clusters, which reflects an arrest in various vesicle maturation and sorting stages. These ultrastructural phenotypes are not observed in mutants for genes with predominant roles in exocytosis.

Compared with WT synapses, the total vesicle number in *syd-9(ju49)* mutants was reduced by 25% (Fig. 6*C*). The distribution of the vesicles was further disrupted, with membrane-contacting vesicles shifting away from the presynaptic specialization and accumulating 300–350 nm distally (Fig. 6). Furthermore, we detected the presence of large and abnormal-sized vesicles, evidenced by increased numbers of cisternae (Fig. 6). The combination of ultrastructural changes in *syd-9* mutants is thus characteristic of perturbations of the classical endocytotic pathway.

***syd-9* Functions in Parallel with *unc-75*/BrunoL.** The speckle-like expression pattern of SYD-9 isoforms suggests that at least some SYD-9 isoforms may function through RNA-binding and mRNA processing. Only two RNA-binding proteins, UNC-75 and EXC-7, have been shown to function in synaptic transmission in *C. elegans*, and both display speckle-like expression patterns (34). UNC-75 is the *C. elegans* homologue of the CELF/Bruno protein family that regulates tissue-specific alternative splicing in mammals and specific mRNA translation repression in *Drosophila* oocytes. *unc-75(e950)* null mutants display uncoordinated locomotion (34) but are nonetheless healthy, with close to normal brood size. All *syd-9;unc-75(e950)* double mutants were paralyzed and arrested upon hatching. This synergistic genetic interaction suggests that the *syd-9* gene functions in parallel with *unc-75* to regulate synaptic transmission. ELAV is a neuronal-specific alterative splicing factor in *Drosophila*, and most of its mammalian homologues, the Hu family proteins, are RNA processing regulators in the nervous system. In contrast to *unc-75* mutations, *exc-7* mutants did not enhance or affect *syd-9* behavioral or developmental defects, which supports the specificity of the genetic interactions between *unc-75* and *syd-9*.

Discussion

We report the identification of the SYD-9 nuclear proteins that regulate synaptic transmission: specifically, endocytosis in *C. elegans*. In *syd-9* mutants, we were unable to detect obvious defects in the differentiation of neurons and muscles. Terminal differentiation at the synapse also takes place; however, the distribution and level of several synaptic proteins are abnormal, which suggests that the function of SYD-9 is highly specific for developmental maintenance of synaptic structures and functions.

Using both mosaic analysis and RNAi, we specifically eliminated the expression of SYD-9 proteins in muscle cells. Neither manipulation resulted in obvious developmental and behavioral defects, suggesting that the muscle expression of SYD-9 is largely indispensable in larvae. It remains possible that there are subtle changes that we did not analyze for (such as the level of GABA receptors) or that the remaining SYD-9 proteins in muscles, although at a much reduced level, are sufficient for development. However, the potential defects in muscles did not contribute greatly to defects in *syd-9* larvae, because neuronal-specific expression of SYD-9 can rescue *syd-9(ju49)* mutants to a similar degree as the *syd-9* genomic fragment. Treatment of *hpIs6* animals with RNAi against *syd-9* led to "Egl" adults that displayed a delay in egg-laying, most likely caused by a down-regulation of hermaphrodite-specific neuron activity (Movies 1 and 2 and Fig. 10, which are published as supporting information on the PNAS web site). *syd-9*, therefore, is predominantly required in neurons to regulate synaptic function.

The phenotype of *syd-9* mutants and the genetic interactions between *syd-9* and several endocytotic mutants strongly suggest that

syd-9 functions through, or in parallel to, clathrin-mediated endocytosis during synaptic transmission. C2H2 domains have been implicated in binding DNA, RNA, and protein motifs. Because SYD-9 protein localizes to nuclear speckles, it is possible that SYD-9 regulates the posttranscriptional processing of transcripts for proteins that are essential for endocytosis. We determined that at a gross level, SNT-1 and several other presynaptic proteins in *syd-9(ju49)* animals (Fig. 11, which is published as supporting information on the PNAS web site) were not significantly altered. Therefore, it is unlikely that SYD-9 directly regulates the transcription of the most obvious synaptic regulators.

SYD-9 proteins likely function together or in parallel with UNC-75, a BrunoL family pre-mRNA processor, to regulate the transcription of proteins that are specifically required for endocytosis. Many RNA-binding proteins and proteins that regulate pre-mRNA processing display speckle-like patterns located at the interchromatin regions (56). Biochemical studies in mammalian systems have identified several C2H2 zinc-finger motif-containing proteins that localize to nuclear speckles and play a role in transcription or posttranscriptional mRNA processing events, (54, 55, 61). Interestingly, both ZNF74 and Glis2, the human proteins containing zinc-finger domains highly homologous to those in SYD-9 proteins, also localize to discrete speckles in nuclei. ZNF74 directly interacts with the hyperphosphorylated form of RNA polymerase II and colocalizes with splicing factors in the nuclear matrix, implicating its potential role in mRNA processing (54, 55). We speculate that at least certain isoforms of SYD-9 function as splicing factors or premRNA processors that specifically regulate the splicing and translation of RNA transcripts that are essential to mediate endocytosis.

Recent studies have just begun to reveal the specific roles for transcriptional and translational regulation in synaptic function. NOVA, BrunoL/UNC-75, and ELAV/EXC-7 were the only reported mRNA processing proteins that specifically regulate synaptic functions. Our studies revealed SYD-9 as a previously uncharacterized factor that functions in parallel to UNC-75 and regulates endocytosis through mRNA processing. *syd-9*, *unc-75*, and *exc-7* mutants provide powerful genetic tools for uncovering other components and targets of the regulatory machinery specific for synapse function.

Methods

***C. elegans* Genetics and Molecular Biology.** See *Supporting Methods*, which is published as supporting information on the PNAS web site.

RNAi Against *syd-9*. pJH147 was transformed in HT115, induced by 1 mM isopropyl β-D-thiogalactoside (IPTG) for 2 h and seeded on nematode growth medium plates with carbenicillin (25 μg/ml) and IPTG (1 mM). L4 animals were propagated on these plates at 15°C.

Generation of Transgenic Lines. *hpIs6* was generated by integrating pJH35 and Lin-15 plasmid into the *lin-15(n765)* animals.

Aldicarb Tests. Twenty adults were transferred to plates containing 0.1–1.5 mM aldicarb and scored for lethality every hour for 4 h. An animal was scored as dead when it appeared hypercontracted, felt flaccid, and did not respond when poked.

Electrophysiology and Ultrastructural Analysis. See *Supporting Methods.*

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