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The Immunoglobulin Superfamily Protein SYG-1 Determines the Location of Specific Synapses in *C. elegans*

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Summary

During nervous system development, neurons form reproducible synapses onto specific targets. Here, we analyze the development of stereotyped synapses of the *C. elegans* HSNL neuron in vivo. Postsynaptic neurons and muscles were not required for accurate synaptic vesicle clustering in HSNL. Instead, vulval epithelial cells that contact HSNL act as synaptic guidepost cells that direct HSNL presynaptic vesicles to adjacent regions. The mutant *syg-1(ky652)* has defects in synapse formation that resemble those in animals that lack vulval epithelial cells: HSNL synaptic vesicles fail to accumulate at normal synaptic locations and form ectopic anterior clusters. *syg-1* encodes an immunoglobulin superfamily protein that acts in the presynaptic HSNL axon. SYG-1 protein is localized to the site of future synapses, where it initiates synapse formation and localizes synaptic connections in response to the epithelial signal. SYG-1 is related to *Drosophila* IrreC and vertebrate NEPH1 proteins, which mediate cell-cell recognition in diverse developmental contexts.

Introduction

The nervous system consists of many neurons that communicate through synaptic connections. Anatomical and physiological evidence suggests that the synapses formed between neurons in local circuits are specific and stereotyped (Gupta et al., 2000; Dantzker and Callaway, 2000; Kozloski et al., 2001). For example, in mammalian hippocampus and cerebral cortex, chandelier cells form synapses only with pyramidal neurons, and these synapses are almost exclusively localized to one subcellular compartment, the axon initial segment (Benson et al., 2001). Thus, after axons are guided to the appropriate target area, additional mechanisms must ensure the formation of synapses between the correct neurons in a local environment. The initial synaptic pattern appears to be specified by activity-independent molecular cues that are later refined by neuronal activity (see review by Goodman and Shatz, 1993). The molecular cues underlying local cell-cell targeting and subcellular targeting are largely unknown.

The molecular control of synapse formation is best characterized in one specific type of synapse, the neuromuscular junction (NMJ) (reviewed by Sanes and Lichtman, 2001). When the motor nerve terminals reach the muscle, motor axons secrete agrin, which acts through the muscle MuSK receptor to cluster acetylcholine receptors. Axon terminals also secrete neuregulin that stimulates the transcription of AChRs in the synaptic nuclei and release acetylcholine that represses AChR expression from extrasynaptic nuclei. Retrograde signals from muscle induce the formation of a mature presynaptic active zone. During the development of NMJs and especially in the post-injury regeneration of NMJs, a third cell type, the Schwann cell, plays an essential role in defining synaptic sites. Thus, NMJ development involves mutual signaling between multiple cell types at the synapse.

The development of central nervous system synapses has been studied mostly in dissociated neuronal cultures, where synapse formation can be initiated by contact between presynaptic axons and postsynaptic neurons. The clustering of the presynaptic vesicle release machinery slightly precedes the clustering of postsynaptic neurotransmitter receptors in hippocampal cultures (Friedman et al., 2000), whereas the accumulation of postsynaptic NMDA receptors can precede the formation of functional presynaptic active zones in cortical glutamatergic synapses (Washbourne et al., 2002). The extent to which primary neuronal cultures preserve the specificity of synapse formation is unclear.

A variety of molecules have been shown to have effects on synaptic strength and number. An interaction between the transmembrane neurexin-neuroligin proteins is sufficient for the development of presynaptic specializations in vitro (Scheiffele et al., 2000). Many other adhesion molecules and signaling molecules, including N-cadherin, protocadherin, FasII, NCAM, synCAM, and Eph receptors have been suggested to play roles in synaptic development and function (Benson et al., 2001; Biederer et al., 2002). However, it is not clear which molecules generate the specificity of synapse formation in vivo: the ability of a presynaptic cell to recognize the correct target dendrites while disregarding irrelevant axons and dendrites.

The nematode *C. elegans* has been useful for analyzing many aspects of synaptic development and function (Nonet et al., 1993; Zhen and Jin, 1999). Its nervous system consists of 302 neurons that are connected by about 5000 chemical synapses, 2000 neuromuscular junctions, and 600 gap junctions that are reproducible between animals (White et al., 1976, 1986). Synapses between neurons are typically formed en passant, so that each cell has multiple presynaptic regions that are dispersed along the length of the axon. This configuration is similar to most synapses in the mammalian central nervous system (e.g., hippocampal CA3-CA1 synapses, cerebellar parallel fiber-Purkinje cell synapses). The presynaptic specialization includes an active zone and clusters of synaptic vesicles, whereas postsynaptic regions are characterized by clusters of neurotransmitter receptors (White et al., 1986, Rongo et al., 1998). A single neuron can have a single synaptic partner or many partners. Synapse formation is not a simple result of physical

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contact, because the number of direct contacts a neuron makes far exceeds the number of synaptic connections.

In order to understand the cellular and molecular mechanisms that underlie synapse formation in vivo, we studied en passant synapses formed by HSNL near the *C. elegans* vulva. We found that vulval epithelial cells have a guidepost role in the formation of these synapses. In a genetic screen, we identified SYG-1, an immunoglobulin superfamily protein, as a potential HSN receptor of the guidepost signal.

Results

Visualization of HSNL En Passant Synapses at the Vulva Region

Egg laying in *C. elegans* is regulated directly by four neurons: HSNL, HSNR, VC4, and VC5. All four of these neurons innervate the vm2 vulval muscles, which contract to open the vulva during egg laying. In the vulval region, the HSN neurons also form synapses onto the VC neurons (White et al., 1986). The HSN cell bodies are situated just posterior to the vulva. Each HSN axon migrates ventrally to the midline to join the ipsilateral ventral cord, where it extends to the nerve ring in the head. As they pass the vulva, the HSN axons defasciculate dorsally, branch, and form en passant synapses onto the VC neurons and vm2 muscle (Figures 1A–1D). These synapses contain serotonin and acetylcholine and mediate egg-laying behavior. HSN neurons also synapse onto a variety of targets in the nerve ring (White et al., 1986).

Presynaptic vesicles in HSNL were visualized using a SNB-1::YFP fusion gene driven by a partial *unc-86* promoter (Baumeister et al., 1996). SNB-1 is a synaptic vesicle protein that labels synaptic vesicle clusters in *C. elegans* and other animals (Nonet, 1999). In HSNL, SNB-1::YFP expression in the body was restricted to a short region where the axon contacts vm2 and the processes of VCs (Figures 1C–1F). SNB-1::YFP expression in HSNL colocalized with a LIN-10::DsRED fusion protein expressed in the VC neurons (Figures 1C–1F). LIN-10 clusters glutamate receptors in glutamatergic synapses in *C. elegans* (Rongo et al., 1998) and probably localizes to the postsynaptic specializations of VC neurons at HSN-to-VC synapses. Several results suggested that the SNB-1::YFP clusters are HSNL synaptic vesicles. First, the position of the vesicles was in close agreement with the position of HSN synapses inferred from serial section electron microscopy (White et al., 1986). Second, the vesicles were absent in the *unc-104* mutant background (Figures 1G and 1H). UNC-104 encodes a kinesin that transports synaptic vesicles from the neuronal cell body to synaptic sites (Hall and Hedgecock, 1991). Third, another synaptic vesicle protein, CAT-1::GFP, had the same expression pattern as SNB-1::YFP when expressed in HSNL (Figures 1I and 1J). CAT-1 encodes a vesicular monoamine transporter that loads serotonin into synaptic vesicles of HSNL (Duerr et al., 1999).

HSNL Synaptic Vesicles Are Patterned by Vulval Epithelial Cells

The requirements for the development of HSN synapses were assessed by genetic and surgical manipulation of animals expressing SNB-1::YFP in HSNL. We first asked whether contact between the presynaptic HSNL neuron and its postsynaptic targets VC and vm2 determines the location of their synapses. The two postsynaptic targets were eliminated during the L1 stage by killing the M cell, the precursor to all vulval muscles, in *lin-39(n709ts)* mutant animals, in which the VC neurons undergo programmed cell death in the L1 stage (Sulston and Horvitz, 1977; Clark et al., 1993). HSNL axon outgrowth occurs during L2 and L3 stages, and HSNL synapse formation occurs in the L4 stage, so it is likely that HSNL never contacts its normal postsynaptic targets in the ablated animals. Surprisingly, in 32 out of 32 ablated animals, the position and intensity of HSNL SNB-1-labeled synaptic vesicles were indistinguishable from those of wild-type animals (Figures 2A–2D). This result indicates that the postsynaptic targets of HSNL are dispensable for the normal pattern of synaptic vesicle clustering.

If HSNL synapses are not induced by the postsynaptic targets, they could be localized by an intrinsic mechanism that places them at a particular location in HSNL or by extrinsic cues. SNB-1 clusters were examined in *mig-1* mutants, in which the cell body position of HSNL is posteriorly displaced due to cell migration defects (Desai et al., 1988). In *mig-1* mutant animals, the SNB-1 clusters were still localized near the vulval opening despite its abnormally long distance from the HSNL cell body (Figures 2E and 2F, $n = 82$). This result suggests that the location of the synapses is not intrinsically specified by HSNL. To confirm this observation, we examined HSNL synapses in *dig-1* mutants, in which the cell body position of HSNL is normal but the vulva, gonad, and vulval muscles are anteriorly displaced (Thomas et al., 1990). In *dig-1* animals, SNB-1 clusters were anteriorly displaced to the vulval opening (Figures 2G and 2H, $n = 78$). These results indicate that extrinsic factors determine the location of HSNL SNB-1 clusters, probably factors made by the gonad or vulval epithelium.

Cells in the somatic gonad initiate a cascade of cell interactions that patterns the vulval epithelium, directs vulval muscle migration, and stimulates branching of HSNs and VCs (Kimble and White, 1981; Thomas et al., 1990; Li and Chalfie, 1990; Garriga et al., 1993). To ask whether the gonad localizes HSNL synapses, the somatic gonad precursor cells Z1 and Z4 were ablated in the L1 stage. These animals lack the entire gonad because germ line proliferation requires Z1 and Z4 (Kimble and White, 1981). They also lack vulval epithelium, because the induction of vulval cell fates is strictly dependent on secretion of the EGF homolog LIN-3 by the somatic gonad (Hill and Sternberg, 1992). In the Z1, Z4 ablated animals, SNB-1::YFP failed to localize in punctate clusters at the normal location (Figures 2I and 2J, $n = 35$); instead, it was displaced anteriorly in a diffuse pattern. These results indicate that the gonad or a second tissue induced by the gonad is required for correct localization of HSNL synapses.

The vulval epithelium is likely to be the direct inducer of synaptic vesicle clustering. *lin-3* mutants, in which the gonad is present but the vulval epithelium is absent due to a loss of EGF signaling, had abnormal anteriorly displaced SNB-1 clusters (Figures 2K and 2L, $n = 75$).

Vulval epithelial cells could provide either a permissive signal for HSN differentiation or a specific signal that

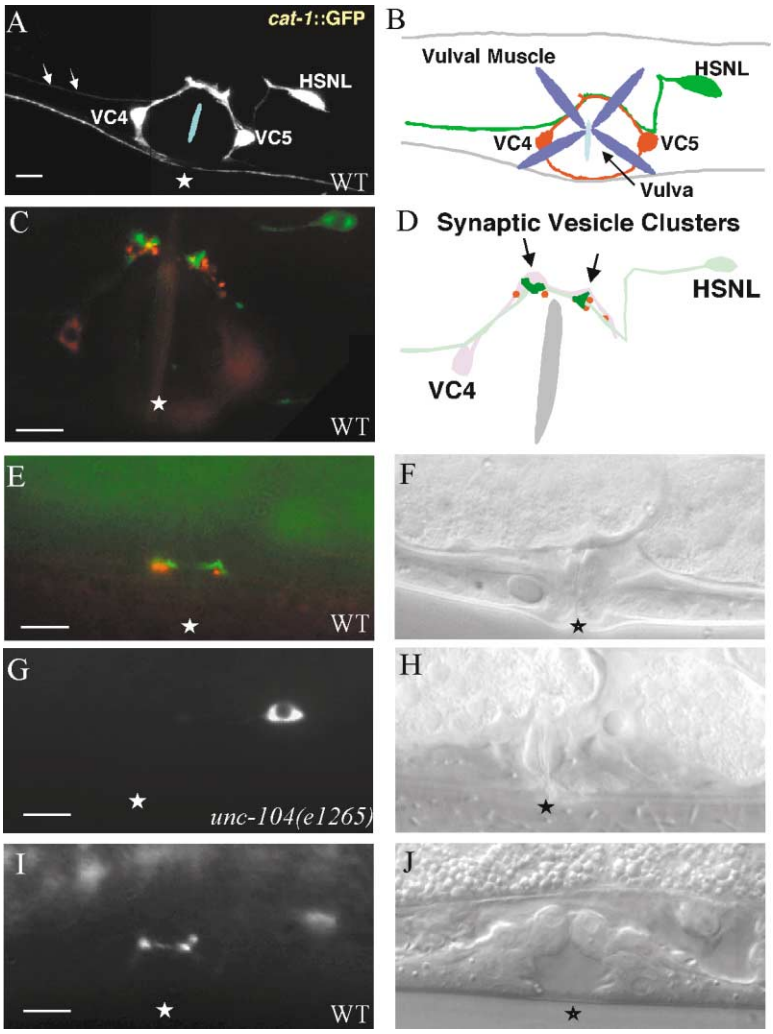


Figure 1. HSNL Forms En Passant Synapses near the Vulva

(A) Ventral view of an animal expressing a *cat-1::GFP* transgene in HSNL, VC4, and VC5. Arrows point to the segment of HSNL axon anterior to the vulva. Blue slit represents the vulval opening.

(B) Schematic representation of (A). Vulval muscles are included. Anterior is to the left and ventral right is down.

(C) Ventral view of a *kyIs235* animal with *unc-86::snb-1::yfp* colored in green and *unc-4::lin-10::dsred* colored in red. Synaptic vesicles are clustered near the vulval area.

(D) Schematic representation of (C). Arrows point to synaptic vesicle clusters. Anterior is to the left and right is down.

(E and F) Epifluorescence (E) and DIC (F) images of a *kyIs235* animal, lateral view.

(G and H) YFP epifluorescence (G) and DIC (H) images of an *unc-104(e1265); kyIs235* animal, lateral view. Note the absence of vesicles at the vulva.

(I and J) Epifluorescence (I) and DIC (J) images of an L4 *nuIs26* (*cat-1::cat-1::gfp*) animal, lateral view.

In (E–J), anterior is to the left and ventral is down. Asterisks indicates the location of the vulva. Scale bars are equal to 5μm.

determines the location of synapses. To distinguish between these possibilities, we examined SNB-1 clusters in *lin-15* mutants, which have vulval epithelium at multiple ectopic pseudovulvae in addition to the normal vulva. In these animals, multiple SNB-1::YFP clusters formed at the ectopic pseudovulvae (Figures 2M and 2N, n = 50). This result indicates that the location of vulval epithelium actively defines the location of synaptic vesicle clusters. Moreover, the vulval epithelium appears to be sufficient for this activity: when the gonad was killed in a *lin-15* mutant, SNB-1::YFP still clustered at the ectopic pseudovulvae (n = 25).

The signal from the epithelium is likely to be a cell-associated or short-range signal, since *unc-40* and *unc-6* mutants, in which the HSNL fails to contact the vulval epithelial cells due to axon guidance defects (Hedgecock et al., 1990), had an abnormal synaptic vesicle pattern similar to that of *lin-3* animals (n = 85 for *unc-40*, n = 95 for *unc-6*). In a small fraction of *unc-40* and *unc-6* mutants, HSNL axon guidance is normal due to redundant axon guidance mechanisms. SNB-1::YFP was localized to the vulva in those animals, suggesting that *unc-40* and *unc-6* affect SNB-1 localization in HSNL indirectly through their effects on axon guidance. The signal from the vulval epithelium appeared to be distinct from known signaling molecules. The vulval cells secret FGF, but synaptic vesicle clustering was normal in *egl-15* and *egl-17* mutants which disrupt the FGF receptor and FGF, respectively (DeVore et al., 1995; Burdine et al., 1997; our data not shown). Clustering was also normal in *cdh-3* mutants, which lack a vulval cadherin protein (data not shown).

A Mutation in *syg-1* Disrupts Synaptic Vesicle Clustering

To understand the mechanisms that lead to the specification of individual synapses, we performed a visual screen for mutants that affect SNB-1::YFP localization in HSNL. From this screen, we isolated the *syg-1(ky652)* mutant. 100% of *syg-1* mutants had ectopic anterior HSNL vesicles in a pattern similar to that of *lin-3* mutants (Figures 3A, 3B, 3E, and 3F) (n = 120). In 68% of *syg-1* mutants, SNB-1::YFP fluorescence was reduced or absent at the normal synaptic location (n = 120).

syg-1(ky652) mutants were viable, fertile, and coordinated. The vulval epithelium was examined by differential interference contrast (DIC) microscopy and appeared to be normal at several developmental stages

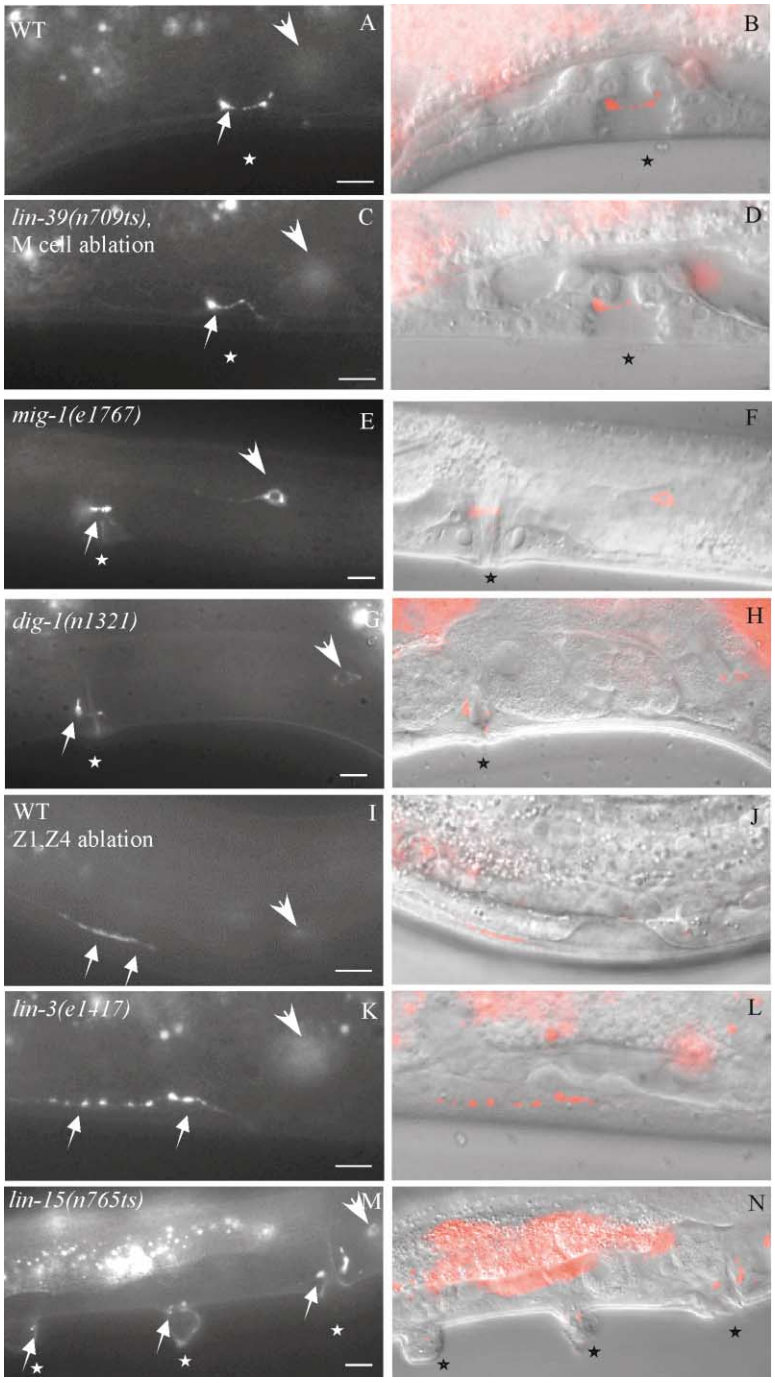


Figure 2. Synaptic Vesicle Localization Is Determined by Vulval Epithelial Cells

Paired fluorescence (left) and DIC (right) images of the vulval region. HSNL SNB-1::YFP fluorescence from *kyIs235* is shown; fluorescence is false-colored red in DIC images to show alignment. Arrows indicate synaptic vesicles, arrowheads indicate the location of the HSNL cell bodies, asterisks indicate the vulva. All images are lateral views with anterior to the left and ventral down. Scale bars are equal to 5μm.

(A and B) L4 *kyIs235* animal showing synaptic vesicles. Synaptic vesicles are positioned within the developing vulva (cavity visible by DIC).

(C and D) L4 *lin-39(n709ts); kyIs235* animal, grown at the restrictive temperature, with the M cell killed during the early L1 stage.

(E and F) Adult *mig-1(e1767); kyIs235* animal. The distance between the HSNL cell body and the vulva is increased, and synaptic vesicles cluster near the vulva.

(G and H) Adult *dig-1(n1321);kyIs235* animal. Vesicles cluster at the anterior vulva.

(I and J) L4 *kyIs235* animal in which the Z1and Z4 cells were ablated in the L1 stage. Vulval development is abnormal and diffuse synaptic vesicles are present in the anterior.

(K and L) L4 *lin-3(e1417); kyIs235* animal. Vulval epithelium is absent and synaptic vesicles are displaced anteriorly.

(M and N) L4 *kyIs235; lin-15(n765ts)* animal grown at the restrictive temperature. Synaptic vesicle clusters are present at the multiple ectopic pseudovulvae.

(Figures 3F and 3J). *syg-1* mutants had no obvious egg-laying defect, suggesting that the vulval epithelium and vulval muscles developed appropriately, and that some HSN function and neurotransmitter release persisted even with the displaced vesicles. The VC4 and 5 neurons (visualized by *cat-1::gfp*) had normal axons and branched at the vulva as in wild-type animals (data not shown). Close examination of HSN axon morphology revealed that HSN branching at the vulva was defective. 85% of wild-type animals have one or two dorsal HSN branches at the vulva (Figure 3G, n = 100), whereas only 10% of the HSNs branched in *syg-1(ky652)* mutants (Figure 3H, n = 100). In HSNL, the dorsal branch is not a major site of synapses, but like synaptic vesicle clusters, the dorsal branch is induced by cells of the vulval epithelium (White et al., 1986; Garriga et al., 1993).

The ectopic anterior SNB-1 clusters in *syg-1* mutants were present at early stages of synapse formation (Figures 3E and 3F) and persisted to the adult stage (Figure 3A). These ectopic clusters have properties suggesting that they represent displaced synaptic vesicles. For example, both SNB-1::YFP and the vesicular monoamine transporter CAT-1 were present on these ectopic clusters (Figures 3I and 3J). In addition, in *unc-104; syg-*

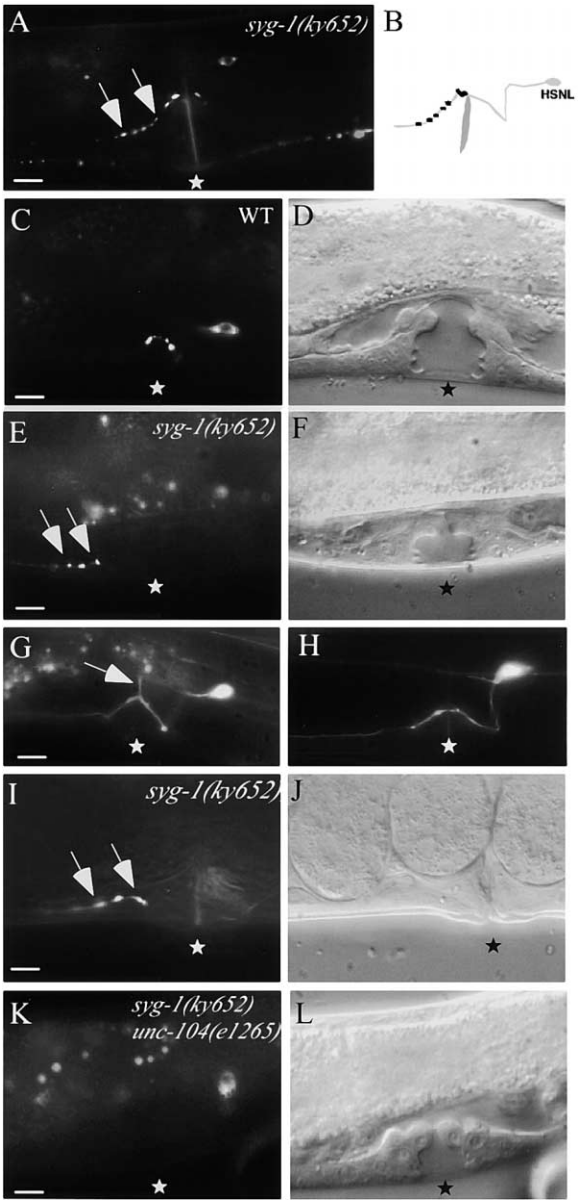


Figure 3. Synaptic Vesicle Patterns Are Abnormal in *syg-1* Mutants

(A) Ventral view of HSNL SNB-1::YFP fluorescence in an adult *kyIs235; syg-1(ky652)* animal. Arrows point to ectopic vesicle clusters. Anterior is to the left and right is down.

(B) Schematic representation of (A).

(C and D) Wild-type L4 *kyIs235* animal showing normal synaptic vesicles positioned within the developing vulva.

(E and F) L4 *kyIs235; syg-1(ky652)* animal showing vesicle clusters anterior to the developing vulva.

(G) Young adult *kyIs179 (unc-86::gfp)* animal. The arrow points to the HSN branch at the vulva.

(H) Young adult *kyIs179; syg-1(ky652)* animal. The HSN branch is missing.

(I and J) Adult *syg-1(ky652) nuIs26 (cat-1::cat-1::gfp)* animal showing CAT-1 vesicular transporter expression anterior to the vulva.

(K and L) L4 *unc-104(e1265); kyIs235; syg-1(ky652)* animal. Vesicle clusters are absent from the axon.

(C–L) Lateral views of the vulval region with anterior at left and ventral down. Asterisks indicate the location of the vulva. Scale bars are equal to 5μm.

1(ky652) double mutants, SNB-1::YFP was lost both from the normal and the ectopic locations, suggesting that the ectopic vesicles are transported by the UNC-104 synaptic vesicle kinesin (Figures 3K and 3L, n = 60). Electron micrographs of HSNL in *syg-1* mutants revealed clusters of ectopic synaptic vesicles associated with active zones at aberrant anterior locations (R. Fetter and C.I.B., unpublished data).

SYG-1 Is a Member of the Immunoglobulin Superfamily

syg-1 was identified as the predicted gene K02E10.8 by genetic mapping and transformation rescue of its mutant phenotype (Figure 4A). cDNA clones for *syg-1* were isolated from the *C. elegans* EST project (a generous gift of Yuji Kohara) and by RT–PCR (see Experimental Procedures). The cDNA of *syg-1* was predicted to encode a 727 amino acid protein (Figure 4C).

syg-1 encodes a novel transmembrane protein in the immunoglobulin superfamily. The SYG-1 protein is predicted to contain a hydrophobic signal sequence, an extracellular domain with four immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic domain that ends with a consensus binding sequence for a type I PDZ domain (Figures 4B and 4C). SYG-1 shares this domain structure with the *Drosophila* Rst/Irrec and Kirre/Duf proteins and a subset of immunoglobulin superfamily proteins in vertebrates including human NEPH1. SYG-1 is 28% identical to Rst/Irrec and 26% identical to NEPH1 (Figure 4D).

To confirm that this open reading frame represented the *syg-1* gene, we identified the molecular lesion in the *syg-1* allele (Figure 4B). A single nucleotide deletion in *syg-1(ky652)* is predicted to cause a frameshift and a translational stop before the first immunoglobulin domain of the protein. It is likely that *syg-1(ky652)* represents a molecular null allele.

A genomic SYG-1 clone with the upstream region and the entire coding region of SYG-1 fused to GFP was expressed in head motorneurons, occasionally in HSN neurons, and weakly in other cells in the vulval region (data not shown). Because expression was weak, variable, and not localized to cell bodies, we could not define the complete group of cells that expressed SYG-1.

SYG-1 Can Act Cell Autonomously in HSNL to Specify Synapse Location

To determine where *syg-1* functions to specify synapse formation, we expressed a *syg-1* cDNA in different cells and asked which transgenes rescued the HSNL synaptic phenotype in *syg-1* mutants. Cell-specific expression was achieved using an *unc-4* promoter that is expressed in the VC neurons (Miller et al., 1992); an *egl-17* promoter that is expressed in a subset of vulval epithelial cells (Burdine et al., 1998); a *lin-11B* promoter that is expressed in VC neurons and vulval epithelial cells (Hobert et al., 1998); a *sra-6* promoter that is expressed in PVQ neurons (Troemel et al., 1995); and an *unc-86* promoter that is expressed in HSN neurons (Baumeister et al., 1996). *syg-1* synaptic vesicle defects were only rescued when the expression of SYG-1 was driven by the *unc-86* promoter, where rescue was observed in all transgenic lines (10 out of 10) (Figure 5D). The synaptic vesicle

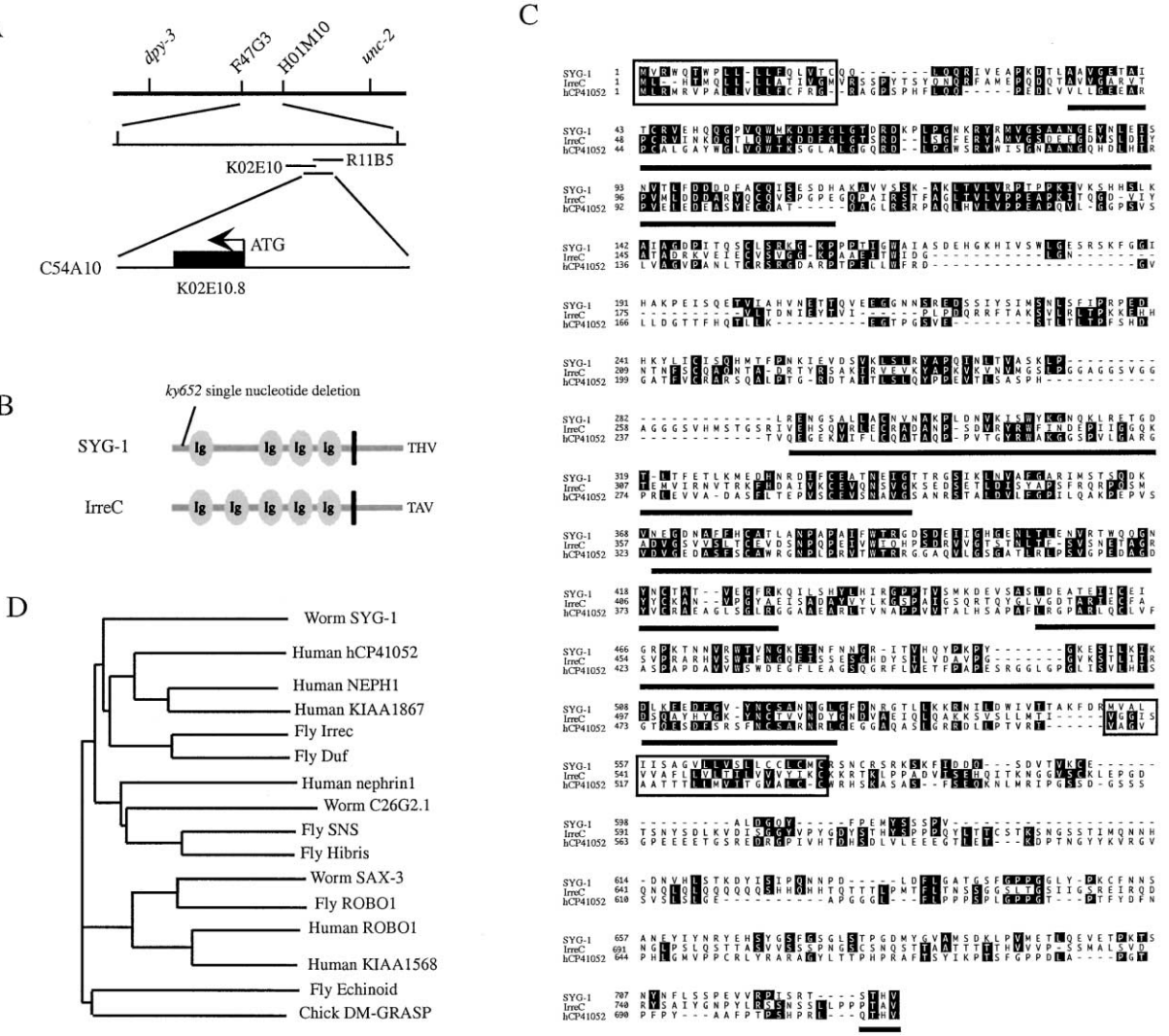


Figure 4. *syg-1* Encodes an Immunoglobulin Superfamily Protein

(A) The *syg-1*(*ky652*) mutation was mapped between *dpy-3* and *unc-2* on the left arm of the X chromosome, and further mapped between two SNP markers on F47G3 and H01M10. The cosmid C54A10 rescued the HSN SNB-1::YFP defects of *syg-1(ky652)*.

(B and C) A full-length cDNA corresponding to K02E10.8 predicts a 727 amino acid protein with homology to the *Drosophila* protein IrreC/Rst. The *ky652* allele was associated with a single nucleotide deletion at residue 38263 of cosmid K02E10.

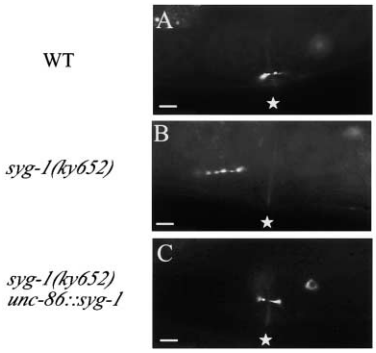
(C) Sequence alignment of SYG-1, Irrec/Rst, and a human homolog hCP41052. The signal peptide sequence and the predicted transmembrane domains are boxed. Predicted Ig domains of SYG-1 are underlined, and the conserved PDZ binding consensus at the C terminus is underlined.

(D) Phylogenetic analysis of SYG-1 and related proteins. DM-GRASP is also known as BEN/SC-1.

clusters in rescued animals were indistinguishable from those of wild-type animals (Figures 5A and 5C). Since *unc-86* expression near the vulva is restricted to HSNs, this result strongly suggests that SYG-1 can act cell autonomously in the presynaptic HSNL neuron to specify the location of its synapses.

SYG-1 Accumulates at Synapses during Synaptogenesis

To further understand the function of SYG-1, the subcellular localization of SYG-1 was examined in HSNL using a reporter gene fusion. GFP was inserted into the carboxyl terminus of a full-length *syg-1* cDNA under the control of the *unc-86* promoter. This transgene expressed SYG-1::GFP in HSNL from the L2 stage until adulthood. Since HSN axons first grow out in the L2 and form synapses in the L4 stage, this period should span the normal time of HSN synapse formation. In the L2 stage, SYG-1::GFP was ubiquitously localized along the short developing HSNL axon (data not shown). In late L3, SYG-1::GFP accumulated at a specific segment of the HSNL axon near the developing vulva epithelial cells (Figures 6A and 6B). At this stage, SNB-1::YFP vesicle clusters were not yet detectable (Figures 6C and 6D). By early L4 stage, SYG-1::GFP was tightly localized to a short stretch of axon where synaptic vesicle clusters form (Figures 6E–6H). A quantitative analysis of synaptic enrichment of SYG-1 and SNB-1 during development showed that accumulation of SYG-1 preceded the clustering of synaptic vesicles (Figure 6O). In this analysis,



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promoter	expression	Lines rescued
unc-4	VCs	0/15
lin-11B	VCs +Vulval Epithelium	0/15
egl-17	Vulval Epithelium	0/14
sra-6	PVQs and ASHs	0/12
unc-86	HSNs and other neurons	10/10

Figure 5. *syg-1* Can Function Cell Autonomously in the Presynaptic HSNL Neuron

(A) HSNL SNB-1::YFP clusters in wild-type animal.
(B) HSNL SNB-1::YFP clusters in *syg-1(ky652)* mutant.
(C) HSNL SNB-1::YFP clusters in *syg-1(ky652)* mutant carrying *kyEx642 (unc-86::syg-1)*.
(D) Summary table of rescue experiments. Only cells in the vulval region are included; all promoters are also expressed in additional cells distant from the vulva.
Asterisks indicate the location of the vulva. Anterior is to the left and ventral is down. Scale bars are equal to 5μm.

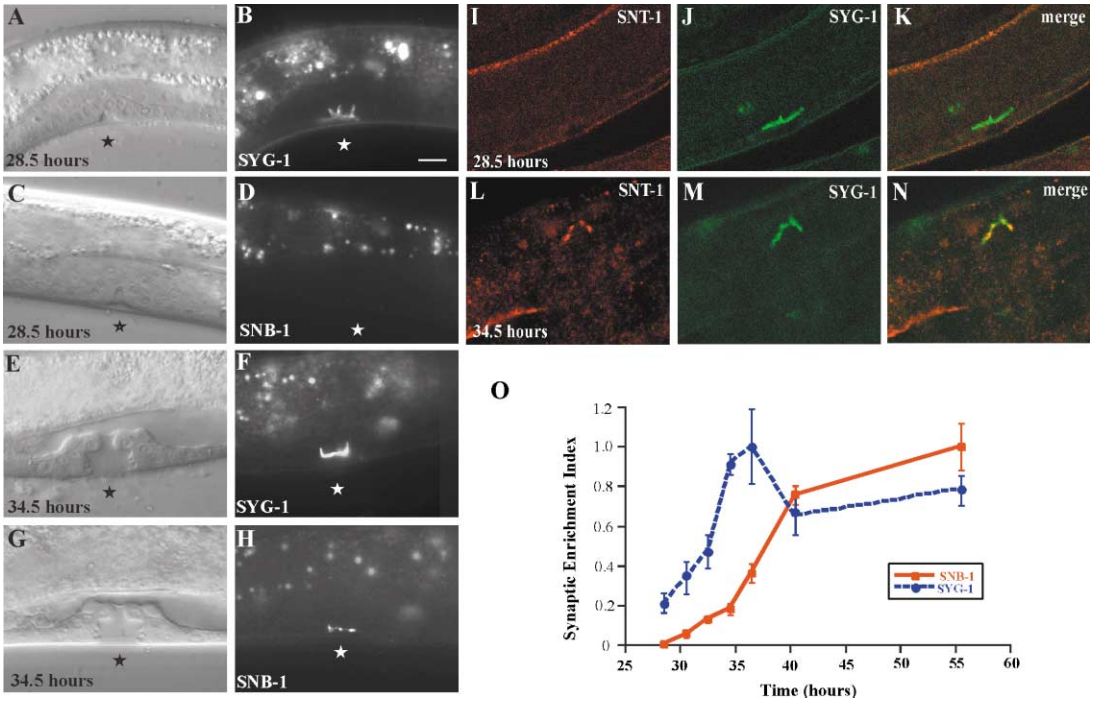

Figure 6. Synaptic Accumulation of SYG-1 Precedes Accumulation of Synaptic Vesicles

(A) DIC image of a *kyEx648* (*unc-86::syg-1::gfp*) animal at late L3 stage. Developing vulval epithelial cells are beginning to invaginate.
(B) Epifluorescence image of the same animal as in (A). SYG-1::GFP fluorescence is enriched at the developing vulva.
(C) DIC image of *kyIs235 (unc-86::snb-1::yfp)* at late L3 stage.
(D) Epifluorescence image of HSNL SNB-1::YFP in the same animal as in (C). No SNB-1 fluorescence is detectable at the vulva.
(E and F) DIC (E) and epifluorescence (F) image of a *kyEx648* (*unc-86::syg-1::gfp*) animal in mid-L4 stage. SYG-1::GFP labels the region of the HSNL axon where synapses form.
(G and H) DIC (G) and epifluorescence (H) image of a *kyIs235* animal in mid-L4 stage. SNB-1::YFP vesicles are localized to the developing vulva.
(I, J, and K) Double-labeling of synaptotagmin (SNT-1) and SYG-1. anti-SNT-1 immunofluorescence (I), SYG-1::GFP epifluorescence (*kyIs288)*(J) and merged image (K) of a late L3 stage animal. SYG-1::GFP is present but no SNT-1 immunofluorescence is detectable near the developing vulva.
(L, M, and N) Double-labeling of synaptotagmin (SNT-1) and SYG-1. anti-SNT-1 immunofluorescence (L), SYG-1::GFP epifluorescence *(kyIs288)* (M) and merged image (N) of an early L4 stage animal. SYG-1::GFP colocalizes with SNT-1 immunofluorescence near the developing vulva.
(O) Quantitative analysis of the synaptic enrichment of SNB-1::YFP and SYG-1::GFP during development. Time of development represents hours after synchronized L1 stage animals were plated on food. The Y axis represents synaptic enrichment of SNB-1::YFP and SYG-1::GFP measured from *kyIs235* and *kyEx648* (see Experimental Procedures).

both SYG-1 and SNB-1 were expressed from the same *unc-86* promoter, so differential accumulation most likely reflected the behavior of the proteins in HSNL.

To confirm the observation that SYG-1 could accumulate at synaptic sites before synaptic vesicles were present, we examined the endogenous synaptic vesicle protein synaptotagmin (SNT-1) (Nonet et al., 1993). Synaptic vesicles were stained with an anti-SNT-1 antibody and compared to the pattern of SYG-1::GFP fluorescence in the same animals. In 19 of 20 late L3-stage animals, SYG-1 was enriched at the developing vulva but the synaptic vesicle protein was absent (Figures 6I–6K). In 23 of 25 early L4 animals, SNT-1 protein colocalized with SYG-1 at developing synapses (Figures 6L–6N). SYG-1 localization was normal in an *unc-104* background, demonstrating that the localization of SYG-1 is independent of the presence of synaptic vesicles (data not shown). These results indicate that SYG-1 localizes near HSNL synapses and can precede and predict the future localization of synaptic vesicles. However, all of these experiments were conducted using the *unc-86* promoter, and the normal pattern of SYG-1 expression from its own promoter could differ in timing or levels from the pattern observed here.

SYG-1 Localization Is Patterned by the Vulva Epithelial Cells

If SYG-1 protein accumulation determines the location of HSNL synapses, then the vulval epithelium should specify the localization of SYG-1 in development. This prediction was tested by examining the subcellular localization of SYG-1 in mutants with altered vulval position or cell fate. In vulvaless animals created by somatic gonad ablation (Figures 7A and 7B, n = 23) and in *lin-3* mutants (Figures 7C and 7D, n = 80), SYG-1 was diffusely distributed across the HSNL axon. Thus, the synaptic accumulation of SYG-1 was lost under conditions in which the vulval epithelium did not signal to HSNL. In *dig-1* mutant animals in which the vulval epithelium was anteriorly displaced, SYG-1 was localized to the displaced vulva (Figures 7E and 7F, n = 60). In multivulval *lin-15* mutants, multiple SYG-1 clusters formed on the HSN axon in positions corresponding to the future pseudovulvae (Figures 7G and 7H, n = 55). These results suggest that SYG-1 in the presynaptic cell responds to the signals from the vulval epithelium that initiates synapse formation.

Discussion

Specific neuronal connections are an intriguing feature of the nervous system. A neuron may make connections in one layer of a target region, onto one target cell type, or onto one subregion of a target dendrite or axon (Benson et al., 2001). Our results demonstrate that a specific set of synapses from the HSN neuron are localized by epithelial guidepost cells adjacent to the HSN axon. The immunoglobulin superfamily protein SYG-1 is required in the presynaptic HSN neuron to specify these synapses and is a candidate receptor for the signal from the epithelial cells.

Vulval Epithelial Cells Are Synaptic Guidepost Cells

The HSN axon forms synapses onto vulval muscles and VC neurons en passant (in passing) early in its trajectory and forms additional synapses when it reaches the nerve ring in the head (White et al., 1986). The vulval epithelial cells act as guidepost cells for the formation of the vulval synapses, driving their accurate placement and suppressing the formation of inappropriate vesicle clusters in alternative locations. The formation of HSN synapses is one aspect of the development of the egg-laying circuit, which is coordinated temporally and spatially through multiple cell interactions (Kimble and White, 1981; Li and Chalfie, 1990; Garriga et al., 1993; Burdine et al., 1998; Wang and Sternberg, 2001). The gonad induces the specialized vulval fate in underlying epithelial cells and attracts migrating sex muscles to the vulva. The vulval epithelial cells communicate with each other to diversity their cell fates and induce HSN defasciculation and branching, VC branching, and HSN synapse formation. Communication between the cells involved in egg-laying is mediated by EGF, Notch/Delta, FGF, and Wnt signaling pathways, as well as a distinct epithelial signal for synapse formation. Although its molecular nature is unknown, the epithelial signal is probably membrane-associated, since it is incapable of inducing synapse formation in *unc-6* and *unc-40* mutants whose HSNL axon is displaced only 5 μm from vulval epithelial cells. It is likely that direct contact between HSNL and the vulval epithelial cells is required for accurate synapse formation.

The guidepost role of the vulval epithelial cells in HSN synapse formation is reminiscent of the guidepost role of Cajal-Retzius cells in the developing hippocampus. Cajal-Retzius cells are transient targets of entorhinal projections that are essential for the normal innervation of dentate granule and SLM layers by entorhinal axons (Super et al., 1998). The synapses on Cajal-Retzius cells appear to act as placeholders until the hippocampal dendrites grow into the SLM layer. Similarly, a guidepost population of cortical subplate neurons are transient targets of thalamic axons until their target cortical cells differentiate (Ghosh and Shatz, 1992), and neurons form transient synapses with glial processes in the developing spinal cord (Henrikson and Vaughn, 1974). A temporal discrepancy between the differentiation of the presynaptic and postsynaptic neurons is a component of both entorhinal and thalamocortical projections and appears to be a component of HSN synapse formation as well (our unpublished data); guidepost cells might be particularly important in such circumstances. Transient guidepost or intermediate cells are common in axon guidance, where intermediate targets like the floorplate of the spinal cord or the optic chiasm direct axons through a specific step in their trajectory (O'Connor, 1999). We speculate that guidepost functions may act in many developmental situations to establish the precise connectivity of the nervous system.

The Immunoglobulin Superfamily Protein SYG-1 May Be a Receptor for the Epithelial Signal

The *syg-1* gene is required for the accumulation of synaptic vesicle clusters at the vulva and inhibits the accu-

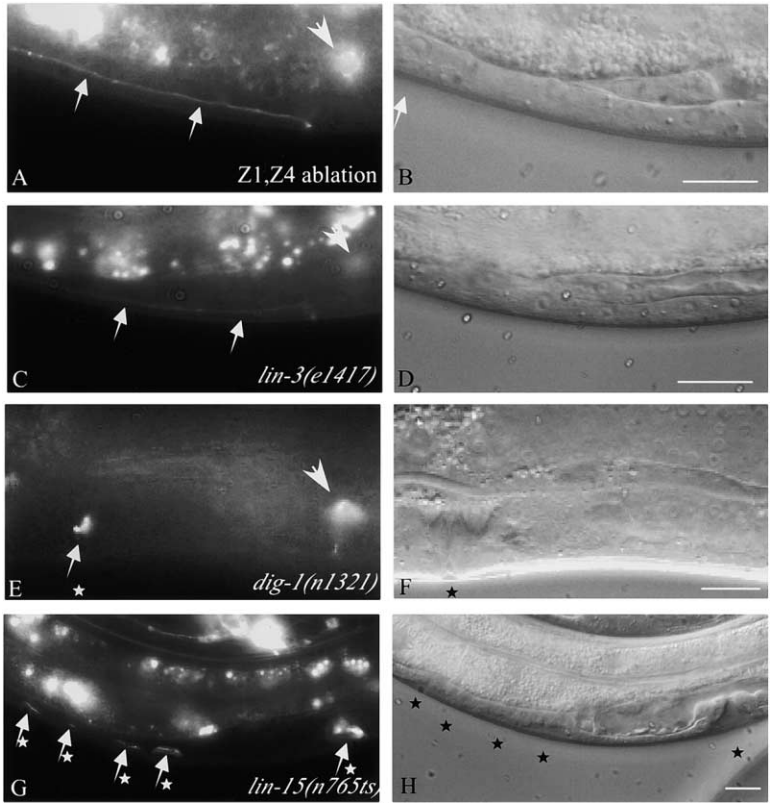

Figure 7. SYG-1 Localization Is Determined by Vulval Epithelial Cells

(A and B) Epifluorescence image (A) and DIC image (B) of a *kyEx648* (*unc-86::syg-1::gfp*) animal in which the Z1 and Z4 gonad cells were ablated at L1 stage. SYG-1::GFP is diffusely distributed.

(C and D) Epifluorescence image (C) and DIC image (D) of a *lin-3(e1417); kyEx648* animal. SYG-1::GFP is diffusely distributed.

(E and F) Epifluorescence image (E) and DIC image (F) of a *dig-1(n1321); kyEx648* animal. SYG-1::GFP is localized to the anteriorly displaced vulva.

(G and H) Epifluorescence image (G) and DIC image (H) of a *lin-15(n765ts); kyEx648* animal. Multiple SYG-1::GFP clusters form on the HSNL axon. The positions of the clusters match the positions of developing ectopic pseudovulvae. Asterisks indicate the location of the vulva(e); arrows indicate the position of SYG-1::GFP clusters. Anterior is to the left and ventral is down. Scale bars are equal to 5 μm.

mulation of synaptic markers at inappropriate anterior locations. The synaptic phenotype of *syg-1* mutants is indistinguishable from the synaptic phenotype of animals that lack vulval epithelium, but the vulval epithelium in the mutants is normal by morphological and functional criteria. Thus, *syg-1* appears to be required for the communication between the vulval epithelium and the presynaptic HSN neuron. Expression of *syg-1* in HSN rescues the synaptic defect of a *syg-1* null mutant, but expression in vulval epithelial cells does not. *syg-1* encodes a transmembrane protein with four immunoglobulin domains, and thus is a candidate receptor for the vulval signal. The SYG-1 protein accumulates in the HSN axon at regions adjacent to the vulval epithelium that define the site of future synapses. The epithelial signal may act predominantly by stimulating the local aggregation of SYG-1 in the HSN, or it may induce signal transduction through SYG-1 to trigger the accumulation of synaptic vesicles.

The vulval epithelial cells induce dorsal branch formation in the HSN and VC neurites near the vulva a few hours after the initial clustering of SNB-1 in HSN (Li and Chalfie, 1990; Garriga et al., 1993; our unpublished data). HSN branching, but not VC branching, requires SYG-1. Thus, SYG-1 coordinates two different aspects of synapse formation, axon branching at the target and localized vesicle clustering.

SYG-1 is closely related to two *Drosophila* proteins, Rst/IrreC and Kirre/Duf. Although they have not been characterized in synapse formation, these proteins act in a variety of developmental events that involve specific cell recognition. Rst/IrreC mutants have defects in cell adhesion in the retina, the roughest phenotype, and in axon pathfinding at higher steps of visual processing, the irregular chiasm phenotype (Schneider et al., 1995). Rst/IrreC and Kirre/Duf also play essential roles in muscle fusion (Ruiz-Gomez et al., 2000, Strunkelnberg et al., 2001). During development, founder cells for each muscle attract and fuse with fusion-competent cells. Recognition between the muscle founder and fusion-competent cells is mediated by Rst/IrreC and Kirre on the founder cells, and Sticks and Stones (Sns) and Hibris (Hib) on fusion-competent cells (Bour et al., 2000; Artero et al., 2001). Muscle fusion occurs through a characteristic prefusion complex, a tight adhesion between cells associated with alignment of paired vesicles at the cell junctions (Doberstein et al., 1997). Several structural features of the prefusion complex are similar to features of synapses, including tight local asymmetric adhesions between cells and the recruitment of vesicles. Sns and Hib are transmembrane proteins with eight immunoglobulin domains that have heterophilic interactions with the Kirre/Duf protein (Dworak and Sink, 2002). A predicted ORF in the *C. elegans* genome, C26G2.1, shows strong homology to SNS and Hibris, suggesting that similar heterophilic recognition mechanisms could also exist in *C. elegans*.

SYG-1 bears similarity to many vertebrate immunoglobulin superfamily members, and strongest similarity to three proteins that are only partly characterized, NEPH1, KIAA1867, and hCP41052. One of these genes, NEPH1, is regionally expressed in the mammalian brain, but its function there is unknown (Donoviel et al., 2001). NEPH1 is required for kidney function and the develop-

ment of the podocyte slit membrane in mice (Donoviel et al., 2001). A human ortholog of Sns/Hib, Nephrin, is also required for kidney function and development of the podocyte slit membrane, a tight epithelial cell adhesion complex that plays a role in glomerular filtration (Tryggvason, 1999). The adhesion complexes of the mammalian podocyte slit membrane, a future *C. elegans* synapse, and a *Drosophila* muscle prefusion complex appear to be initiated by similar molecular interactions that are interpreted in cell-type specific contexts.

SYG-1 has more distant similarity to the vertebrate Ig protein BEN/SC-1/DM-GRASP. BEN is a homophilic adhesion molecule that has been suggested to play a role in neuromuscular junction formation and axon outgrowth (Fournier-Thibault et al., 1999, Pourquie et al., 1992).

Synaptic Specificity, Synaptic Growth, and Cell Recognition

The final pattern of synapses that an axon makes reflects a series of developmental events: topographic mapping across the surface of the target region, invasion and arborization in one or more layers, or laminae, and selection of a specific target neuron. In different regions of the nervous system, different processes may be more or less important in determining synaptic specificity. For example, in highly layered structures like the retina, laminar subtargeting may be the most important step in correct synapse formation, whereas the extensive intermixing of cells and processes in the cortex could require discrimination between cells in one lamina.

Cell adhesion molecules in the immunoglobulin superfamily (IgSF) can act in many steps of synapse formation. Two homophilic synaptic IgSF molecules, the Sidekicks, can act as specific mediators of laminar targeting in the mammalian retina (Yamagata et al., 2002). The homophilic IgSF adhesion molecules *Aplysia* ApCAM and *Drosophila* fasciclin II are strongly implicated in determining the strength and number of synaptic connections (Benson et al., 2001). The mammalian SynCAM molecule (also known as TSLC1, a tumor suppressor gene in non-small cell lung cancer) may have a similar function, since it is a homophilic adhesion molecule that is widely expressed during synaptic growth (Kuramochi et al., 2001; Biederer et al., 2002). Many non-IgSF adhesion molecules have also been suggested to function in synaptic assembly and maintenance, including neurexin-neuroligin, classical N-cadherins, and protocadherins (Benson et al., 2001). Neuroligin-expressing or synCAM-expressing HEK293 epithelial cells can induce the formation of presynaptic specializations in hippocampal neurons (Scheiffele et al., 2000; Biederer et al., 2002). Although the in vivo functions of these molecules are unknown, a combination of different proteins is likely to generate the complex circuits of the nervous system.

SYG-1 protein can accumulate at the site of future HSN synapses a few hours before synaptic vesicles are clustered. When the vulval epithelium is absent, SYG-1 distributes diffusely across the HSNL axon, whereas the synaptic vesicles cluster in areas distinct from their normal location. Therefore, in the absence of *syg-1* function, other mechanisms can drive synaptic vesicle clustering, and perhaps the formation of synapses, at ectopic locations. During wild-type development, SYG-1-mediated mechanisms dominate HSN synaptogenesis at the vulva and suppress alternative sites, but the presence of ectopic vesicle clusters in the *syg-1* mutant suggests that HSN has alternative strategies that may support synapse formation with different targets. The final synaptic choice might result from competition among a hierarchy of alternative synaptogenic mechanisms.

Most studies of mammalian synapse formation have been conducted in cultured neurons. Although it is not clear whether all forms of synaptic specificity are represented under these circumstances, there appears to be a hierarchy of preferred synaptic partners in these mixed primary cultures. Neurons prefer their normal target cell type, but will synapse onto other cells if the target is not available, and will even form aberrant synapses onto themselves (autapses) if cultured in isolation. Similarly, when the correct synaptic target is absent in vivo, neurons can be observed to synapse onto incorrect targets (Cash et al., 1992). In some cases, presynaptic structures can form in the absence of postsynaptic cells (Prokop et al., 1996). Highly specific molecules like SYG-1 may represent the first choice for a correct synapse, but it appears that a neuron's drive to form a synapse will eventually override the absence of the correct partner.

Experimental Procedures

Strains and Genetics

Wild-type animals were *C. elegans* variety Bristol, strain N2. Strains were maintained using standard methods (Brenner, 1974). Animals were grown at 20°C. Some strains were provided by the *Caenorhabditis* Genetic Center.

Molecular Biology

Standard molecular biology techniques were used (details available on request). To identify the mutations in *syg-1*, the open reading frame and splice junctions of the mutant alleles were amplified from two separate genomic DNA preparations of the mutant strains by PCR. PCR fragments were sequenced on both strands using an ABI sequencing machine (UCSF HHMI DNA facility).

Partial cDNAs (yk410d1, yk76d2) corresponding to the predicted ORF K02E10.8 were obtained from Yuji Kohara. The 5′ end of the *syg-1* coding region was isolated by RT-PCR from wild-type N2 RNA. The three most C-terminal exons are different from those predicted by the *C. elegans* Sequencing Consortium.

Expression clones were made in the pSM vector, a derivative of pPD49.26 with extra cloning sites (S. McCarroll and C.I.B., unpublished data). Promoter sequences were *unc-4* (4 kb) (Miller et al., 1992); *egl-17* (2.5 kb) (Burdine et al., 1998); *lin-11B* (4 kb) (Hobert et al., 1998); *sra-6* (4.2 kb) (Troemel et al., 1995); and *unc-86* (5 kb) (Baumeister et al., 1996). The full-length *syg-1* cDNA was generated by PCR and sequenced to avoid unwanted mutations. For *unc-86::syg-1::gfp,* sequences encoding GFP followed by the C-terminal STHV residues of SYG-1 were fused to the C terminus of the *syg-1* cDNA.

Mutant Isolation

Wild-type animals were injected with *unc-86::snb-1::yfp* at 1 ng/μl, *unc-4::lin-10::dsred* at 50 ng/μl and *odr-1::dsred* as a coinjection marker at 10 ng/μl. The transgenic array was integrated into the genome using trimethylpsoralen/UV mutagenesis. *ky652* was isolated as a mutation in one of the integrants. The mutation was separated from the integrated transgene, outcrossed four times, and crossed into the integrant *kyIs235* for further analysis. Mutant phenotypes were indistinguishable in two different integrants of *unc-86::snb-1::yfp unc-4::lin-10::dsred*.

Germline Transformation

Transgenic strains were created as previously described (Mello and Fire, 1995). Multiple lines from each injection were characterized for

rescue of the SNB-1::YFP phenotype. Cosmids were injected at 20 ng/μl, the coinjection markers *odr-1::gfp* and *odr-1::dsred* at 10 ng/μl, and expression plasmids at 100 ng/μl, except for *unc-86* fusion genes (1 ng/μl).

Light Microscopy

HSNL vesicle clusters were visualized in *kyIs235* animals. HSN and VC axons were visualized with the *cat-1::gfp* transgene *cyIs4* (a gift from A. Colavita and M. Tessier-Lavigne). All images except Figure 1A and Figures 6I–6N were taken on a Zeiss Axioplan 2 with an AxioCam camera. Figures 1A and 6I–6N were taken on a Biorad MRC-1024 confocal microscope. Images were processed in NIH Image and Adobe Photoshop.

Quantitation of Synaptic Enrichment of SYG-1 and SNB-1

Fluorescence intensity of SYG-1::GFP or SNB-1::YFP was measured on the HSNL axon in the vulval region. The vulval region was defined using the boundary of P6p cells (L3) or the Christmas tree pattern of invaginating vulval cells (L4). These values were then compared to the average fluorescence intensity measured on the HSNL axon between the vulva and the HSNL cell body of the same animals to generate a ratio for synaptic enrichment (weak fluorescence is always present in the HSNL axon). The ratio values for each protein were normalized against the maximum value for that protein in the same developmental series (set at 1.0) to generate the Synaptic Enrichment Index.

Immunostaining of Synaptic Vesicles

Eggs collected from SYG-1::GFP *kyIs288* animals were allowed to hatch in M9 overnight without food, and L1 animals plated on food for 28.5 or 34.5 hr. L3 or L4 animals were fixed in 2% paraformaldehyde and methanol overnight at −80°C as described (Finney and Ruvkun, 1990) and stained with rabbit anti-SNT-1 antisera at 1:1000 (Nonet et al., 1993). Images were taken using a Biorad confocal microscope.

M Cell Ablation

lin-39(n709ts); kyIs235 mutant animals were grown at 25°C. M cells were identified and laser ablated in the early L1 stage. Synapses in ablated animals were examined at the L4 stage by fluorescence microscopy. The absence of LIN-10 expression confirmed that VC4,5 did not form due to the *lin-39(n709ts)* mutation. These animals were then recovered and scored for the Egl phenotype 24 hr later. All ablated animals showed severe Egl phenotypes, which suggested that the M cell ablation was successful.

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