

A Conserved Mechanism of Synaptogyrin Localization

Hongjuan Zhao and Michael L. Nonet*

Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

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We have studied the localization of synaptogyrin family members *in vivo*. Both native and green fluorescent protein (GFP)-tagged *Caenorhabditis elegans* synaptogyrin (SNG-1) are expressed in neurons and synaptically localized. Deletion and mutational analysis with the use of GFP-tagged SNG-1 has defined a 38 amino acid sequence within the C terminus of SNG-1 and a single arginine in the cytoplasmic loop between transmembrane domain 2 and 3 that are required for SNG-1 localization. These domains may represent components of signals that target synaptogyrin for endocytosis from the plasma membrane and direct synaptogyrin to synaptic vesicles, respectively. In chimeric studies, these regions were sufficient to relocalize cellugyrin, a nonneuronal form of synaptogyrin, from nonsynaptic regions such as the sensory dendrites and the cell body to synaptic vesicles. Furthermore, GFP-tagged rat synaptogyrin is synaptically localized in neurons of *C. elegans* and in cultured hippocampal neurons. Similarly, the C-terminal domain of rat synaptogyrin is necessary for localization in hippocampal neurons. Our study suggests that the mechanisms for synaptogyrin localization are likely to be conserved from *C. elegans* to vertebrates.

INTRODUCTION

Synaptic vesicles (SVs) contain a restricted set of membrane proteins important for neuronal function (Südhof, 1995; Calakos and Scheller, 1996; Fernandez-Chacon and Südhof, 1999). The mechanisms responsible for targeting these proteins specifically to the SV membrane are poorly understood. Integral membrane proteins are synthesized on the rough endoplasmic reticulum and traffic through the Golgi network. Proteins exiting the *trans*-Golgi network (TGN) are sorted to different types of transport vesicles. SV proteins are sorted to synaptic vesicle precursors (preSVs), which differ from mature SVs both in morphology and protein composition (Tsukita and Ishikawa, 1980; Okada *et al.*, 1995). Evidence supports both direct routing of SV proteins from TGN to preSVs and indirect routing via the plasma membrane (reviewed by Hannah *et al.*, 1999). preSVs are subsequently transported along axonal processes to the nerve terminal by motor proteins of the kinesin superfamily (reviewed by Hirokawa, 1998). How preSVs mature after delivery to the nerve terminal is unknown. preSVs might fuse with an endosomal compartment and SVs generated by budding from the endosome. A second, but not mutually exclusive possibility, is that preSVs are delivered to the presynaptic plasma membrane at the nerve terminal and then retrieved by the same mechanism(s) used to recycle SVs after regulated exocytosis (Hannah *et al.*, 1999; reviewed by Buckley *et al.*, 2000). Regardless of the exact route of trafficking to the mature organelle, SV proteins must be sorted away from

other membrane proteins at several stages during their life cycle.

Signal sequences resident in proteins are thought to mediate the sorting of many proteins to cellular compartments. Several studies have identified domains and motifs necessary for correct localization of SV proteins. Targeting and sorting signals have been identified in synaptophysin (Linstedt and Kelly, 1991), synaptobrevin (Grote *et al.*, 1995; Grote and Kelly, 1996), synaptotagmin (Blagoveshchenskaya *et al.*, 1999; Krasnov and Enikolopov, 2000), and vesicular neurotransmitter transporters (Tan *et al.*, 1998; Varoqui and Erickson, 1998). However, no common motif that could serve as a universal targeting signal has been found by experimental studies or by direct comparison of the primary sequences of SV proteins. Although tyrosine-based signals are one of the major sorting signals used by clathrin pit-mediated endocytosis (Davis *et al.*, 1986, 1987; Collawn *et al.*, 1990; Peters *et al.*, 1990; Thies *et al.*, 1990; Letourneur and Klausner, 1992), only one protein, P-selectin, has been shown to use a tyrosine-based signal to be targeted to synaptic-like microvesicles (SLMVs) in PC12 cells (Blagoveshchenskaya *et al.*, 1999).

The absence of a common sorting signal element in SV proteins contrasts to targeting to other organelles where many distinct proteins use the same sorting signal (Stanley, 1996). At least two different mechanisms of SV protein sorting could account for the lack of a universal SV targeting signal. First, distinct components of SVs may be sorted by independent sorting mechanisms. Such an idea is also supported by other findings. For example, the SV proteins synaptotagmin, synaptophysin, and SV2 are sorted into differ-

* Corresponding author. E-mail address: nonetm@thalamus.wustl.edu.

ent classes of organelles when expressed in nonneuronal Chinese hamster ovary cells (Feany *et al.*, 1993). In vivo, SV2 and synaptophysin associate with different kinesin motor proteins and consequently probably reside in distinct vesicle populations (Okada *et al.*, 1995). In addition, in the *Caenorhabditis elegans unc-11* (AP180 homolog) mutant, synaptobrevin alone is mislocalized, whereas other SV proteins are unaffected (Nonet *et al.*, 1999). Furthermore, the protein stoned is required selectively for the retrieval of synaptotagmin I from the plasma membrane in *Drosophila* (Fergestad *et al.*, 1999; Fergestad and Broadie, 2001), perhaps via its interaction with the C2B domain of synaptotagmin I (Littleton *et al.*, 2001). Another possibility is that sorting information is only present in a few proteins, and other components form complexes with these proteins and are secondarily targeted to SVs (Bennett *et al.*, 1992). More likely, a combination of these two mechanisms is used to target SV proteins. Defining distinct trafficking signals for all SV proteins will be complex and ultimately may require defining multiple protein-protein interactions that act coordinately.

Caenorhabditis elegans has several features that make it a valuable system for studying both synaptic function and SV protein localization in vivo. Both forward and reverse genetics have been used to generate mutations in multiple synaptic components, many of which are evolutionarily conserved (Fernandez-Chacon and Südhof, 1999; Nonet, 1999). The unique life cycle of *C. elegans* allows many mutants (and double and triple mutants) that are severely compromised in neuronal function to survive simplifying detailed analysis of the role of these genes (Nonet *et al.*, 1993, 1997, 1999; Harris *et al.*, 2000). The compact organization and completed sequence description of the genome greatly facilitates the identification and manipulation of existing and novel members of protein families known to have important functions in other organisms (Bargmann, 1998; The *C. elegans* Sequencing Consortium, 1998). Qualitative and quantitative analysis of the distribution of GFP-tagged protein in live animals under fluorescent microscopy is simple owing to the transparent nature of the organism (Labrousse *et al.*, 1998; Nonet, 1999). Finally, genes that are involved in protein localization can be isolated in classical genetic screens with the use of GFP-tagged transgenes (reviewed by Koushika and Nonet, 2000).

In this study we have characterized signals required for localization of members of the synaptogyrin family, which currently consists of three human genes, three mouse genes, two rat genes and one *C. elegans* gene (Stenius *et al.*, 1995; Janz and Südhof, 1998; Kedra *et al.*, 1998; Nonet *et al.*, 1999). The first cloned member of this family, rat synaptogyrin (p29), is highly expressed in neurons and neuroendocrine cells where it colocalizes with synaptophysin on SVs and SLMVs (Baumert *et al.*, 1990; Stenius *et al.*, 1995). Synaptogyrin together with synaptophysin, a family distantly related to synaptogyrin, accounts for >10% of the total SV protein content (Jahn *et al.*, 1985; Wiedenmann and Franke, 1985; Baumert *et al.*, 1990; Jahn and Südhof, 1993; Stenius *et al.*, 1995). Moreover, both synaptogyrin and synaptophysin families contain nonneuronal isoforms that are ubiquitously expressed (Haass *et al.*, 1996; Janz and Südhof, 1998; Kedra *et al.*, 1998; Takeshima *et al.*, 1998). Synaptophysin and synaptogyrin share similar membrane topologies with four transmembrane domains where their N and C termini face the cytoplasm (Johnston *et al.*, 1989; Stenius *et al.*, 1995). In PC12

cells, overexpressing synaptogyrin and synaptophysin inhibits exocytosis (Sugita *et al.*, 1999). Double mutant mice lacking both synaptogyrin and synaptophysin show severe reductions in both short-term and long-term synaptic plasticity, suggesting that they are regulators of exocytosis (Janz *et al.*, 1999). *sng-1*, the *C. elegans* member, has 30% identity to the rat synaptogyrin and a similar hydrophobicity profile (Nonet, 1999). Analysis of animals expressing green fluorescent protein (GFP)-tagged synaptogyrin (SNG-1) suggests that SNG-1 is localized to synaptic regions (Nonet, 1999). Thus far, no other *C. elegans* synaptogyrin family members have been identified.

We have identified two primary sequences in SNG-1 that are necessary for its synaptic localization, a C-terminal region containing 38 amino acids and an arginine in the loop facing the cytoplasm. An SNG-1 sequence containing these elements is sufficient to localize rat cellugyrin that is normally not restricted to synaptic regions in *C. elegans*. Furthermore, rat synaptogyrin is localized to synaptic regions in both *C. elegans* and hippocampal neurons in culture and the C terminus is necessary for synaptic localization in each system. Our results suggest that the mechanisms used for synaptic localization among synaptogyrin family members are evolutionarily conserved.

MATERIALS AND METHODS

Nematode Strains and Culture

Bristol strain N2, mutants, and transgenic animals were grown at 22.5°C on solid medium as described by Sulston and Hodgkin (1988).

Microscopy and Image Analysis

Live animals were anesthetized with 10 mM sodium azide, mounted on 2% agarose pads, and examined under epifluorescence with the use of an Olympus BX60. Time-averaged images were collected with the use of a DAGE SIT68 camera and a CG-7 frame grabber (Scion Image). Confocal images were taken in a series of 0.5 μM optical sections with the use of an Olympus Fluoview microscope and Fluoview software. A three-dimensional representation of a 50- μm segment of sublateral ventral nerve cord just anterior to the posterior end point of the cord near the tail (boxed area in Figure 4B) was made for quantitative analysis. Total intensity in the boxed area was measured with the use of Fluoview software. Background fluorescence was subtracted as the average intensity of areas adjacent to the boxed region. Total intensity in puncta (closed circles in Figure 4B), which were defined as discrete bright fluorescent patches, was calculated by summing the intensity in each punctum. The percentage of fluorescence in puncta was calculated by dividing the total intensity in the puncta by the total intensity in the boxed area after background subtraction.

Immunocytochemistry

Immunocytochemistry with antibodies against synaptotagmin (SNT-1) (Nonet *et al.*, 1993) and GFP (mouse monoclonal; CLONTECH, Palo Alto, CA) was performed as described previously (Saifee *et al.*, 1998). The polyclonal antibody against SNG-1 was generated in chicken with a purified bacterial product representing amino acids 164–248 fused to a his6 tag. The fusion product was purified under denaturing conditions (8 M urea) with the use of Ni-NTA agarose (Qiagen, Chatsworth, CA), and dialyzed into phosphate-buffered saline. The resulting serum was then immunoadsorbed against the SNG-1 fusion protein bound to nitrocellulose as described by Smith and Fisher (1984). Immunocytochemistry with

an antibody against rat synaptophysin (gift of P. De Camilli, Yale University, New Haven, CT) was performed as previously described (Rao *et al.*, 1998).

Plasmid Construction

All deletions were made with the use of Pfu polymerase and DpnI digestion as previously described (Fisher and Pei, 1997). All oligonucleotides listed below are 5' to 3'.

pSY1: Oligonucleotides TGAGTTGTATTGCATTCCAGATCTAG and GTCCGG ATCCATAAGCACGCACGTTC were used in a polymerase chain reaction (PCR) to amplify the *sng-1* promoter, a 1.8-kb sequence upstream of the start codon. The PCR product was digested with *Bgl*III and *Bam*HI, and was ligated with pPD95.69 digested with *Bam*HI. A. Fire, J. Ahnn, G. Seydoux, and S. Xu kindly provided pPD95.69. Detailed description of pPD95.69 available at ftp.ciwemb.edu/PNF:byName:/FireLabWeb/FireLabInfo/FireLab-Vectors/1995_Vector_Kit/

pSY12: Oligonucleotides CATTCTCAGATCTATGGAAGGGGG-TGCGTACGGAGC and CATTACCGGTCCGTAGCCCTGCGACTGGTAGCCC corresponding to the beginning and the end of the coding region of rat synaptogyrin were used in a PCR to amplify the gene from pCMV-Sgyr, a clone containing rat synaptogyrin cDNA (Stenius *et al.*, 1995). The PCR product was digested with *Bgl*III and Age I, and was ligated with pSY1 digested with *Bam*HI and Age I.

pSY14: Oligonucleotides CATTCTCAGATCTATGCCCTTGAGG-GTCGGCGGGC and CATTACCGGTCCGTACTGCGGGGAGGCTGGTAG corresponding to the beginning and the end of the coding region of rat cellugyrin were used in a PCR to amplify the gene from pCMV-Cgyr, a clone containing the rat cellugyrin cDNA (Janz and Südhof, 1998). The PCR product was digested with *Bgl*III and Age I, and was ligated with pSY1 digested with *Bam*HI and Age I.

pSY17: Oligonucleotides CTTTTATTTAGCATGCCCGCGGTT-TGTTTACAGG and CCTGAACAAAACCGCGGGCATGTAAAAT-AAAAG were designed to delete the region encoding amino acids 2–24 in pSY3, a clone containing the *sng-1* genomic DNA, including the coding region and the promoter (Nonet, 1999).

pSY18: Oligonucleotides GCATTTTTCGCATGGCGGCCGCTA-GAAAAATGAGandCTCATTTTTTCTAGCGGCCCATGCGA-AAAATGC were designed to delete the region encoding amino acids 173–248 in pSY3.

pSY26: Oligonucleotides GAAGATCTCGCCACCATGGAAGGG-GGTGCGTAC and CATTACCGGTCCGTAGCCCTGCGACTGG-TAGCCC corresponding to the beginning and the end of the coding region of rat synaptogyrin were used in a PCR to amplify the gene from pCMV-Sgyr. The PCR product was digested with *Bgl*III and Age I, and was ligated with pEGFP (CLONTECH), a clone containing human CMV promoter and the EGFP gene, digested with the same enzymes.

pSY31: Oligonucleotides CGG GAT CCC CGT CGT TAC GAA GAA GG and CGA CCG GTC CAT AAC CAT ATC CTT CCG corresponding to the beginning and the end of the coding region of the C-terminal domain of SNG-1 were used to PCR from pSCG110a, a clone contain partial SNG-1 cDNA. The PCR product was digested with *Bam*HI and Age I and was ligated with pSY14 digested with the same enzymes.

pSY36: Oligonucleotides GGAGGAGACTCAACCCGGGTACCGG-TAG and CTACCGGTACCCGGTTGAGTCTCTCC were designed to delete the region encoding amino acids 211–248 in pSY3.

pSY39: Oligonucleotides CTTTTTCGCATGGATCGGACATGT-TGG and CCAACATGTCGATCCATGCGAAAAATG were designed to delete the region encoding amino acids 173–210 in pSY3.

pSY42: Oligonucleotides GCATTTTTCGCATGGCAAGTGTGCA-CAGAC and GTCTGTGACACTTGCCATGCGAAAAATGC were designed to delete the region encoding amino acids 173–191 in pSY3.

pSY43: Oligonucleotides GATGAACATTTTGGACATGTTG-GCGCACCT and AGGTGCGCCAACATGTCCAAAATGTTTCATC were designed to delete the region encoding amino acids 192–210 in pSY3.

pSY44: Oligonucleotides GAAATCAAGCAACTGGATACG-GAGGAGAC and GTCTCCTCCGTATCCAGTTGCTTGATTTC were designed to delete the region encoding amino acids 182–200 in pSY3.

pSY45: Oligonucleotides GATGAACATTTTGGACAAAACCATG-CAACAAC and GTTGTTCATGGTTTGTCCAAAATGTTTCATC were designed to delete the region encoding amino acids 191–229 in pSY3.

pSY46: Oligonucleotides CAGCGGTATCAGATTGGACCGGTC-G-CCACC and GGTGGCGACCGTCCAATCTGATACCGCTG were designed to delete the region encoding amino acids 176–235 in pSY26.

pSY47: Oligonucleotides GCCGTGCTAGCCTTGGGACCGGTAG-TAAAAA and TTTTCTACCGGTCCGAAGGCTAGCACGGC were designed to delete the region encoding amino acids 179–243 in pSY12.

pSY57: Oligonucleotides CTTTTATTTAGCATGCCCGCGGA-CAAGAAGGAGAGCTG and CAGCTCTCCTTCTTGCCGGCGG-ATGCTAAAATAAAAAG were designed to delete the region encoding amino acids 1–99 of SNG-1 in pSY3 and a *Sac*II restriction site was engineered into the deletion site. The resulting plasmid is designated pSY54. Oligonucleotides CAGATAAGCAATGC-CCC CGGATGAGTAAAGGAGAAG and CTCTCTTTACTCA-TCCGCG-GGGCATTGCTTATCT were used to delete the region encoding amino acids 109–234 of cellugyrin in pSY14 and a *Sac*II restriction site was engineered into the deletion site. The resulting plasmid is designated pSY53. A *Hind*III and *Sac*II fragment from pSY54 was inserted into pSY53 digested with the same enzymes.

pSY59: Oligonucleotides GATCTCGCCACCATGACCATCCT-GCGCGTC and GACGCGCAGGATGGTCATGGTGGCGAGATC were designed to delete the sequences encoding amino acids 2–26 in pSY26.

pSY60: Oligonucleotides GACGTGCAAGATGGTGCAGGAGCG-GGAGGAGACTCAACC and GGTTGAGTCTCTCCCGCTCTG-CACCATCTTGCACGTC were designed to engineer the changes Y201A and Y203A in pSY3. Oligonucleotides CGCATGGCGTGT-GCAGAAGAAGGAAATC and GATTTCCTTCTTCTGCACGACG-CCATGCG were designed to engineer the change Y174A into the resulting plasmid.

pSY62: Oligonucleotides CCAACAAGAAGGGCAGCTGTCTTA-GCAGAT and ATCTGCTAGGACAGCTGCCCTTCTTGTGTGG were designed to engineer the R104A change in pSY3.

Germline Transformation

Nematodes were transformed with the use of the method described by Mello *et al.* (1991). Plasmids were injected at 5 ng/μl in conjunction with the dominant *rol-6(su1003)* transformation marker (Kramer *et al.*, 1990) plasmid pRF4 at 140 ng/μl. Several independent transformed lines were obtained for each construct examined.

Neuron Culture and Transfection

Hippocampal neuronal cultures were prepared as previously described (Banker and Cowan, 1977; Goslin and Banker, 1989). After dissociation and before plating, neurons were transfected by a lipid-mediated gene transfer method with the use of the Effectene kit (Qiagen). Approximately 5×10^5 cells were incubated with 1 μg of DNA for 2h at 37°C in the presence of the transfection reagents and were then plated onto coverslips in fresh medium at a density of 18,000 cells/cm². This procedure resulted in a 0.01–0.05% transfection efficiency. Neurons 5–7 d old were fixed and immunostained with an antibody against synaptophysin.

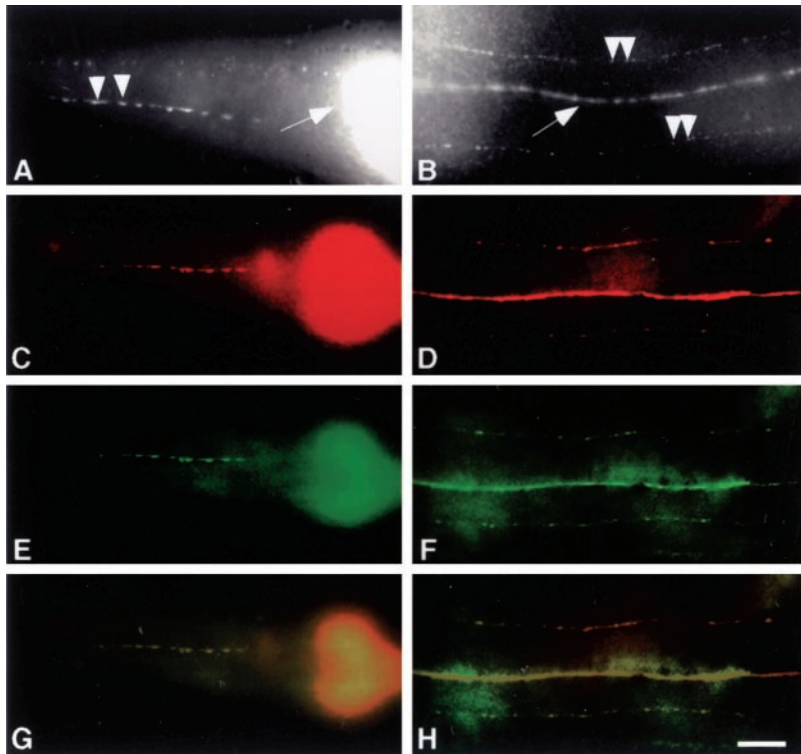


Figure 1. SNG-1::GFP is localized to synaptic regions in *C. elegans*. (A and B) Whole mount immunocytochemistry of adult wild-type animals stained with a primary antibody directed against SNG-1 and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. (A) Lateral view of the head illustrates the synaptic varicosities along an SAB motor neuron axon (arrowheads). The nerve ring (arrow) of the animal is also visible. (B) Dorsal view shows the dorsal nerve cord (arrow) and small fluorescence puncta (arrowheads) in dorsal sublateral nerve cords. (C–F) Whole mount immunocytochemistry of adult *jsIs219* animals visualized by incubation with primary antibodies directed against SNT-1 (C and D) and GFP (E and F). Cy3- and FITC-conjugated secondary antibodies are used respectively. (G and H) Merged images. (C, E, and G) Lateral views of the nerve ring and the presynaptic varicosities of SAB neurons. (D, F, and H) Dorsal views of the dorsal and dorsal sublateral nerve cords ([C–H] genotype: *jsIs219*). Bar, 10 μ m.

RESULTS

Endogenous SNG-1 and GFP-tagged SNG-1 Are Localized to SVs in *C. elegans*

To assess the localization of endogenous SNG-1, an antibody was raised against the C terminus of the protein. Immunostaining wild-type animals with the use of this antibody revealed a typical localization pattern observed for other SV proteins, including SNT-1, SNB-1 (synaptobrevin), and RAB-3 (Nonet *et al.*, 1993, 1997, 1998). Immunoreactivity was concentrated in nervous system regions rich in synapses, including the nerve ring (Figure 1A), the dorsal nerve cord (Figure 1B) and the ventral nerve cord (not shown). Discrete puncta were found in the ventral and dorsal sublateral process bundles (Figure 1B; our unpublished results). The SNG-1 protein also accumulated in the presynaptic varicosities of SAB motor neurons innervating the head muscle (Figure 1A). Finally, the endogenous protein was undetectable in neuronal cell bodies, commissures that run circumferentially along the body, and the dendrites of sensory neurons in the nose. Wild-type animals double immunolabeled with antibodies against SNG-1 and SNT-1 showed that native SNG-1 colocalized with SNT-1 (our unpublished results). Taken together, these results suggest that endogenous SNG-1 is localized to SVs in *C. elegans*, although localization to another membrane such as synaptic endosomes cannot be excluded.

We next asked whether GFP-tagged SNG-1 fusion protein is localized like the endogenous protein. *jsIs219* animals harbor a GFP fusion to the C terminus of *sng-1* under the control of the *sng-1* promoter (SNG-1::GFP) stably integrated into the genome (Nonet, 1999). No behavioral defects have

been observed in *jsIs219* animals. In *jsIs219*, the pattern of GFP fluorescence observed was similar to the typical localization pattern seen in immunolocalization experiments with endogenous SNG-1, except that faint GFP fluorescence was detected in the neuronal cell bodies in the head ganglia and the ventral nerve cord (Figure 2B). Double staining *jsIs219* with antibodies against GFP (Figure 1, E and F) and SNT-1 (Figure 1, C and D) revealed similar localization pattern in the nerve ring, nerve cords, and the SAB axons. The merged images indicate that SNG-1::GFP colocalizes with SNT-1 (Figure 1, G and H). These results strongly suggest that SNG-1::GFP is localized to SVs, similar to endogenous SNG-1.

An additional line of evidence supporting this conclusion is the dependence of the synaptic localization of SNG-1::GFP on the *unc-104* gene. The *unc-104* gene encodes a kinesin-like molecule that is required for the axonal transport of SVs (Hall and Hedgecock, 1991; Otsuka *et al.*, 1991). Mutations in this gene result in the accumulation of SVs in neuronal cell bodies (Hall and Hedgecock, 1991). SV proteins such as RAB-3, SNB-1, and SNT-1 all require *unc-104* for synaptic localization (Nonet *et al.*, 1993, 1997, 1998). In contrast, localization of nonvesicular localized proteins such as SYD-2, RIM, UNC-11, and UNC-13 does not require *unc-104* (Nonet *et al.*, 1999; Zhen and Jin, 1999; Kohn *et al.*, 2000; Hadwiger and Nonet, unpublished observations). In *unc-104(e1265)* mutants, SNG-1::GFP was concentrated in neuronal cell bodies in both the head ganglia and the ventral nerve cord (Figure 2, C and D). GFP fluorescence was greatly diminished in ventral axonal regions (Figure 2D) and absent from sublateral nerve cords, SAB axons and the dorsal nerve cord (our unpublished results). This result demonstrates that

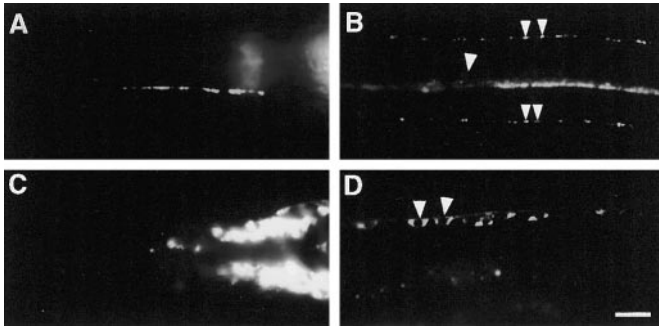


Figure 2. SNG-1::GFP localization depends on *unc-104*. (A–D) Images of live anesthetized animals expressing GFP-tagged SNG-1. (A) Lateral view of the head of a *jsIs219* animal showing an SAB axon with presynaptic varicosities. (B) Ventral view of the ventral nerve cord and ventral sublateral nerve cords of a *jsIs219* animal. A punctate pattern of fluorescence is seen along all three nerve cords. Individual puncta are seen in the sublateral cords (small arrowheads). Neuronal cell bodies are barely detectable in the ventral nerve cord (large arrowhead). (C) Lateral view of the head of the axonal transport mutant *unc-104(e1265)* showing fluorescence accumulations in the head ganglia. (D) Ventral view of the ventral nerve cord of an *unc-104(e1265)* hermaphrodite. Fluorescence accumulates in the neuronal cell bodies (arrowheads) along the nerve cord [genotype of A and B, *jsIs219*; C and D, *unc-104(e1265) jsIs219*]. Bar, 10 μm .

SNG-1::GFP localization requires the UNC-104 kinesin like other SV proteins, suggesting that it is localized to SVs.

For the purposes of this study, any GFP fusion protein that is considered to be localized to SVs has the following features: 1) strong punctate signal along the nerve cords; 2) accumulation of GFP fluorescence in the nerve ring and presynaptic varicosities of SAB neurons with little process staining; 3) no signal in sensory dendrites in the nose and commissures along the body, and neuronal cell bodies undetectable or barely visible; and 4) a dependency of the localization pattern on the *unc-104* gene.

SNG-1::GFP Localization Does Not Require Several Synaptic Proteins We Examined

Biochemical studies have revealed many protein–protein interactions between different SV proteins (Bennett *et al.*,

1992; Calakos and Scheller, 1994; Galli *et al.*, 1996; Schivell *et al.*, 1996). To assess whether localization of SNG-1 is mediated by protein–protein interactions with other SV protein(s), we examined SNG-1::GFP or native SNG-1 localization in mutants lacking distinct SV-associated proteins, including RAB-3 and RBF-1 (rabphilin), SNB-1, and SNT-1 (Table 1; Nonet *et al.*, 1993, 1997, 1998; Staunton, Ganetzky, and Nonet, unpublished observations). In these single mutant backgrounds, the localization of SNG-1::GFP or native SNG-1 was indistinguishable from that in *jsIs219* or wild-type animals (our unpublished results). We also determined whether SNG-1::GFP localization depends on *unc-11* and *aex-3* genes, which are required for the localization of SNB-1 and RAB-3 in *C. elegans*, respectively (Table 1; Iwasaki *et al.*, 1997; Nonet *et al.*, 1999). The localization of SNG-1::GFP was not affected by mutations in either gene. In addition, the gross localization pattern of SNG-1::GFP was not affected by genes that have been implicated in SV endocytosis such as *unc-26* and *dpy-23* (Table 1; Harris *et al.*, 2000; Baum and Garriga, personal communication). For all mutants analyzed, except *aex-3*, null alleles were used to examine the localization of SNG-1::GFP or native SNG-1. Thus, SNG-1::GFP/SNG-1 localization does not depend on the individual SV proteins considered, suggesting that SNG-1 localization to synaptic regions might be mediated by information in its own sequence.

A Synaptic Localization Signal Resides in C-Terminal Domain of SNG-1

The topology of rat synaptogyrin suggests that the N terminus, C terminus, and the small loop between the second and the third transmembrane domain of SNG-1 reside in the cytoplasm (Stenius *et al.*, 1995; Nonet, 1999). Reasoning that the localization of SNG-1 might be mediated by interactions with cytoplasmic factors, we deleted cytoplasmic domains of SNG-1 and assessed the ability of the lesioned proteins to localize *in vivo* (Figure 4C). As shown in Figure 3, A and B, when the N terminus was deleted, the localization of the mutated protein was similar to the full-length protein. However, when the C terminus was deleted, the pattern of GFP fluorescence was altered. SNG-1 ($\Delta 173$ –248)::GFP decorated the entire cell in a distribution likely to reflect localization to the plasma membrane. Fluorescence was observed in the sensory dendrites in the nose (Figure 3E) and in many

Table 1. Mutants in which SNG-1 localization is examined

Gene	Protein homolog	Function	Allele used
<i>snt-1</i>	Synaptotagmin	Regulating Ca^{2+} -triggered fusion	<i>md290</i> , null
<i>snb-1</i> ^a	Synaptobrevin	v-Snare, essential for fusion	<i>js124</i> , null
<i>rab-3</i> ^a	RAB-3	Regulating synaptic vesicle docking	<i>js49</i> , null
<i>rbf-1</i> ^a	Rabphilin	RAB-3 binding protein, potentiate SNARE complex formation independent of RAB-3	<i>js232</i> , null
<i>unc-11</i>	AP180	Required for SNB-1 localization	<i>e47</i> , null
<i>aex-3</i>	DENN	GDP/GTP exchange factor, required for RAB-3 localization	<i>y255</i> , severe
<i>unc-26</i>	Synaptojanin	Phosphatidylinositol-5-phosphatase, required for endocytosis	<i>s1710</i> , null
<i>dpy-23</i>	AP-2 subunit $\mu 2$	Required for endocytosis	<i>e840</i> , null

^a SNG-1 localization is examined by incubation with primary antibody against SNG-1 and FITC-conjugated secondary antibody. Others were examined by fluorescence of GFP-tagged SNG-1.

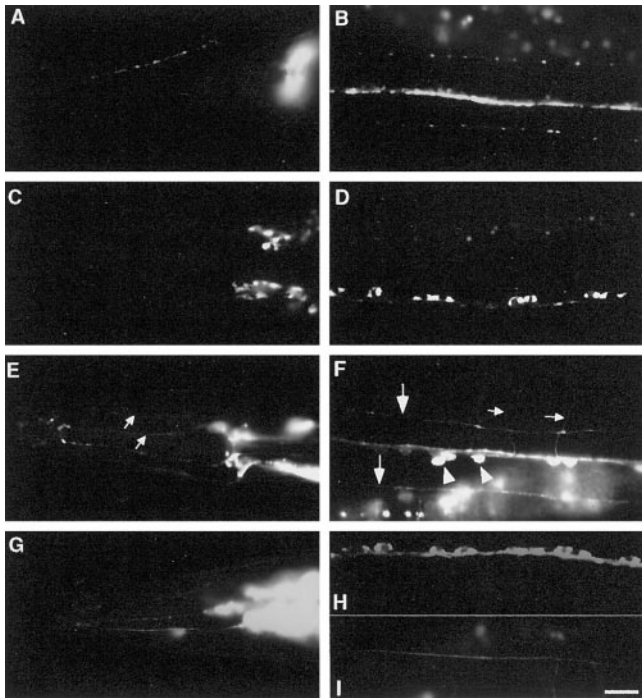


Figure 3. Deletion of C-, but not N-terminal domain causes mislocalization of SNG-1::GFP. (A–I) Images of live anesthetized transgenic animals expressing mutated GFP-tagged SNG-1. (A, C, E, and G) Lateral views of the head. (B, D, F, and H) Ventral views of the ventral nerve cords. (I) Dorsal view of the dorsal nerve cords. (A and B) SNG-1($\Delta 2$ –24)::GFP accumulates in presynaptic varicosities along an SAB axon (A) and shows a punctate pattern along all three nerve cords in a wild-type background (genotype: *jsEx353*, plasmid: pSY17). (C and D) SNG-1($\Delta 2$ –24)::GFP accumulates in neural cell bodies in head ganglia (C) and along the ventral nerve cord (D) in an *unc-104(e1265)* background [genotype: *unc-104(e1265); jsEx353*, plasmid: pSY17]. (E and F) SNG-1($\Delta 173$ –248)::GFP is observed in sensory dendrites (small arrows in E) and commissures (small arrows in F) in a wild-type background. Fluorescence is distributed diffusely along ventral sublateral cords (large arrows in F). GFP also accumulates in the neuronal cell bodies along the ventral nerve cord (arrowheads in F) (genotype: *jsEx355*, plasmid: pSY18). (G–I) SNG-1($\Delta 173$ –248)::GFP shows a similar pattern in an *unc-104(e1265)* background as in a wild-type background. Fluorescence decorates sensory dendrites (G), commissures, and accumulates in neuronal cell bodies along the ventral nerve cord (H). The dorsal nerve cords are also visible (I) [genotype: *unc-104(e1265); jsEx355*, plasmid pSY18]. Bar, 10 μ m.

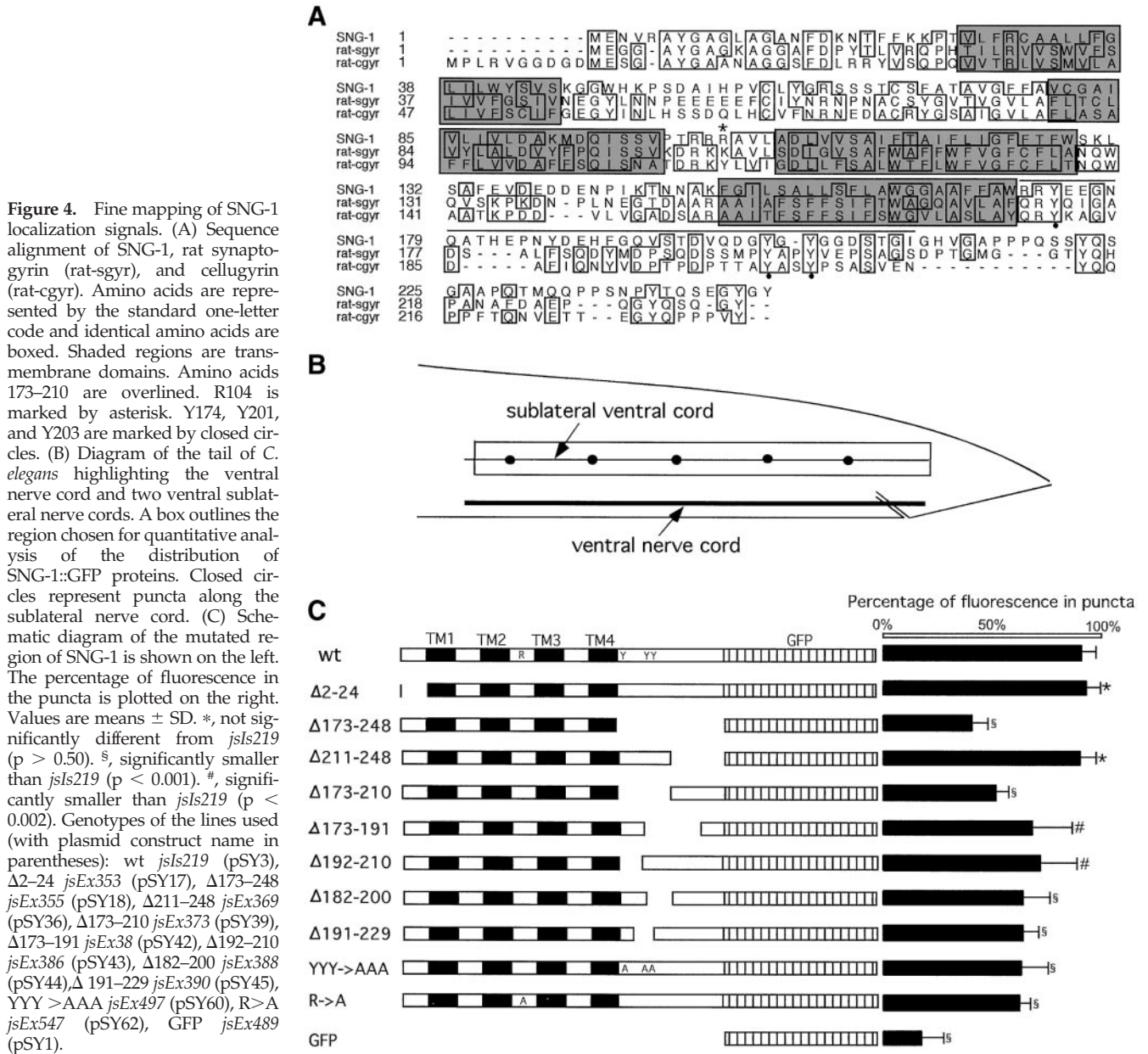
commissures along the body (Figure 3F). Moreover, fluorescence was more diffuse and less punctate along neuronal processes (Figure 3F). The fraction of GFP in the puncta of the ventral sublateral cord was dramatically decreased and the process regions were more clearly visible (Figures 3F and 4C). The process regions of SAB neurons were also much brighter compared with those in *jsIs219* (our unpublished results). Finally, bright fluorescence was seen in neuronal cell bodies along the ventral nerve cord and in the head ganglia (Figure 3, E and F). These results suggest that the C-terminal domain of SNG-1 is required for the correct localization of SNG-1, whereas the N-terminal domain is dispensable.

To provide additional support that the C-terminal deletion localizes to a cellular compartment distinct from SVs, we examined the localization of the mutant proteins in a *unc-104* background. The GFP fluorescence pattern of the fusion protein lacking the N terminus in *unc-104(e1265)* was similar to that of full-length SNG-1::GFP, namely, GFP accumulated in the neuronal cell bodies of the ventral nerve cord and the head ganglia (Figure 3, C and D) and was absent from the dorsal nerve cord and the varicosities of SAB neurons. In contrast, the C-terminal truncated protein showed a similar distribution pattern in both an *unc-104(e1265)* and a wild-type background (Figure 3, G–I). To eliminate the possibility that GFP is detached from the SNG-1($\Delta 173$ –248)::GFP protein, an eight amino acid FLAG tag was placed at the N terminus of the mutant protein [FLAG::SNG-1($\Delta 173$ –248)::GFP]. An identical distribution pattern of FLAG::SNG-1($\Delta 173$ –248)::GFP was revealed by antibodies directed against each of the FLAG and GFP tags (our unpublished results), demonstrating that the fusion protein is intact and the localization pattern of the epitopes reflects the distribution pattern of the fusion protein. Immunodetection of FLAG::SNG-1::GFP with the use of both anti-FLAG and anti-GFP showed that the FLAG tag had no effect on localization (our unpublished results). Taken together, these data demonstrate that the C-terminal region of SNG-1 is necessary for its localization to SVs.

Fine Mapping of Localization Signal within C Terminus of SNG-1

To further map the localization signal within the C-terminal domain, we performed a systematic deletion analysis within the C terminus. Figure 4C shows a schematic diagram of the mutated regions. A quantitative approach was used to determine the degree of *in vivo* synaptic localization of each mutated protein (see MATERIALS AND METHODS). The percentage of the fluorescence intensity in the puncta within a segment of the sublateral ventral cord just anterior of the tail was used as an index for synaptic localization. The localization index of GFP alone was determined as a control.

As shown in Figure 4C, when the whole C-terminal domain was deleted, the localization index was dramatically decreased compared with the full-length fusion protein. However, when amino acids 211–248 were deleted, the localization index was unchanged. In contrast, when amino acids 173–210 were deleted, the localization index was decreased almost to the same extent as when the whole C-terminal domain was deleted. Further division of this region led to partial localization. These results were consistent with the distribution patterns observed by fluorescence microscopy, indicating that the localization signal is broadly distributed within amino acids 173–210 in the C terminus. There are three tyrosines conserved between rat synaptogyrin and SNG-1 within this defined region (Figure 4A). We investigated the role of these tyrosine residues in the localization of SNG-1 because such tyrosine-based signals have been shown to mediate endocytosis (Davis *et al.*, 1986, 1987; Collawn *et al.*, 1990; Peters *et al.*, 1990; Thies *et al.*, 1990; Letourneur and Klausner, 1992; Haucke and De Camilli, 1999). The Y174A Y201A Y203A triple mutant protein was partially mislocalized (Figure 4C). This suggests that the three tyrosines in the C terminus play a role in SNG-1 localization although they are not solely responsible for it. In



summary, our data are consistent with a role for the C terminus in targeting SNG-1 for endocytosis from the plasma membrane. However, they do not exclude a role of the C terminus in the sorting of SNG-1 to SV precursors at the level of the *trans*-Golgi.

A Single Arginine in Small Cytoplasmic Loop Is Involved in Proper Localization of SNG-1

The cytoplasmic loop between transmembrane domain 2 and 3 of SNG-1 is well conserved among members of the synaptogyrin family (Figure 4A). Because rat synaptogyrin and SNG-1 are localized in *C. elegans* (Figure 6), but rat cellugyrin is not (Figure 5), we focused our attention on

differences in this domain because they might identify a signal regulating the selective targeting of synaptogyrin to SVs, rather than to other transport vesicles. Specifically, we noted that a tyrosine in cellugyrin that is substituted by a positively charged amino acid in both synaptogyrin and SNG-1. Hence, we mutated the corresponding amino acid, arginine, in SNG-1 to an alanine by site-directed mutagenesis. In transgenic animals, R104A mutant SNG-1::GFP was partially mislocalized. The fraction of GFP signal in synaptic puncta was decreased (Figure 4C). In addition, GFP signal was retained in the neuronal cell bodies and was present in the commissures and sensory dendrites (Figure 5, K and L). Although this finding does not directly address which sort-

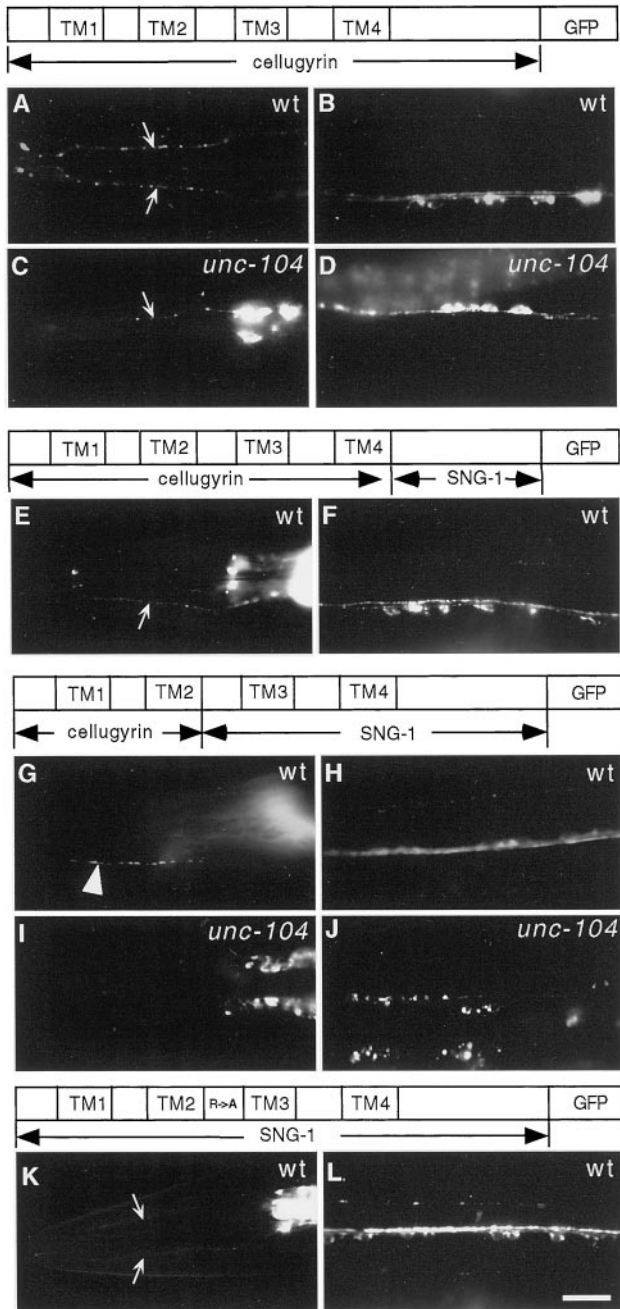


Figure 5. Localization signals of SNG-1 target rat cellugyryn to synaptic regions in *C. elegans*. Images of live anesthetized animals expressing diagrammed constructs (A, C, E, G, I, and K). Lateral views of the head (B, D, F, H, J, and L). Ventral views of the ventral nerve cords. (A) Cellugyryn is seen in sensory dendrites (labeled with small arrows), commissures, and neural cell bodies (B) in a wild-type background and an *unc-104* background (C and D). A chimera containing cellugyryn sequences to the end of the transmembrane segments and the SNG-1 C-terminal domain shows a similar distribution pattern as cellugyryn (F and G). In a chimera with additional SNG-1 sequences, the protein now behaves like SNG-1 and accumulates in the presynaptic varicosities along an SAB axon (large arrowhead in H) and shows a punctate pattern along the ventral nerve cords (I) in a wild-type background. In an

ing step is disrupted by this domain, our data suggest that it plays a role in sorting synaptogyrin to SVs.

Cellugyryn-SNG-1 Chimera Is Localized to Synaptic Regions

We next assessed whether a region of SNG-1 containing the two identified primary sequence motifs necessary for SNG-1 localization are sufficient to localize rat cellugyryn to synaptic regions. GFP-tagged full-length cellugyryn expressed in *C. elegans* under the control of the *sng-1* promoter showed a very different localization pattern from SNG-1::GFP. Cellugyryn::GFP accumulated in neuronal cell bodies of the head ganglia and the ventral nerve cord (Figure 5B). GFP was also distributed in the sensory dendrites in the nose and commissures running circumferentially along the body (Figure 5, A and B). Along the nerve cords and SAB axons, GFP was much less punctate (our unpublished results). This distribution pattern was not affected by mutation in *unc-104* gene (Figure 5, C and D). These results demonstrate that rat cellugyryn is not restricted to synaptic regions in *C. elegans*, which is consistent with its endogenous localization in rat where it is excluded from SVs (Janz and Südhof, 1998).

When the C-terminal domain of cellugyryn was replaced by the C-terminal domain of SNG-1, the localization pattern of the chimera was indistinguishable from cellugyryn alone (Figure 5, E and F). However, when a region of SNG-1 containing both the C terminus and the cytoplasmic loop was used to replace the corresponding region of cellugyryn, the chimera was localized to synaptic regions in a manner similar to SNG-1::GFP (Figure 5, G and H). The only difference between the two localization patterns was the slightly greater fluorescence intensity in neuronal cell bodies in animals expressing the cellugyryn::GFP chimera compared with animals expressing SNG-1::GFP (Figure 5H). Furthermore, in *unc-104(e1265)* background, the chimeric protein was accumulated in neuronal cell bodies (Figure 5, I and J). The GFP-tagged cellugyryn portion or the SNG-1 portion of the chimeric protein was not localized to synaptic regions by itself, instead, the fusion protein was accumulated in the neuronal cell bodies (not shown). These results suggest that the region of SNG-1 that contains the identified localization motifs is sufficient to localize cellugyryn to synaptic regions. This does not exclude the possibility that the third and fourth transmembrane domain and the second intravesicular loop of SNG-1 may aid in the localization of the chimera.

Mechanisms for Synaptogyrin Localization Are Evolutionarily Conserved

To determine whether mechanisms of synaptogyrin localization are evolutionarily conserved, we assessed whether

unc-104(e1265) background, the chimera accumulates in neural cell bodies in the head ganglia (J) and the ventral nerve cord (K). SNG-1(R104A) is partially mislocalized because fluorescence is present in sensory dendrites (K) and commissures as well as cell bodies (L). Genotype of strains used (with plasmid construct in parentheses): A and B, *jsEx375* (pSY14); C and D, *unc-104(e1265); jsEx375* (pSY14); E and F, *jsEx357* (pSY31); J and H, *jsEx498* (pSY57); I and J, *unc-104(e1265); jsEx498* (pSY57); K and L, *jsEx547* (pSY62). Bar, 10 μ m.

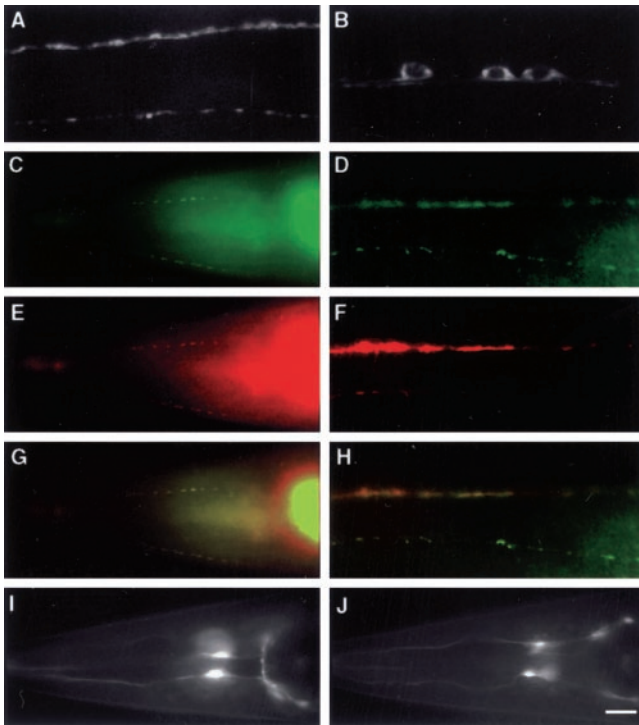


Figure 6. Rat synaptogyrin is localized to synaptic regions in *C. elegans*. (A and B) Images of live anesthetized animals expressing GFP-tagged rat synaptogyrin. (A) Ventral view of a wild-type animal showing a punctate GFP pattern in the ventral nerve cords. (B) Ventral view of an *unc-104(e1265)* animal showing GFP accumulations in neuronal cell bodies along the ventral nerve cord. (C–H) Images of wild-type animals expressing GFP-tagged synaptogyrin double stained with antibodies against GFP (C and D) and SNG-1 (E and F). FITC- and Cy-3-conjugated secondary antibody are used respectively. (G and H) Merged images. (C, E, and G) Lateral views of the head. (D, F, and H) Ventral views of the ventral nerve cords. (I and J) Lateral views of the head of wild-type animals expressing GFP-tagged rat synaptogyrin lacking the C-terminal domain. Fluorescence accumulates in selective processes in the nerve ring (I) and neuronal cell bodies of the head ganglia (I and J). GFP is also present in sensory dendrites in the nose (I and J). Genotypes of strains used (plasmid construct in parentheses): A, C–H, *jsEx403* (pSY12); B, *unc-104(e1265); jsEx403* (pSY12); I and J, *jsEx421* (pSY47). Bar, 10 μ m.

the localization signal(s) of rat synaptogyrin can be recognized in *C. elegans*. A GFP fusion to the C terminus of rat synaptogyrin was expressed in a wild-type genetic background under the control of the *sng-1* promoter. In transgenic animals, GFP-tagged rat synaptogyrin showed a punctate pattern along the major nerve cords and the sublateral nerve cords (Figure 6A). GFP was concentrated in the nerve ring and in the presynaptic varicosities of SAB neurons (our unpublished results). In contrast, in an *unc-104(e1265)* mutant background, fluorescence accumulated in the cell bodies along the ventral nerve cord (Figure 6B) and in the head ganglia (our unpublished results). Furthermore, GFP-tagged rat synaptogyrin colocalized with native SNG-1 shown by double immunostaining with the use of antibodies against GFP and SNG-1 (SNG-1 antibody did not cross-react with rat synaptogyrin) (Figure 6, C–H). These results suggest that

rat synaptogyrin is localized to synaptic regions in *C. elegans*, indicating that the localization signal(s) of rat synaptogyrin are recognized by the *C. elegans* SNG-1 localization machinery. We next asked whether the C terminus of rat synaptogyrin is necessary for its localization in *C. elegans*. GFP-tagged synaptogyrin lacking the C terminus was expressed in wild-type animals. Fluorescence was detected in neuronal cell bodies in the head ganglia and the sensory dendrites in the nose (Figure 6, I and J), but not synaptic regions, suggesting the C terminus of synaptogyrin is necessary for its localization in *C. elegans*.

To determine whether rat synaptogyrin was localized similarly to other SV proteins in mammalian neurons, we examined the localization of synaptogyrin in hippocampal neurons. First, GFP was fused to the C terminus of full-length synaptogyrin and the fusion gene was transfected into cultured hippocampal primary neurons. Then 5- to 7-day-old neurons were fixed to preserve GFP fluorescence and stained with an antibody against synaptophysin. Figure 7B shows that the full-length GFP-tagged synaptogyrin exhibited a punctate pattern along the axonal process of the hippocampal neuron, similar to the pattern observed by anti-synaptophysin immunostaining (Figure 7, A and C). GFP was also seen in neuronal cell bodies and dendritic processes, where it was evenly distributed with no obvious puncta (our unpublished results). This aberrant localization may be the result of overexpression of the fusion protein. These results suggest that axonal GFP-tagged synaptogyrin is largely localized to synaptic regions in hippocampal neurons.

To identify whether the localization signals used by *C. elegans* SNG-1 and rat synaptogyrin were similar we examined the localization of GFP-tagged rat synaptogyrin lacking either the N- or C-terminal domain in cultured hippocampal neurons. Synaptogyrin lacking its N terminus (Figure 7, I–L) showed a distribution pattern similar to the full-length rat synaptogyrin (Figure 7, A–D), suggesting that the N-terminal domain is not required for its correct localization. However, when the C-terminal domain was deleted from rat synaptogyrin, GFP fluorescence was no longer punctate, but showed a diffuse pattern along the axonal processes of cultured hippocampal neurons (Figure 7, E and F). Furthermore, this mutant protein no longer colocalized with synaptophysin immunostaining (Figure 7G) because GFP was present in regions of processes where synaptophysin was absent (Figure 7H). This distribution pattern was similar as that of GFP alone expressed in hippocampal neurons (Figure 7, M–P). These results suggest that the C-terminal domain of synaptogyrin contains a targeting signal that is required for its synaptic localization in mammalian neurons.

DISCUSSION

We have identified the primary sequences that are required for the subcellular localization of both *C. elegans* and rat synaptogyrin. Synaptic localization of SNG-1 depends upon two sequence motifs located in the cytoplasmic loop separating the second and third transmembrane domains and in the C-terminal domain. These two domains may define signals required for targeting SNG-1 selectively to SVs and for selective endocytosis from the plasma membrane. These regions define bona fide localization signals because they are

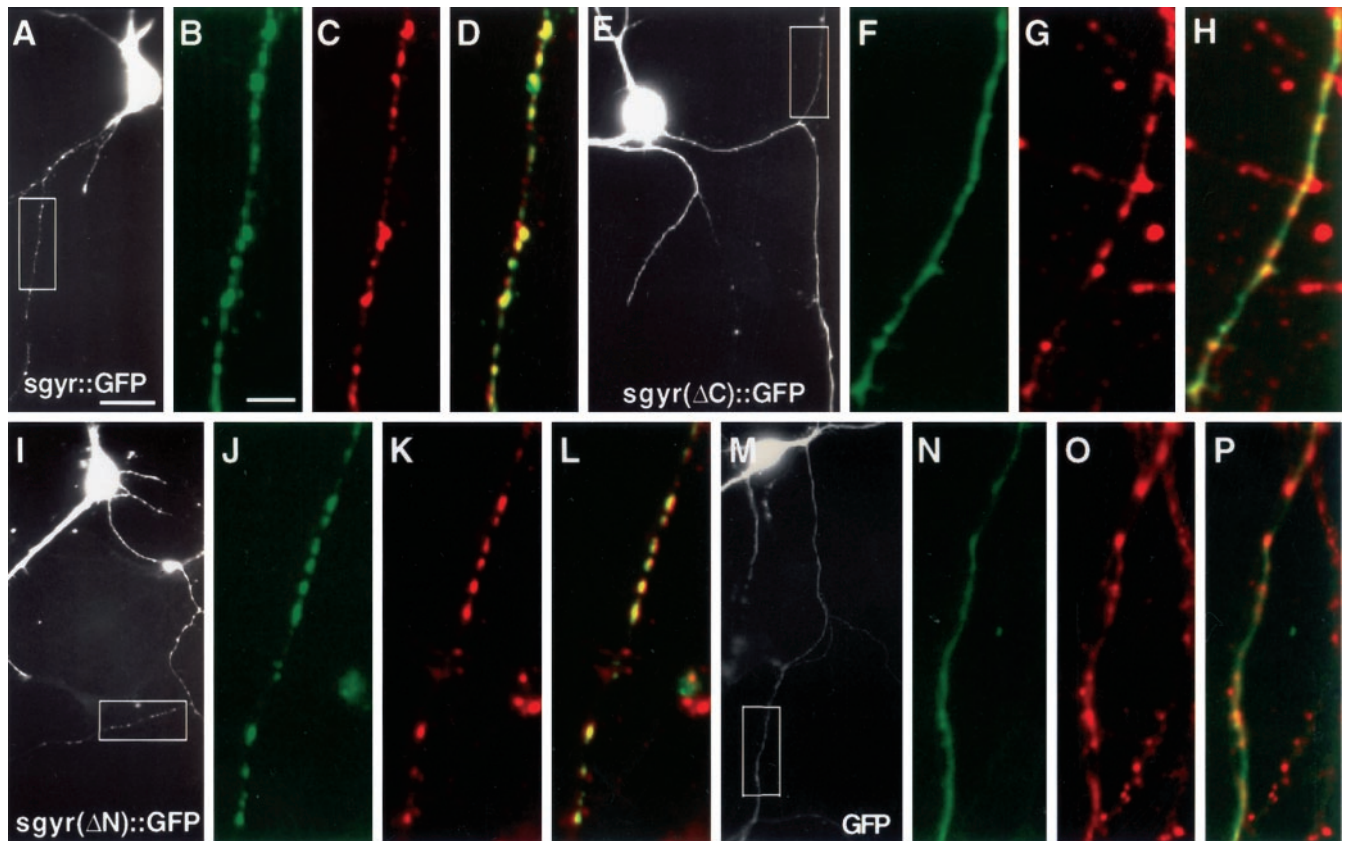


Figure 7. Deletion of C-terminal domain of rat synaptogyrin causes mislocalization in hippocampal neurons. (A, E, I, and M) Images of hippocampal neurons expressing GFP or GFP-tagged synaptogyrin visualized by GFP. Boxed areas are regions shown in after high magnification images. (B–D, F–H, J–L, N–P) High-magnification images of axonal processes visualized by GFP (B, F, J, and N) and incubation with a primary antibody against synaptophysin and an LRSC-conjugated secondary antibody (C, G, K, and L). (D, H, L, and P) are merged images. (A–D) GFP-tagged full-length synaptogyrin expressed with the use of plasmid pSY26 colocalizes with synaptophysin in a punctate pattern along the axonal process. (E–H) GFP-tagged synaptogyrin lacking the C terminus expressed with the use of plasmid pSY46 shows a smooth distribution along the axon (F), whereas synaptophysin is localized to synaptic puncta (G). They do not colocalize in the merged image (H). (I–L) GFP-tagged synaptogyrin lacking N terminus expressed with the use of plasmid pSY59 shows a similar distribution pattern as the full-length protein. (M–P) GFP alone is distributed evenly along the axonal process. Bar in A is for A, E, I, and M, 10 μ M. Bar in B is for B–D, F–H, J–L, and N–P, 5 μ m.

also sufficient, in the context of other SNG-1 sequences, to localize rat cellugyrin to synaptic regions in *C. elegans*. The C-terminal domain of rat synaptogyrin contains a similar targeting signal required for localization in hippocampal neurons. Indeed, the rat ortholog is appropriately localized in *C. elegans* and the analogous C-terminal region of rat synaptogyrin is required in this assay. These data suggest that the rat localization signal is recognized by the *C. elegans* trafficking machinery. Thus, the mechanisms for synaptogyrin localization appear to be evolutionarily conserved. These results represent the first set of experiments that define the localization signal of a SV integral membrane protein in a model organism with the use of an in vivo assay.

Caenorhabditis elegans as a Model System to Study Protein Targeting

The criteria we have used to examine localization of a SV protein in *C. elegans* differ considerably from criteria used for

most prior studies in PC12 cells to define SV targeting signals. Our studies use primarily visual criteria to assess localization, whereas studies with the use of PC12 cells rely on biochemical fractionation. Due to the small size of *C. elegans* it is essentially impossible to separate the 2% of neuronal tissue in the *C. elegans* adult before biochemical fractionation. Our attempts to develop biochemical assays for localization have been severely hampered by this limitation. Despite the limitations, the availability of mutants permit us to examine the dependence of localization on specific trafficking components. For example, examination of the targeting behavior in *unc-104* kinesin mutants acts as an indirect assay for sorting to preSVs. As the study of trafficking identifies genes essential for other trafficking steps, these assays will become a powerful tool in our analysis. Our extensive reliance on visual assays does limit the interpretation of some of our in vivo findings. Specifically, although N-terminal-deleted SNG-1 still targets to regions rich in syn-

apses, it is possible that the protein is still absent from mature SVs, and instead accumulates in another vesicular structure in synaptic neuropil (such as synaptic endosomes). It is also difficult to assess in which cellular compartments our mistargeted SNG-1 mutants are accumulating.

Although our assays have limitations, they examine trafficking in functioning neurons in an intact animal. In other studies, we have observed significant differences between the role of targeting signals in PC12 cells and in our *in vivo* assays. Specifically, we examined the role of previously defined SV targeting signals of synaptobrevin (Grote *et al.*, 1995). We introduced into the *C. elegans* synaptobrevin gene two specific lesions (M46A and N49A), which altered targeting of synaptobrevin in PC12 cells. Genomic clones containing each lesion completely rescued the lethal phenotype of *C. elegans* synaptobrevin mutants and the mutant protein localized indistinguishably from wild-type (Wei and Nonet, unpublished observations). These observations imply that the identified sequences do not play as critical roles in synaptobrevin sorting in neurons *in vivo*. This dichotomy could be explained either by differences between how PC12 cells and neurons sort synaptic proteins, or in differences between synaptobrevin sorting mechanisms in vertebrates and invertebrates. However, our data demonstrating the conservation of synaptogyrin sorting signals through the metazoan lineage suggest that differences between PC12 cells and neurons may be more significant than species differences.

Our *in vivo* observations also provide a framework within which to interpret functional studies in PC12 cells. Previous experiments reported that C-terminal sequences adjacent to the transmembrane region in rat synaptogyrin are required for its inhibitory effect on Ca^{2+} dependent exocytosis in PC12 cells (Sugita *et al.*, 1999). Our data suggest that deleting this region probably disrupts protein localization; this in turn could indirectly lead to the reported effects on exocytosis. Our system provides a relatively simple method of examining the role of identified targeting signals *in vivo* that complement biochemical studies with the use of PC12 cells. Furthermore, in cases where mutants disrupting the gene encoding the protein of interest have been identified, the role of these signals can also be assessed functionally.

The potential to saturate or alter sorting pathways as in response to changes in expression levels of introduced genes is a difficult issue to control for in both this system and in PC12 cells. However, we believe it is unlikely that expression levels are significantly affecting our assays. In *C. elegans*, expression levels in transgenic animals are often manipulated by varying the ratio of input experimental and marker DNAs (Mello and Fire, 1995). The mutated SNG-1 constructs were injected at a DNA concentration several fold below levels where wild-type SNG-1 protein remains localized indistinguishably from the native protein (our unpublished results). In addition, multiple transgenic lines were created for each construct we examined, and several with similar expression levels behaved identically. Finally, the fluorescent intensity did not vary dramatically in animals expressing different mutant SNG-1 proteins. Taken together, our data suggest that expression levels of the various mutant SNG-1 proteins do not contribute significantly to their mislocalization.

Synaptic Localization Signals in Other Systems

Most of the studies of trafficking pathways of SV proteins have examined the targeting of proteins to SLMVs in PC12 cells. Experimental evidence suggests that SV protein sorting occurs by at least three pathways at a number of distinct stages during the SV life cycle (Calakos and Scheller, 1996; Hannah *et al.*, 1999). SVs are probably not formed directly by budding from the TGN; rather mature SVs probably bud off from another membrane compartment. Experimental evidence supports both the plasma membrane and endosomal intermediates as the donor compartment (Regnier-Vigouroux *et al.*, 1991; Bauerfeind and Huttner, 1993). Pulse chase studies of synaptophysin trafficking in PC12 cells suggest that synaptophysin is routed through the plasma membrane before inclusion into SLMVs. This route is apparently adaptor protein complex 2- (AP-2), clathrin-, and dynamin-dependent and brefeldin A-insensitive (Takei *et al.*, 1996; Shupliakov *et al.*, 1997; Schmidt and Huttner, 1998; Shi *et al.*, 1998). *In vitro* studies have demonstrated that SVs can be budded from the endosomal compartment with the use of AP-3 and the small GTPase ADP ribosylation factor 1 (Faundez *et al.*, 1997, 1998) as well as being brefeldin A-sensitive (Shi *et al.*, 1998). However, AP-3 δ subunit mutants in mouse are viable and contain an abundance of normal SVs, suggesting this is not an obligate pathway (Kantheti *et al.*, 1998). In addition, recent data have documented that a third route of SLMV formation, from the late endosome, also exists in PC12 cells. In this pathway, P-selectin passes through early and late endosomal intermediates sequentially en route to both the lysosome and SLMVs (Blagoveshchenskaya and Cutler, 2000).

At least in neuroendocrine PC12 cells, different proteins use different pathways to differing extents to traffic to the same destination vesicle, the SLMV (Shi *et al.*, 1998; Blagoveshchenskaya *et al.*, 1999a,b). This suggests that each SV protein contains multiple signals that control the flux of the protein through more than one pathway. These signals may mediate different sorting steps at the TGN, plasma membrane, and early or late endosome. In keeping with this idea multiple signal sequences have been found within the cytoplasmic domain of synaptobrevin and synaptotagmin. In neuroendocrine PC12 cells, a sorting signal of synaptobrevin consisting of residues 41–50 probably functions at the plasma membrane because its deletion prevented endocytosis and SLMV targeting (Grote *et al.*, 1995; Grote and Kelly, 1996). In contrast, residues 31–38 of synaptobrevin are required at a sorting stage other than endocytosis at the plasma membrane because a deletion mutant lacking these amino acids was endocytosed normally but excluded from SLMV (Grote *et al.*, 1995). Although these regions are within the amphipathic helix required for biochemically defined synaptobrevin functions (SNARE complex formation), studies by Hao *et al.* (1997) revealed little correlation between either endocytosis or SLMV targeting activity and complex formation activity. Furthermore, West *et al.* (1997) showed that the cytoplasmic sequences of synaptobrevin were sufficient to target synaptobrevin to synaptic sites in hippocampal cultures, but not into SVs, suggesting that the transmembrane sequences may also contain targeting information. Although multiple sorting motifs have been found in the C-terminal domain of synaptotagmin, a detailed biochemical study is lacking that would identify the precise steps in

which they play an important role (Blagoveshchenskaya *et al.*, 1999; Krasnov and Enikolopov, 2000).

SNG-1 Sorting Signals in *C. elegans*

In the *C. elegans* SNG-1 protein we have also found multiple elements that are required for its proper targeting, namely, a 38 amino acid element in the C-terminal domain and a single amino acid in the cytoplasmic loop. Mutations in these sequences could affect SNG-1 sorting at the TGN, plasma membrane, or endosomal compartments. We observe an accumulation of SNG-1 at the periphery of neuronal cell bodies, suggesting that these elements may be required for endocytosis at the plasma membrane. Lesioning the three tyrosines in the C-terminal targeting domain also resulted in accumulation of mutant protein in the periphery of the soma. Thus, one component of the signal may be a tyrosine-based endocytosis signal. In addition, the distribution of the mutant proteins is not significantly altered by a reduction in SV axonal transport caused by the *unc-104* mutant, suggesting that SNG-1 lacking these elements is distributed to vesicles other than preSVs at the TGN. One simple model explaining the behavior of our mutants implicates two signals. First, a TGN signal targeting proteins to preSVs. In absence of this signal synaptogyrin traffics to the plasma membrane in the cell body. The protein accumulates in the plasma membrane rather than maturing via an endocytic pathway in the soma because of the absence of a second signal, perhaps tyrosine-based, which mediates an AP-2-dependent endocytosis. Although our data are consistent with this model, in absence of a clearer understanding of the cellular compartments where targeting mutants accumulate, it is difficult to eliminate many other consistent models.

What Can Diverse Sorting Signals Achieve?

The signal sequences identified in SNG-1 are unique and do not share any obvious homology to other SV protein-sorting sequences. Other sequences identified in other systems also do not show any similarity to each other. This strongly supports the idea that no common primary sequence motif serves as a universal SV targeting signal. What would be the advantage of having each SV protein sorted with the use of distinct targeting signals, instead of with the use of one common signal? First, multiple signals may aid in regulating the stoichiometry of individual proteins in the SV, a small organelle containing only a few molecules of certain critical protein constituents. Therefore, achieving vesicular homogeneity may require that SV formation be tightly controlled at the single protein level. During the sorting process, "adaptor" proteins that interact with multiple vesicle proteins with the use of distinct targeting sequences could ensure a specific protein stoichiometry in each vesicle. In contrast, if different proteins compete with the use of the same targeting signal, the relative stoichiometry may be more stochastic. Second, several different types of related secretory vesicles are present in neurons and secretory cells with unique, but related membrane protein composition (Winkler, 1997). These include peptide-containing dense core secretory vesicles as well as zinc-containing vesicles (Perez-Clausell and Danscher, 1985). Multiple targeting signals may be needed to target the same protein to different classes of related vesicles. Finally, it may be beneficial for

certain classes of vesicles to be nonfunctional with respect to secretion until they reach their final destination. Trafficking of distinct secretory vesicle components to the synapse with the use of different pathways is one mechanism of ensuring that preSVs are largely fusion incompetent.

How Is Sorting Achieved?

Identifying *trans*-acting factors that interact with identified sorting signals is critical to understanding complex sorting processes that are likely to be dependent on both protein-protein and protein-lipid interactions. We have used two approaches to attempt to identify *trans*-acting factors. First, we have examined the localization of SNG-1 in other mutants lacking specific SV proteins. In particular, we examined the localization of SNG-1 in a synaptobrevin mutant background. In vertebrates, synaptophysin (related to synaptogyrin) interacts with synaptobrevin (Calakos and Scheller, 1994; Galli *et al.*, 1996), and formation of this complex correlates with maturation of synapses (Becher *et al.*, 1999). In *C. elegans*, synaptogyrin localization was not dependent upon synaptobrevin, nor on the presence of any other SV protein we examined (Table 1). Although our experiments would not detect interactions that are mediated by the synergistic action of several SV proteins, they argue against a piggyback targeting mechanism for synaptogyrin. In a second attempt to identify other proteins that aid in SNG-1 localization we carried out a yeast two-hybrid analysis with the C termini of both SNG-1 and rat synaptogyrin, without success. The focus of our current and future work is identifying mutants that fail to efficiently target SNG-1::GFP with the use of classical genetic mutant screens.

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