Sorting Nexin 9 Interacts with Dynamin 1 and N-WASP and Coordinates Synaptic Vesicle Endocytosis*

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Sorting nexin 9 (SNX9) is a member of the sorting nexin family of proteins, each of which contains a characteristic Phox homology domain. SNX9 is widely expressed and plays a role in clathrin-mediated endocytosis, but it is not known if it is present in neuronal cells. We report that SNX9 is expressed in the presynaptic compartment of cultured hippocampal neurons, where it binds to dynamin-1 and N-WASP. Overexpression of full-length SNX9 or a C-terminal truncated version caused severe defects in synaptic vesicle endocytosis during, as well as after, stimulation. Knockdown of SNX9 with short interfering RNA also reduced synaptic vesicle endocytosis, and the W39A mutation of SNX9 abolished the inhibitory effect of SNX9 on endocytosis. Rescue experiments showed that most of the effect of SNX9 on endocytosis results from its interaction with dynamin 1, although its interaction with N-WASP contributes in some degree. We further showed that SNX9 dimerizes through its C-terminal domain, suggesting that it may interact simultaneously with dynamin 1 and N-WASP. We propose that SNX9 interacts with dynamin-1 and N-WASP in presynaptic terminals, where it links actin dynamics and synaptic vesicle endocytosis.

Sorting nexin 9 (SNX9), also known as SH3PX1, is a member of the sorting nexin superfamily characterized by the presence of a phospholipid-binding motif, the PX domain. Sorting nexin family proteins contribute to protein sorting in cells by their ability to bind specific lipids and to form protein-protein complexes. SNX9, initially identified as a protein interacting with the metalloproteases MDC9 and MDC15 (1), is composed of an N-terminal Src homology 3 domain, a low complexity region, a PX domain, and a C-terminal Bin/Amphiphysin/Rvs (BAR) domain (2–4). It forms a complex with dynamin-2 and regulates the recruitment of dynamin-2 to the membrane (5). It also enhances the assembly of dynamin and increases its GTPase activity (6). Other endocytic molecules, namely AP-2 (adaptor protein complex 2) and clathrin, also bind to the low complexity region of SNX9 in a cooperative manner (2). Through these interactions, SNX9 plays an important role in clathrin-mediated endocytosis in non-neuronal cells (2, 6).

Dynamin is centrally involved in clathrin-mediated endocytosis (7, 8). It self-assembles around the necks of invaginated clathrin-coated pits and releases vesicles from the membrane via GTP hydrolysis (9). It is composed of several domains. The N-terminal nucleotide-binding domain is responsible for GTP hydrolysis, and the C-terminal proline-rich domain (PRD) links it to several SH3 domain-containing proteins such as Grb2, amphiphysin, and endophilin (10–12). The central pleckstrin homology domain controls its binding to membrane phospholipids (13), and a coiled-coil domain (also called the GTPase effector domain) is involved in its self-assembly and in regulating its GTPase activity.

The affinity between the pleckstrin homology domain of dynamin and lipids is not high enough to translocate dynamin from the cytosol to the plasma membrane (14). Rather, truncation of the PRD of dynamin blocks endocytosis because of mislocation of the protein (13) suggesting that it is the interaction of the PRD with SH3-containing domains of other endocytic molecules that is the key to the correct localization and functioning of dynamin.

Because of the mechanistic similarity between clathrin-mediated endocytosis in non-neuronal cells and synaptic vesicle endocytosis, SNX9 is suspected of having a role in synaptic vesicle endocytosis. However, the presence of SNX9 in neuronal cells and the functional interplay between it and its presynaptic binding partners in synaptic vesicle endocytosis have never been studied. Here we report that SNX9 is expressed in the presynaptic compartment of cultured hippocampal neurons where it associates with dynamin-1 as well as N-WASP, and that it plays a regulatory role in synaptic vesicle endocytosis. Because SNX9 can be dimerized, and binds dynamin at one end and N-WASP at the other, our data raise the possibility that it links actin dynamics and synaptic vesicle endocytosis.
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EXPERIMENTAL PROCEDURES

GST Pulldown Assays—The GST-SNX9, GST-SNX9-SH3, GST-SNX9ΔSH3, and GST-virgin vector plasmids were transformed into Escherichia coli BL-21, and the transformants were cultured in LB medium supplemented with ampicillin. After overnight induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 25 °C, the cultures were sonicated in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 1 mM EGTA, 0.1 mM PMSF) and centrifuged for 15 min at 12,000 rpm, and the supernatants were incubated with glutathione-agarose-4B beads (Amersham Biosciences) at 4 °C for 30 min. After washing three times with lysis buffer, the beads were incubated for 2 h at 4 °C with a brain lysate in lysis buffer. The beads were then washed extensively with lysis buffer and analyzed by SDS-PAGE and immunoblotting.

Co-immunoprecipitation—To detect SNX9 binding to dynamin-1 in vivo, COS-7 cells were transfected with FLAG-SNX9 and FLAG-SNX9ΔSH3 together with GFP-dynamin-1 using Liopofectamine-2000 (Invitrogen). The cells were washed twice with cold PBS and extracted for 1 h at 4 °C in a modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM apro tinin). They were then clarified by centrifugation at 12,000 rpm for 10 min, and protein concentrations were determined with a Bradford protein assay reagent kit (Bio-Rad). Samples containing 1 mg of total protein were immunoprecipitated for 4 h with anti-FLAG antibody, followed by an additional 3 h at 4 °C with protein G-Sepharose beads (Amersham Biosciences). The immunoprecipitates were extensively washed with lysis buffer and subjected to SDS-PAGE and immunoblot analysis with anti-FLAG and anti-GFP antibodies.

In-gel Digestion and Peptide Sample Preparation—The SDS-polyacrylamide gels were silver-stained, and protein bands were excised. The resulting samples were washed three times with a 1:1 (v/v) solution of acetonitrile/deionized water for 10 min, dehydrated with 100% acetonitrile, washed with a 1:1 (v/v) solution of 100% acetonitrile, 100 mM ammonium bicarbonate, and dried using a SpeedVac. Then they were reduced with 10 mM dithiothreitol at 37 °C for 14–18 h. The resulting peptides were extracted sequentially by agitation for 20 min with 45% acetonitrile in 20 mM ammonium bicarbonate, 45% acetonitrile in 0.5% trifluoroacetic acid, and 75% acetonitrile in 0.25% trifluoroacetic acid. The extracts containing tryptic peptides were pooled and evaporated under vacuum.

Micro- LC-MS/MS Analysis and Protein Data Base Search—In gel digested proteins were loaded onto fused silica capillary columns (100-μm inner diameter, 360-μm outer diameter) containing 8 cm of 5-μm particle size Aqua C18 reverse-phase column material. The columns were placed in line with an Agilent HP 1100 quaternary LC pump, and a splitter system was used to achieve a flow rate of 250 nL/min. Buffer A (5% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid) were used to make a 90-min gradient. The gradient profile started with 5 min of 100% buffer A, followed by a 60-min gradient from 0 to 55% buffer B, a 25-min gradient from 55 to 100% buffer B, and a 5-min gradient of 100% buffer B. Eluted peptides were directly electrosprayed into an LTQ linear ion trap mass spectrometer (ThermoFinnigan, Palo Alto, CA) by applying 2.3 kV of DC voltage. Data-dependent scans consisting of one full MS scan (400–1,400 m/z) and five data-dependent MS/MS scans were used to generate MS/MS spectra of the eluted peptides. A normalized collision energy of 35% was used throughout data acquisition. MS/MS spectra were searched against an NCBI rat protein sequence database using Bioworks version 3.1 and Sequest Cluster System (14 nodes). DTASelect was used to filter the search results, and the following Xcorr values were applied to the different charge states of peptides: 1.8 for singly charged peptides, 2.2 for doubly charged peptides, and 3.2 for triply charged peptides. Fragment ions in each MS/MS spectrum were manually assigned to confirm the database search results.

Cell Culture—E-18 primary rat hippocampal neurons were prepared as described (15). Briefly, hippocampi were dissected from embryonic day 18 Sprague-Dawley fetal rats, dissociated with papain, and triturated with a polished half-bore Pasteur pipette. Cells (250,000) in minimum Eagle’s medium supplemented with 0.6% glucose, 1 mM pyruvate, 2 mM l-glutamine, 10% fetal bovine serum, and antibiotics were plated on polystyrene-coated glass coverslips in a 60-mm Petri dish. Four hours after plating, the medium was replaced with basal media Eagle’s (Invitrogen) supplemented with 2% B-27, 10 mM HEPES, and 0.5 mM pyruvate or Neurobasal (Invitrogen) supplemented with 2% B-27, 0.5 mM l-glutamine. 4 μM of 1-β-d-xylosine-arabinofuranoside (Ara-C, Sigma) was added as needed.

Transfection—Neurons were transfected using calcium-phosphate (15). Briefly, synaptophysin or spH (spH) alone or with either FLAG-tagged full-length SNX9 or its truncated variants were transfected at 10 DIV for the endocytosis assays. The spH and SNX9 constructs were cotransfected in a ratio of 1:1-2:1. After the endocytosis assays, the cells were fixed and doubly stained with anti-GFP and anti-FLAG antibodies to confirm cotransfection, and only immunopositive neurons were included in the analysis. The amount of spH construct transfected was fixed for constant fluorescent signals.

Immunoblot Analysis—Hippocampal neurons (~3,000,000) were plated on 100-mm tissue culture dishes coated with poly-D-lysine and grown for 3, 7, 14, or 21 days. They were lysed in a lysis buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 10 mM leupeptin, 1.5 mM pepstatin, and 1 mM apro tinin) and scratched out, boiled for 5 min, and clarified by centrifugation at 12,000 rpm for 10 min. Protein concentrations were measured with a bicinchoninic acid protein assay reagent kit (Pierce). Constant amounts of proteins were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h with 5% nonfat dry milk in TBS/T (10 mM
Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20), after which they were incubated with the respective primary antibodies, anti-SNX9, or anti-dynamin-1 (ABR, Golden, CO), and then with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch). The antigen-antibody complexes were detected with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences). Blots were stripped by heating to 60 °C for 30 min in a stripping buffer (100 mM mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) and re-probed with anti-tubulin (Sigma) as a control.

**Immunocytochemistry**—Immunocytochemistry was performed as described previously (16). Cells were fixed in 4% formaldehyde, 4% sucrose, PBS for 15 min, permeabilized for 5 min in 0.25% Triton X-100, PBS and blocked for 30 min in 10% BSA, PBS at 37 °C. The cells were incubated with primary antibodies, 3% BSA, PBS for 2 h at 37 °C or overnight at 4 °C, washed in PBS, and incubated with secondary antibodies, 3% BSA, PBS for 45 min at 37 °C. Primary antibodies used were as follows: anti-SNX9 (provided by Dr. Sven R. Carlsson, Umeå University, Sweden, or Santa Cruz Biotechnology, Santa Cruz, CA), anti-synaptophysin, anti-synaptobrevin2, anti-synaptotagmin, anti-synaptotagminan (Synaptic Systems, Göttingen, Germany), anti-GFP (Abcam, Cambridge, UK), anti-FLAG (Sigma), anti-dynamin-1 (ABR), anti-Hudy2 (Upstate Biotechnology, Lake Placid, NY), anti-GFAP (Chemicon, Temecula, CA), and anti-type β-III tubulin (Chemicon). Secondary antibodies were obtained from Jackson ImmunoResearch.

**SynaptopHluorin Endo-exocytosis Assay**—Coverslips were mounted in a perfusion/stimulation chamber equipped with platinum-iridium field stimulus electrodes (EC-S-10, LCI, Seoul, Korea) on the stage of an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan) with 40 × 1.0 or 60 × 1.4 NA oil lenses. Cells were continuously perfused at room temperature with Tyrode solution (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM HEPES, 30 mM glucose, pH 7.4). 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione and 50 μM of dl-2-aminoo-5-phosphonovaleric acid were added to the Tyrode solution to reduce spontaneous activity and prevent recurrent excitation during stimulation. Time-lapse images were acquired every 10 s for 4 min using a CoolSNAP-ES CCD camera (Roper Scientific, Tucson, AZ) driven by MetaMorph Imaging software (Universal Imaging Corp., West Chester, PA) with a GFP optimized filter set (Omega Optical, Brattleboro, VT). From the 4th frame, cells were stimulated (1 ms, 20–50 V, bipolar) for 30 s at 20 Hz or 30 s at 10 Hz using a Grass SD9 stimulator (Grass-Telefactor, West Warwick, RI). Quantitative measurements of the fluorescence intensity at individual boutons were obtained by averaging a selected area of pixel intensities using MetaMorph software. Individual regions were selected by hand, and rectangular regions of interest were drawn around the synaptic boutons, and average intensities were calculated. Large puncta, typically representative of clusters of smaller synapses, were rejected during the selection procedure. The center of intensity of each synapse was calculated to correct for any image shift over the course of the experiment. Fluorescence was expressed in intensity units that correspond to fluorescence values averaged over all pixels within the region of interest. Light from a mercury lamp was shuttered using a VMM1.
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A. α-SNX9 and IgG

B. Mouse Brain Lysate

C. Mouse Brain Lysate

D. GST, GST-SN9, GST-SN9ΔSH3, GST-SN9ΔSH3

E. IB: α-Dyn 1

F. IB: α-SJ

G. Dynamin

H. IB: GFP

I. Anti-SNX9

J. Anti-Dynamin1

K. Merge
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Uniblitz shutter (Vincent Associates, Rochester, NY). Net fluorescence changes were obtained by subtracting the average intensity of the first four frames ($F_0$) from the intensity of each frame ($F_i$) for individual boutons. They were then normalized to the maximum fluorescence intensity ($F_{max}$ - $F_0$) and averaged. The decay of fluorescence was fitted with a single exponential. All fitting was done using individual error bars to weight the fit, using SigmaPlot 6.0. In some experiments where fluorescence decay deviated from single exponential behavior, we obtained the best fitting single exponential function from the early portion of the decay. Data are presented as means ± S.E. For the rescue experiments, FLAG-SNX9 was coexpressed with HA-dynamin-1 or Myc-N-WASP; FLAG-SNX9-D BAR was coexpressed with HA-dynamin-1 or Myc-N-WASP, and FLAG-SNX9-SH3 was coexpressed with HA-dynamin-1-PRD. For the exocytosis assays, control neurons expressing shH4, or neurons expressing FLAG-SNX9, FLAG-SNX9-D BAR, or FLAG-SNX9-SH3 with shH4, were preincubated with bafilomycin A$_1$ for 60 s and stimulated for 30 s at 10 Hz. Net fluorescence changes were obtained by subtracting the average intensity of the first four frames ($F_0$) from the intensity of each frame ($F_i$) for individual boutons, then normalizing to the maximum fluorescence intensity ($F_{max}$ - $F_0$), and averaging bafilomycin A$_1$ (Calbiochem) was dissolved in Me$_2$SO to 0.2 mM and diluted to a final concentration of 0.5 µM prior to the experiments. Bafilomycin was applied throughout the fluorescence measurements. Expression of each construct was confirmed by retrospective immunostaining with specific antibodies (rabbit anti-Myc, mouse anti-FLAG, and rat-anti-HA antibodies), and only immunopositive neurons were included in the analysis. Statistical analysis was carried out with SigmaStat (Systat Software, Point Richmond, CA). For multiple conditions, we compared means by analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test or Fisher’s LSD test (depending on the number of groups).

**FM 4-64 Endocytosis Assay—**FM 4-64 was used at a concentration of 15 µM in the above Tyrode solution. Pools of synaptic vesicles were labeled during electrical stimulation for 30 s at 10 Hz in the presence of FM 4-64. After 10 min of washing in dye-free Tyrode, images were taken, and neurons were stimulated for 2 min at 10 Hz to unload the FM 4-64. A fully unloaded image was then taken. Net fluorescence changes were obtained by subtracting the intensity of the unloaded image from the intensity of the loaded image. A 15-min rest period was inserted between the end of the first unloading stimulus train and the start of the second loading stimulus. Images were acquired using a CoolSNAP-ES CCD camera driven by MetaMorph Imaging software with a FM 4-64 optimized filter set (Omega Optical). Statistical analysis was carried out using SigmaStat (Systat Software). Data are presented as means ± S.E.

**RNA Interference—**SNX9-specific siRNAs were designed from the rat SNX9 cDNA sequence acquired by Blast search, targeting to the region of nucleotides 1183–1203 (siRNA 1, gi[2985617] and 1365–1385 (siRNA 2, gi[294287]). A pair of complementary oligonucleotides was synthesized separately with the addition of an Apal site at the 5’ end and an EcoRI site at the 3’ end. For the primary forward primer sequences were 5’-ATAGAACAGATTGTAGACGTTCACACTCTGTTCTAATTTTTTTTTT-3’ and 5’-GGAGAGACGGACCTTAAACATTCAAGAGATTGTAAGGTCCGTCTCCCTCTCTCTCTCTCT-3’ (the underlined letters are the SNX9-siRNA sequences). The annealed cDNA fragment was cloned into the Apal–EcoRI sites of pSilencer 1.0-U6 vector (Ambion, Austin, TX) modified by inserting an mRFP tag at the C terminus. The knockdown efficiency of the siRNA was tested in Rat-1 cells of fibroblast origin. Hippocampal neurons were transfected with SNX9-specific siRNAs using calcium phosphate.

**RESULTS**

**SNX9 Is Expressed in Cultured Hippocampal Neurons and Its Expression Increases with Developmental Stage—**A previous Northern blot analysis showed that SNX9 was expressed in many tissues (1). To see whether it is expressed in cultured hippocampal neurons, we performed a Western blot analysis using an SNX9 antibody. We used three different culture conditions to test the expression of SNX9 as follows: a pure glial cell culture obtained by 2 h of treatment with 200 µM of glutamate to remove neurons; a serum-free Neurobasal culture completely depleted of glial cells with Ara-C, and a Neurobasal culture without Ara-C treatment, containing mostly neurons and only a few glial cells. The glial cells expressed SNX9 as expected. The hippocampal neurons also expressed considerable amounts of SNX9, and expression increased as the neurons matured (Fig. 1, B–D).

FIGURE 3. SNX9 binds dynamin-1 and N-WASP via its SH3 domain in vitro and in vivo. A, brain lysates were immunoprecipitated with anti-SNX9 antibody, and SDS-polyacrylamide gels were silver-stained. Protein bands were excised from the stained gels, analyzed by micro- LC-MS/MS, and identified by a protein data base search. Band 1 includes dynamin-1, clathrin heavy chain, translational endoplasmic reticulum ATPase, synaptojanin, and N-a/K-ATPase a3 subunit. Band 2 includes sorting nexin 9, amphiphysin, N-WASP, and dihydroxyproline-related protein-2. Band 3 includes ß-actin, aldolase-1, and tubulin a-1 chain. Proteins that were unknown or not yet unambiguously identified are not included. B, brain lysates were incubated with GST, GST-SNX9-SH3, or GST-endophilin-SH3, and SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue (B) or with silver staining (C, arrow indicates synaptojanin). The proteins were transferred to a polyvinylidene difluoride membrane and immunoblotted with anti-dynamin-1 antibody (B) or anti-synaptojanin antibody (C, a-sJ). The last lane in B is a 1% input of the total cell lysates. D and E, GST fusions of SNX9, SNX9-SH3, SNX9-SH3, or GST alone were incubated with rat brain lysates. 10% of each lysate was used for the pulldown, and the complexes were resolved by SDS-PAGE, and immunoblotted with anti-dynamin-1 and anti-N-WASP antibodies. F, COS-7 cells were cotransfected with GFP-dynamin-1 or GFP-N-WASP, and FLAG-SNX9 or FLAG-SNX9-SH3. 24 h after transfection, the cells were lysed and immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (B) with anti-GFP antibody or anti-FLAG antibody, respectively. G, hippocampal neurons at 14 DIV were stimulated with high KCl, lysed immediately, analyzed by SDS-PAGE, and immunoblotted with anti-dynamin-1 or anti-SNX9 antibody. TCT, total cell lysate; NRS, normal rabbit serum; IP, immunoprecipitate. H, HEK 293T cells were cotransfected with FLAG-tagged SNX9-BAR and the indicated GFP-SNX9 truncated mutants, and the resulting immunoprecipitates were immunoblotted with either anti-GFP or anti-FLAG antibody. The right panel shows total cell lysates immunoblotted with anti-GFP antibody to verify expression of each truncated mutant. J, hippocampal neurons at 14 DIV were fixed, permeabilized, and immunostained with anti-SNX9 antibody and anti-Hu2y antibody. The secondary antibodies used were Oregon Green-conjugated anti-rabbit IgG for SNX9, and Texas Red-conjugated anti-mouse IgG for dynamin-1. Middle panels are merged images of immunostaining of SNX9 and dynamin-1, showing high magnification views of the regions enclosed in rectangles. Scale bars: low magnification, 20 µm; high magnification, 5 µm.
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SNX9 Is Present at the Presynaptic Terminals of Hippocampal Neurons Where It Binds to Dynamin-1—To study the distribution and subcellular localization of SNX9 in the neurons, we performed immunocytochemistry. SNX9 was found to be present diffusely all along the neurites, and formed many clusters in the axons, and the majority of it colocalized with the presynaptic vesicle markers synaptobrevin2, synaptoxin, and synaptotagmin (Fig. 2, A–I).

Because SNX9 contains an SH3 domain and is present at presynaptic nerve terminals, it could bind PRD-containing proteins. Dynamin-1 is the major SH3-containing protein in the presynaptic compartment. To test whether SNX9 and dynamin-1 bind to each other via an SH3-PRD interaction, we performed a micro-LC-MS/MS analysis to identify SNX9-interacting proteins (Fig. 3A). Brain lysates were immunoprecipitated with an SNX9 antibody, and SDS-polyacrylamide gels were silver-stained. After in-gel digestion, micro-LC-MS/MS, and a protein data base search, dynamin-1 was identified as one of the endogenous binding partners (Fig. 3A). Binding of SNX9SH3 to dynamin-1 was as strong as that of endophilin-SH3 (Fig. 3B).

We carried out a series of GST pulldown assays. Dynamin-1 was found to interact with full-length SNX9 and with its SH3 domain, but not with SNX9 lacking the SH3 domain (SNX9ΔSH3) (Fig. 3, B and E). Co-immunoprecipitation analysis of lysates of cells overexpressing FLAG-SNX9 and GFP-dynamin-1 confirmed that SNX9 associated with dynamin-1 in vivo in an SH3 domain-dependent interaction (Fig. 3F). We did not observe any significant change in this interaction in response to high KCl (Fig. 3G). Interaction of SNX9 with dynamin-1 was further confirmed by immunocytochemistry. Neurons were doubly stained with SNX9 and dynamin-1 antibodies. Although SNX9 exhibited rather diffuse cytosolic staining, it colocalized with dynamin-1 throughout the neurites (Fig. 3I).

SNX9 Interacts with N-WASP and Synaptojanin—N-WASP is the most potent and best characterized activator of actin nucleation by the Arp2/3 complex (17), and it is also enriched at nerve terminals, where it links the dynamin-mediated processes of endocytosis to rearrangements of the actin cytoskeleton within nerve terminals. Drosophila SNX9 (dSH3PX1) is known to interact with the Drosophila orthologue of WASP (18). We also showed that SNX9 interacts with N-WASP using micro-LC-MS/MS analysis (Fig. 3A). Although we could not detect N-WASP in GST pulldown followed by Coomassie staining, immunoblotting with anti-N-WASP antibody revealed that N-WASP interacted with full-length SNX9 and with its SH3 domain, but not with SNX9 lacking the SH3 domain (SNX9ΔSH3) (Fig. 3E). Co-immunoprecipitation of lysates of cells overexpressing FLAG-SNX9 and GFP-N-WASP cells confirmed that SNX9 indeed interacts with N-WASP in vivo in an SH3 domain-dependent interaction (Fig. 3F).

Previous study showed that SNX9 interacts with synaptojanin (4). Synaptojanin is a polyphosphoinositide phosphatase implicated in synaptic vesicle recycling and vesicle trafficking (19). Synaptojanin, however, was not detected in Coomassie staining (Fig. 3B) and only faintly visible in silver staining (Fig. 3C, arrow), suggesting very weak binding. The interaction of synaptojanin with SH3 domain of SNX9 further confirmed by immunoblotting with anti-synaptojanin antibody (Fig. 3C).

SNX9 contains a single SH3 domain at its N terminus, by which it interacts with dynamin-1 and N-WASP. This raised the possibility that it dimerizes. Fig. 3H shows that SNX9 indeed dimerizes and that it does so via its BAR domain. Thus, our results suggest that SNX9 can dimerize, thus binding dynamin-1 and N-WASP simultaneously and coordinating clathrin-mediated endocytosis with the actin cytoskeleton.

Overexpression of SNX9 Impairs Clathrin-mediated Synaptic Vesicle Endocytosis—We investigated whether the interaction between SNX9 and dynamin-1 could be involved in synaptic vesicle endocytosis. To measure synaptic vesicle endocytosis in the cultured hippocampal neurons we used synaptopHluorin (spH) (Fig. 4A). spH is a VAMP-2/synaptobrevin-2 fused with a pH-sensitive variant of GFP. The fluorescence of spH is quenched once synaptic vesicles are endocytosed and re-acidiﬁed (from external pH ~7.4 to an internal pH of a synaptic vesicle ~5.5). It has been proved that re-acidiﬁcation is not a rate-limiting step; thus the fluorescence change to synaptoPHluorin reliably reﬂects the kinetics of endocytosis (20).

To investigate the effect of SNX9 on endocytosis, FLAG-tagged SNX9 or truncated SNX9 variants were cotransfected with spH into hippocampal neurons. We conﬁrmed by retrospective immunostaining with anti-FLAG antibody at the end of each experiment that the neurons expressing spH also expressed FLAG-tagged SNX9 or the truncated variants of SNX9.

When neurons were stimulated electrically (600 action potentials/20 Hz), the fluorescence intensity of individual spH boutons increased rapidly, reached a peak, and then decayed with an exponential time course (Fig. 4A). In boutons expressing SNX9, synaptic vesicle endocytosis occurred much more slowly than in nonexpressing boutons (τ = 182.4 ± 28.9 for SNX9 expression; τ = 39.2 ± 3.4 for control; Fig. 4, A, C, and D). In previous studies, inhibition of endocytosis was observed with the SH3-containing truncated variant (SNX9ΔBAR) (2, 6). To test whether this variant also affected synaptic vesicle endocytosis, we overexpressed SNX9ΔBAR together with spH. We found that SNX9ΔBAR severely impaired synaptic vesicle endocytosis although not to the same extent as full-length SNX9 (Fig. 4, A–E). Fig. 4E shows histograms of the rates of spH fluorescence decay (measured as the reciprocal of the time constant, 1/τ) from individual puncta in a number of separate experiments, with and without coexpression of truncated variants of SNX9. Expression of SNX9 or SNX9ΔBAR increased the number of boutons in which spH fluorescence decayed slowly and shifted the distribution of endocytosis rates to lower values (Fig. 4E).

We infer from the above results that SNX9 and dynamin-1 bind to each other via an SH3-PRD interaction so that cells expressing SNX9–SH3 have a reduced rate of endocytosis (τ = 120.3 ± 25.4), whereas SNX9ΔSH3 has no effect (Fig. 4, C and D). Histograms of the rates of spH fluorescence decay also demonstrate that the SH3 domain is responsible for the slowing effect of SNX9 on endocytosis (Fig. 4E).

An alternative to the use of spH for measuring synaptic vesicle endocytosis is to employ FM 4-64, a red-shifting fluorescent lipophilic dye. We used the following protocol (Fig. 5A).
After the first loading and unloading of FM 4-64, the second loading was applied 20 s after the onset of electrical stimulation. In that 20 s some vesicles undergo endocytosis and escape being labeled, i.e. the slower the endocytosis, the higher the intensity of FM 4-64 staining (21). In these experiments, the intensity of FM 4-64 staining of SNX9- or SNX9-SH3-transfected synaptic boutons was invariably higher than that of the control boutons (0.61 ± 0.05 for control; 0.77 ± 0.04 for SNX9; 0.73 ± 0.05 for SNX9-SH3; n = 29, p < 0.01; see Fig. 5, B and C). This confirms that endocytosis proceeds more slowly when SNX9 or SNX9-SH3 is overexpressed.

The Endocytic Defect Is Attributable to the Interaction of SNX9 with Dynamin-1 and N-WASP—Because both N-WASP and dynamin-1 bind SNX9 via an SH3-PRD interaction, the endocytosis defects could be due to interaction with dynamin-1 or with N-WASP. To distinguish between these possibilities, we first tested the SNX9-W39A mutant, which cannot bind dynamin-1 (Fig. 6A). Overexpression of SNX9-W39A failed to affect the kinetics of endocytosis (Fig. 6B and C). We next performed a rescue experiment by overexpressing dynamin-1 together with SNX9. Coexpression of dynamin-1 with SNX9 and SNX9-ΔBAR rescued the endocytic defect because of overexpression of SNX9 and SNX9-ΔBAR, respectively, suggesting that the impairment of endocytosis caused by SNX9 and SNX9-ΔBAR is mostly because of their interaction with dynamin-1 (τ = 39.2 ± 3.4 s for control; τ = 54.18 ± 5.68 s for SNX9/dynamin 1 coexpression; see Fig. 6D and E). The endocytic defect caused by SNX9-SH3 was also rescued by coexpression...
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**FIGURE 5. Independent verification of the endocytosis defects induced by SNX9 using FM 4-64.** Comparison of the endocytosis kinetics at SNX9-expressing synaptic boutons and control boutons. Neurons were transfected with GFP-tagged SNX9 or GFP-tagged SH3, and two consecutive load-unload cycles were performed with FM 4-64. A, experimental protocol used to compare endocytosis. For condition 1, the second load of FM 4-64 was identical to the first and was not delayed. For condition 2, the second load was added 20 s after the onset of stimulation. B and C, FM 4-64 intensities are expressed as ratios of the intensities of the first loads and those of the second loads. The dashed line gives the change of intensity ratio for control boutons, and the solid line, the change of intensity ratio for SNX9- or SNX9-SH3-expressing boutons. Condition 1 is as follows: 1.01 ± 0.04 for control, n = 22; 1.00 ± 0.04 for SNX9, n = 25; 0.99 ± 0.03 for control, n = 20; 1.00 ± 0.03 for SNX9-SH3, n = 20. Condition 2 is as follows: 0.61 ± 0.02 for control, n = 22; 0.77 ± 0.04 for SNX9, n = 25; *, p < 0.01; 0.60 ± 0.05 for control, n = 20; 0.73 ± 0.05 for SNX9-SH3, n = 20; *, p < 0.01.

of dynamin-PRD (Fig. 6, D and E). Coexpression of N-WASP with SNX9 or SNX9-ΔBAR, however, resulted in less than ~30% rescue of the endocytic defects (τ = 127.6 ± 16.8 s for SNX9/N-WASP coexpression), confirming that the SH3-PRD interaction between SNX9 or SNX9-ΔBAR and dynamin-1 is primarily responsible for the effects on endocytosis (Fig. 6, D and E) and that the interaction of SNX9 with N-WASP only makes a minor contribution.

We further investigated the specificity of the interaction between SNX9 and dynamin-1 using Nck-SH3SH3, a mutant of Nck that lacks the third SH3 domain. Nck interacts with dynamin via this third SH3 domain, and with synaptopodin via its second SH3 domain (22, 23). Overexpression of Nck-SH3SH3 did not affect the kinetics of synaptic vesicle endocytosis (Fig. 6, F and G).

**Synaptic Vesicle Exocytosis Is Not Affected by SNX9 Overexpression**—To investigate whether SNX9 also has effects on synaptic vesicle exocytosis, we measured the rate of exocytosis when SNX9 was expressed, using spH with bafilomycin A₁ (24). Bafilomycin A₁, a V-type ATPase inhibitor, blocks acidification of endocytosed vesicles and traps them in an alkaline state during recycling, thus enabling one to measure exocytotic events, independent of the speed of endocytosis. The kinetics of exocytosis was measured by the slope of the spH response in the presence of bafilomycin during a 300-AP stimulus. Fig. 7 shows that the rate of synaptic vesicle exocytosis was not affected by expression of SNX9 or SNX9-ΔBAR (the slopes of the linear fits to the data: 0.032 for control, 0.032 for SNX9, 0.033 for SNX9-ΔBAR; p > 0.7; see Fig. 7, A and B), suggesting that the role of C and D), indicating that SNX9 overexpression impairs synaptic vesicle endocytosis not only after stimulation but also during a train of action potentials.

**Knockdown of Endogenous SNX9 Using siRNA Impairs Synaptic Vesicle Endocytosis**—We investigated the effect of knocking down endogenous SNX9 on synaptic vesicle endocytosis. Two independent siRNA constructs were made, and suppression of SNX9 expression by each in Rat-1 fibroblasts was confirmed by immunoblotting (Fig. 8, A and B) as well as by immunofluorescence staining of SNX9 in the presynaptic boutons of siRNA-transfected neurons (Fig. 8C). The expressions of other endocytic proteins such as syndapin, dynamin, endophilin, and Rab5β were not affected (Fig. 8A).

When the mRFP-SNX9-siRNAs were cotransfected into neurons with spH, synaptic vesicle endocytosis was slowed (τ = 38.6 ± 2.3 s for control, n = 155; τ = 91.8 ± 8.9 s for SNX9-siRNA-1, n = 127; and τ = 120.0 ± 8.1 s for SNX9-siRNA-2, n = 149, p < 0.05; see Fig. 8, D and E).

**DISCUSSION**

Originally identified as a protein that binds to PRDs in metalloprotease disintegrins (ADAMs) (1), SNX9 interacts with Dock (the fly orthologue of mammalian Nck) and Dscam (Down syndrome cell adhesion molecule) to form a complex involved in axonal guidance in Drosophila (18). Recent studies show that the PRD of activated Cdc-42-associated kinase-2 (ACK2) binds to the SH3 domain of SNX9 and, upon epidermal growth factor stimulation, forms a complex with clathrin and SNX9, induces the phosphorylation of SNX9, and subsequently SNX9 in synaptic vesicle recycling is restricted to the endocytic realm.

**Synaptic Vesicle Endocytosis during Stimulation Is Also Affected by SNX9 Overexpression**—Synaptic vesicles undergo endocytosis not only after stimulation but also during stimulation. We tested whether SNX9 also affects the rate of endocytosis during a train of action potentials. We took advantage of the effect of bafilomycin and calculated the kinetics of endocytosis during stimulation by simply subtracting the value of spH fluorescence without bafilomycin from that with bafilomycin. The difference gives the time course of endocytosis during stimulation and the slope obtained by linear fit to the 300-AP train is compared with that for the time course of exocytosis. We found that the ratio Endo/Exo was significantly reduced when SNX9 or SNX9-ΔBAR were overexpressed (0.439 for control; 0.1762 for SNX9; 0.206 for SNX9-ΔBAR, p > 1, n = 29 boutons for control, n = 31 boutons for SNX9; see Fig. 7,
promotes the degradation of the epidermal growth factor receptor (25). SNX9 also contributes to clathrin-mediated endocytosis in non-neuronal cells by interacting with components of the clathrin-mediated endocytic machinery, which include adaptor protein complex-2 (AP-2), clathrin, synaptojanin, and dynamin-2 (2–4, 6). We showed here that SNX9 is also expressed in presynaptic compartments, where it interacts with dynamin-1 and regulates synaptic vesicle recycling in neurons.

Synaptic vesicle endocytosis involves the interaction of many proteins at several stages of endocytosis. Dynamin functions as a mechanochemical enzyme that pinches vesicles from the plasma membrane (26), and also participates in regulation of the actin cytoskeleton (27). Although it is not directly associ-
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FIGURE 7. The rate of endocytosis during stimulation, but not the rate of exocytosis, is affected by SNX9 expression. Control, SNX9, and SNX9-ΔBAR (A and B) cells were stimulated in the presence or absence of bafilomycin (Baf). In the absence of bafilomycin, the fluorescence signal reflects the net balance of exocytosis and endocytosis. In the presence of bafilomycin, exocytosis events are trapped in an alkaline state, and the fluorescence signal reflects exocytosis. Fluorescence values were normalized to the peak fluorescence in each experimental condition. ΔF, rate of exocytosis was obtained from the linear fit to the data during a 300-AP stimulus (slopes: 0.032 for control, 0.032 for SNX9, and 0.033 for SNX9-ΔBAR), and the ratios of the slope values (1.03) are indicated (n = 59 for control, n = 62 for SNX9, and 0.033 for SNX9-ΔBAR, p > 0.418, Student’s t test). C, the graphs give the rates of endocytosis (Endo) and exocytosis (Exo) during stimulation with 300 APs at 10 Hz. Rates of endocytosis during stimulation were derived by subtracting the fluorescence trace in the absence of bafilomycin from the trace in its presence (ΔF<sub>endo</sub> = ΔF<sub>exon</sub> - ΔF<sub>exo</sub>). The traces obtained in the presence and in the absence of bafilomycin were normalized to the maximal stable fluorescence signal in the bafilomycin trace. The ratios of the slopes were obtained by linear fits to the 300-AP train. D, ratios of endocytosis/exocytosis in control, SNX9, and SNX9-ΔBAR.

ated with actin, several dynamin-binding proteins interact with actin or with proteins that regulate actin assembly. These include profilin (28), Abp1 (29), cortactin (30), syndapin (31), intersectin (32), Grb2 (33, 34), and Nck (35). SNX9 has also been implicated in actin cytoskeletal events through its interaction with N-WASP, a major regulator of actin polymerization via Arp2/3 (Fig. 3, and D). Because SNX9 can dimerize through its BAR domain (25) and so interact with dynamin and N-WASP at the same time, it may play a role as a linker between actin dynamics and synaptic vesicle endocytosis. Indeed, coexpression of SNX9 with N-WASP resulted in a 30% rescue of the endocytic defects caused by SNX9, although this effect was small compared with that resulting from dynamin coexpression (Fig. 6). SNX9 may play a role in regulating actin assembly via its interaction with N-WASP.

Full-length SNX9, which has the same capacity to bind dynamin-2 as its SH3-containing truncated variants, did not inhibit endocytosis in non-neuronal cells (2). We found, however, that overexpression of full-length SNX9 inhibited synaptic vesicle endocytosis in neurons. This difference may result from a difference between the cell types. Although the basic mechanism of clathrin-mediated endocytosis appears to be similar in non-neuronal cells and neurons, there are some differences; synaptic vesicles are recycled locally, and exo-endocytic recycling is quite tightly controlled in space as well as in time. In addition, we showed here that full-length SNX9 impairs endocytosis not only after, but also during, stimulation. During intense stimulation, synaptic vesicles are known to release neurotransmitters by forming short lived fusion pores without full fusion, and this type of release may not involve the conventional machinery of slow clathrin-mediated recycling. Moreover, the fact that SNX9 also affects endocytosis during stimulation indicates that its interaction with dynamin-1 also has a role in the rapid non-clathrin-mediated recycling pathway. Another possibility is that, because high level expression of full-length SNX9 causes noticeable tubulation in non-neuronal cells, probably via its BAR domain (data not shown), our failure to detect endocytic defects upon overexpression of full-length SNX9 in non-neuronal cells may have been due to a low level of expression of SNX9.

Interestingly, either overexpression or knockdown of SNX9 impaired synaptic vesicle endocytosis and, in non-neuronal cells, both impaired clathrin-mediated endocytosis (2, 6). Too much SNX9 may act in a dominant negative manner to prevent dynamin or N-WASP from interacting with other endocytic proteins. Too little endogenous SNX9 could affect its physiological role in endocytosis. There are other examples of this kind. 1) Overexpression of N-WASP and depletion of endogenous N-WASP both impair endocytosis (36). 2) Overexpression as well as knockdown of SPIN90 impairs synaptic vesicle endocytosis (16). 3) Increases and decreases of phosphatidyl-inositol 4,5-bisphosphate in presynaptic terminals cause similar endocytic defects (19, 37, 38). Apparently abnormal levels of various regulatory proteins can impair the homeostasis of protein networks.

SNX9 bears some structural and functional resemblance to amphiphysin. Both contain an SH3 domain and a BAR domain and bind dynamin, AP-2, clathrin, and lipid, and both are involved in clathrin-mediated endocytosis (2, 5, 39–41). However, we have shown that SNX9 and amphiphysin are not func-
FIGURE 8. Knockdown of endogenous SNX9 slows endocytosis. A, rat-1 fibroblasts were transfected with mRFP-tagged SNX9-specific siRNAs, and endogenous SNX9, syndapin, dynamin, endophilin, and Rab5b were measured with antibodies that are specific for each protein. B, relative band intensities displayed as histograms. C, hippocampal neurons transfected with SNX9 siRNAs were fixed, permeabilized, and immunostained with anti-SNX9 and anti-synaptophysin antibody. The secondary antibodies used were Oregon Green 488 for SNX9 and Cy5 for synaptophysin. Lower panels are high magnification views of the regions enclosed in rectangles. Arrowheads indicate presynaptic boutons. Note that the presynaptic boutons in siRNA transfected neurons showed considerably low immunoreactivities of SNX9 compared with those in the control neurons. Scale bars: low magnification, 30 μm; high magnification, 2 μm. D and E, hippocampal neurons were cotransfected with spH and siRNAs at 11 DIV and endocytosis assays were performed at 15 DIV. The graphs show the normalized average fluorescence intensity profiles, plotted as ΔF/F₀ against time, after stimulation with 600 APs at 20 Hz. The decay kinetics were fitted by a single exponential, with τ = 38.6 ± 2.3 (n = 155) for control; τ = 91.8 ± 8.9 (n = 127) for SNX9-siRNA-1; and τ = 120.0 ± 8.1 (n = 149) for SNX9-siRNA-2. *, p < 0.05, significantly different from the control value, ANOVA, and Tukey’s HSD post hoc test.
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tionally redundant in synaptic vesicle recycling; no significant defects in synaptic vesicle endocytosis were observed in amphiphysin knock-out mice, whereas knocking down of endogenous SNX9 caused severe endocytic defects (40). Besides, as discussed above, SNX9 also has a role in the rapid recycling pathway. Therefore, SNX9 and amphiphysin probably have distinct roles in synaptic vesicle endocytosis. Further investigation is needed to reveal the interplay of dynamin-1 and its binding partners at each step of recycling.

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