Syntaphilin Binds to Dynamin-1 and Inhibits Dynamin-dependent Endocytosis*  

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**Neurotransmitter release involves a series of interactions between the membranes of synaptic vesicles and the presynaptic terminal, culminating in the calcium-dependent maturation of the SNARE complex, fusion of the two membranes and release of the vesicle contents into the synaptic cleft (1, 2). Following fusion, synaptic vesicle membranes and the components of the vesicle release apparatus are recycled at the nerve terminal by clathrin-dependent, dynamin-mediated endocytosis. This process is necessary for efficient neurotransmitter release.**

**In the present study, we have used proteomic, biochemical, and cell biological analyses to show that syntaphilin also binds to dynamin-1 and functions consequently as an independent inhibitor of dynamin-mediated endocytosis.**

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**Syntaphilin is a brain-specific syntaxin-binding partner first characterized as an inhibitor of SNARE complex formation and neurotransmitter release. Here we show that syntaphilin also binds to dynamin-1 and through this interaction inhibits dynamin-mediated endocytosis. Immunoprecipitation studies from cross-linked rat synaptosomes demonstrate that an endogenous syntaphilin-dynamin-1 complex exists independently of dynamin-1 binding to amphiphysin. Functionally, syntaphilin expression inhibits transferrin internalization in COS-7 cells. These data reveal that syntaphilin is an inhibitor of both SNARE-based fusion and dynamin-mediated endocytosis.**

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**Experimental Procedures**

**cDNAs and Antibodies—**Human amphiphasin 1 cDNA was kindly provided by Peter McPherson. Human full-length dynamin-1 in pcDNA3 vector was provided by Sandra Schmid. Dynamin-1 recombinant protein was expressed in transfected 293 T cells, and lysates were used for co-IP and *in vitro* binding studies. Rabbit anti-syntaphilin-65 antibody has been described previously (7). Rabbit anti-syntaphilin-17 antibodies were raised against a C-terminal epitope of the syntaphilin protein in conjunction with Research Genetics/Invitrogen. Monoclonal anti-syntaxin-1A and SNAP-25 antibodies were generous gifts from Masami Takahashi. The following antibodies were obtained from commercial sources: monoclonal anti-GST (Pierce), anti-T7-His (Novagen), anti-dynamin-1 (Hudy-1, Upstate Biotechnology), anti-syntaxinophsin (Chemicon), anti-lactate dehydrogenase (BD Transduction), anti-Na+/K+-ATPase (BD Transduction), anti-amphiphysin (BD Transduction), anti-tubulin (Sigma), nonimmune rabbit and mouse IgGs (Zymed Laboratories). Secondary fluorescent antibodies were obtained from Jackson Laboratories.

**Fusion Proteins and *in Vitro* Binding—**Full-length syntaphilin in the pcDNA vector was used for transfection into 293 T cells for co-IP studies or into COS cells for the transferrin uptake assays. Syntaphilin 1-469, the cytoplasmic domain, was constructed into amino-terminal tagged GST or His fusion vectors (pGEX-4T-1 and pcDNA 3.1 His C, respectively) for expression in bacterial and *in vitro* binding studies, because of the fact that full-length syntaphilin protein with its carboxy-terminal transmembrane domain is difficult to solubilize from bacterial lysates. Dynamin-1 fragments were subcloned into the same GST vector. Translated syntaphilin fragments were also constructed as above for subcloning into the His6 fusion vector pcDNA 3.1 His C (Invitrogen).

**Binding studies were performed as described previously (7). Briefly, GST fusion proteins (~1 μg) were bound to glutathione-Sepharose beads, washed, and then incubated with 750 μg of rat brain homogenates (Fig. 1, A and B) or 250 μg of cell lysates transfected with His-tagged protein vectors, as indicated (Fig. 1, C and D). Proteins were eluted by SDS-PAGE and processed by gel electrophoresis for Simply Blue staining (Invitrogen) or Western blot analysis.**

**Transfection and Immunoprecipitation—**HEK 293 T cells cultured in 100-mm dishes were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen) and 0.5% t-glutamine and were transfected with 5-10 μg of cDNA using LipofectAMINE 2000 (Invitrogen) according to the instruction manual. After 48 h, the cells were harvested and solubilized in Tris-buffered saline with 1% Triton X-100.
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X-100, 5 mM EDTA, and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Cell lysates were centrifuged at 13,000 rpm for 20 min at 4 °C, and the supernatant was used for in vitro binding and immunoprecipitation studies. Brain homogenates were prepared from adult rat brains following brief sonication in 1X PBS with protease inhibitors. For co-immunoprecipitation from heterologous cell lysates and rat brain homogenates, 1% Triton X-100/sodium deoxycholate extracts were prepared, and immunoprecipitation was performed as described previously (7) using antibodies immobilized on protein A-Sepharose beads and 500 μg of total protein in TBST buffer (1X Tris-buffered saline, 0.1% Triton X-100) with protease inhibitors. For chemical cross-linking, crude rat brain synaptosomes were prepared by differential centrifugation of rat brain homogenates as described (7). For the final wash, the synaptosomes were gently resuspended in ice-cold 100 mM sodium borate buffer, pH 8.0, and incubated in 200 μM cross-linking reagent diethyldithiobis(succinimidyl propanone) (DSP) (Pierce) at 4 °C for 2 h. The reaction was terminated with Tris-HCl (pH 7.6) at a final concentration of 100 mM, and the proteins were solubilized by the addition of SDS (final concentration 1%). Following centrifugation at 14,000 × g for 10 min at 4 °C, the supernatant was diluted 5-fold with 150 mM NaCl, 2 mM EDTA, 0.1% protein A-Sepharose beads and 500 μM of total protein in TBST buffer. For chemical cross-linking, crude rat brain synaptosomes were prepared by differential centrifugation of rat brain homogenates as described (7). Following a final wash, the synaptosomes were gently resuspended in ice-cold 100 mM sodium borate buffer, pH 8.0, and incubated in 200 μM cross-linking reagent diethyldithiobis(succinimidyl propanone) (DSP) (Pierce) at 4 °C for 2 h. The reaction was terminated with Tris-HCl (pH 7.6) at a final concentration of 100 mM, and the proteins were solubilized by the addition of SDS (final concentration 1%). Following centrifugation at 14,000 × g for 10 min at 4 °C, the supernatant was diluted 5-fold with 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, and 50 mM Na2SO4 (pH 7.2) prior to immunoprecipitation. Protein complexes were reduced by boiling in 100 μM dithiothreitol and 100 μM mercaptoethanol and processed for Western blot analysis as described above.

Mass Spectrometry Analysis—The identification of dynamin-1 (after pull-down experiments) was performed by the Mass Spectrometry Facility at NINDS, National Institutes of Health. The excised 100-kDa Coomassie Brilliant Blue-stained band was subjected to in-gel tryptic digestion. The resulting tryptic peptides were analyzed by liquid chromatography/mass spectrometry/mass spectrometry on Model Magic 2002 Model capillary high pressure liquid chromatography (Microm BioResources, Auburn, CA) coupled to a Model LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray interface. Data base search of the mass spectrometry/mass spectrometry spectra identified the protein as rat dynamin-1 (GenBank™ accession number P215755).

Transfection—Update Assay—COS-7 cells plated on 100-mm dishes were transfected using the LipofectAMINE (Invitrogen) method with 7.5 μg of DNA encoding syntaphilin or syntaphilin-ΔCC in pcDNA 3.1 His vectors or with vector alone and then incubated overnight in media containing serum. Twenty-four h after transfection, the cells were gently lifted using trypsin digestion and split into five wells of equal volume for the next day in acid-stripping experiments to generate a time course for transferrin internalization. Acid-strip experiments were performed as described (8). Briefly, the cells were incubated in ice-cold superfusion buffer (25 mM HEPES, 119 mM NaCl, 2.5 mM KCl, 2 mM MgCl2, 30 mM glucose, pH 7.4) containing 25 μg/ml biotinylated transferrin (Sigma) for 2, 4, 8, or 12 min. Then, intact cells were exposed to 0.5 M NaCl, 0.2 M acetic acid for 4 min on ice. This procedure selectively strips bound transferrin from the cell surface and leaves intracellular transferrin intact. After acid stripping, cells were washed three times with ice-cold PBS (pH 5.2), permeabilized with 1% Triton X-100 in PBS with protease inhibitors. Protein samples (100 μg of each) were separated by SDS-PAGE and blotted with streptavidin-conjugated horseradish peroxidase (Amersham Biosciences). Transferrin levels were determined by quantification using NIH ImageJ.

Immunocytochemistry—COS-7 cells were plated on coverslips at a density of 25,000 cells/ml. The next day, cells were transfected with syntaphilin, dynamin-1, syntaxin-1A-ATM, or syntaphilin-ΔCC in pcDNA 3.1 or pcDNA 3.1 His vectors and then incubated overnight in medium containing serum. Twenty-four h after transfection, the coverslips were removed and the cells were incubated at 37 °C for 12 min with Dulbecco’s modified Eagle’s medium containing 25 μg/ml biotinylated transferrin, then washed three times in PBS, and processed for immunocytochemistry. For protein staining, cells were fixed in physiologic solution containing 4% paraformaldehyde (119 mM NaCl, 2.5 mM KCl, 2 mM MgCl2, 30 mM glucose, pH 4.4) at room temperature, washed 4 times in PB (pH 8.0) for 20 min, washed in PBS three times for 5 min, permeabilized in 0.2% Triton X-100 for 15 min, and incubated in blocking buffer (1% bovine serum albumin in PBS) for 1 h. Cultures were incubated with primary antibodies in blocking buffer overnight at 4 °C. The cells were then washed four times in PBS for 5 min at room temperature, incubated with secondary antibody in PBS for 2 h, washed again four times in PB, for 5 min, and finally incubated in water. Coverslips were mounted on slides for examination using a Zeiss 510 confocal microscope. Images were obtained through a Zeiss 63× objective using fixed imaging parameters. Cells were imaged using a Z-series consisting of 6–8 images, each averaged twice and taken at 1-μm intervals. Displayed images and images used for quantification were taken from slices intermediate to the nucleus and the uppermost section of the cell. Morphometric measurements were performed using NIH ImageJ software. First, tracings were made around individual cells to quantify the total signal level generated by the transferrin image. Then, tracings were made just inside of the outer membrane internal to staining adherent to the plasma membrane. Transferrin-internalization levels were computed as the dividend of the signal level internal to the second tracing and the total cell transferrin signal level.

RESULTS

Direct Interaction of Syntaphilin and Dynamin-1—To identify proteins that interact with syntaphilin, we utilized a proteomic approach using a GST fusion protein of syntaphilin (residues 1–469) for pull-down studies with rat brain homogenate. Simply Blue staining (Invitrogen) of a pull-down using the syntaphilin fusion protein demonstrated two bands at 35 and 100 kDa not present in the GST control (Fig. 1A). Analysis of the digested 100-kDa band by mass spectrometry revealed a sequence consistent with rat dynamin-1 (GenBank™ accession number P215755). To corroborate the mass spectrometry results, we repeated the pull-down study for Western blot analysis (Fig. 1B) with a dynamin-1 antibody (Hudy-1) again identifying the 100-kDa band as dynamin-1. Sequential immunoblot of the same membrane demonstrated the 35-kDa band to be syntaxin-1A (data not shown), an established syntaphilin-binding partner (7).

To obtain more informative data for in vitro binding between syntaphilin and dynamin-1, we performed a Coomassie-based estimation of the stoichiometry of the syntaphilin-dynamin-1 interaction by incubating equal amounts (1 μg) of GST-syntaphilin with increasing amounts of recombinant dynamin-1 expressed in transfected 293 T cells. Our studies show that, under our experimental conditions, the relative ratio for the interaction of GST-syntaphilin with recombinant dynamin-1 is close to a 1.97 molar ratio as determined by semiquantification with purified recombinant proteins as standards (data not shown). To determine the minimal binding motif involved in their interactions, we constructed truncated fragments of syntaphilin (Fig. 1C) and dynamin-1 (Fig. 1D) for insertion into His-tagged and GST fusion vectors and then used these constructs to make recombinant proteins for in vitro binding studies. Binding of the dynamin-1 carboxyl-terminal half (GST-dynamin-CT) was seen to the full-length (FL), amino-terminal half (NT) and the coiled-coil (CC) domain of syntaphilin (Fig. 1C). The proline-rich (PR) fragment of syntaphilin alone showed no binding to the dynamin-1 fusion protein. In parallel experiments, we found equal binding to syntaphilin by the carboxyl-terminal (CT) half and pleckstrin homology domain (PHD) fragment of dynamin-1 (Fig. 1D).

Association of Syntaphilin and Dynamin-1 in Rat Brain—Given our in vitro results, we sought evidence of an association between syntaphilin and dynamin-1 in a cellular context. From co-transfected HEK 293 cells, dynamin-1 could be readily co-precipitated with His-tagged syntaphilin by a polyclonal syntaphilin antibody (Fig. 2A, philin-65) or a monoclonal antibody against the T7-(His, fusion protein leader peptide)-tag (data not shown). No immunoprecipitation occurred in cell lysates transfected by dynamin-1 alone. To test whether syntaphilin and dynamin-1 are associated in native tissues, Triton X-100/deoxycholate extracts from rat brain homogenates were used for immunoprecipitation studies with syntaphilin antibody. Immunoblot analysis demonstrated that dynamin-1 precipitates with syntaphilin, but that a substantial portion remains in the supernatant (Fig. 2B). Quantification of dynamin-1 immunoprecipitation with saturating levels of syntaphilin antibody suggests that 12.7 ± 1.6% (n = 12) of dynamin-1 is in a...
levels of anti-syntaphilin antibody allowing direct estimation of the amount of protein immunoprecipitated.

To ensure that this complex exists in the native state and is not simply an artifact of solubilization, we repeated our immunoprecipitation studies using SDS extracts of rat brain synaptosomes chemically cross-linked with the reversible homobifunctional agent DSP. Synaptosomes were cross-linked before solubilization and were used instead of brain homogenates because of the relative enrichment of syntaphilin in synaptosomal preparations. Using these extracts, we found that dynamin-1 co-immunoprecipitated with antibodies against two different syntaphilin epitopes (philin-65 and philin-17) but not with control nonimmune rabbit IgG (Fig. 2C).

**Syntaphilin Inhibits the Binding of Dynamin-1 to Amphiphysin**—Using immunoprecipitation studies from DSP cross-linked rat brain synaptosomes, we sought to characterize further the *in vivo* nature of the syntaphilin-dynamin-1 interaction. Both dynamin-1 and syntaxin-1A precipitated with syntaphilin (Fig. 3A). In keeping with previous findings, we found no evidence of SNAP-25 immunoprecipitation with anti-syntaphilin antibody consistent with the hypothesis that syntaphilin binding to syntaxin-1A inhibits the interaction of syntaphilin with SNAP-25. Interestingly, we also found that syntaphilin did not precipitate with amphiphysin, a dynamin-1-binding adaptor protein necessary for efficient synaptic vesicle endocytosis (9). Conversely, immunoprecipitation from the same brain extracts with anti-dynamin-1 antibody pulled down both syntaphilin and amphiphysin but not syntaxin-1A (Fig. 3A). These results suggest that syntaphilin binding to dy-
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To test if the binding of syntaphilin to dynamin-1 leads to a functionally relevant disruption of the dynamin-1-amphiphysin interaction, we examined the effect of syntaphilin overexpression on transferrin internalization in COS-7 cells (10). We chose this system because it allowed us to examine vesicle endocytosis in a manner relatively independent from vesicle fusion, which is a necessity given the known effect of syntaphilin on SNARE assembly. Transferrin internalization is a constitutive endocytotic event normally mediated by dynamin-2; numerous studies have shown, however, that this process can be “knocked out” in heterologous cells by transfection with mutant forms of dynamin-1 or by overexpression of dominant-negative dynamin-binding partners (11–13).

We first examined the expression of syntaphilin in transfected cells. Immunoblot of COS-7 cell lysates and immunocytochemistry of plated cells with anti-syntaphilin antibody showed no detectable endogenous syntaphilin expression. In transfected cells, syntaphilin staining was present in the cytoplasm and extended to the plasma membrane. Dynamin-1 expression in transfected COS-7 cells occurred in a similar pattern with heavy overlap of syntaphilin staining in cotransfected cells. Both proteins localized extensively with Na+/K-ATPase, a marker for the plasma membrane (data not shown).

Next, we studied the time course of transferrin internalization in COS-7 cells following transfection with syntaphilin compared with cells transfected with vector alone. Transferrin internalization was measured using Western blot analysis of cell lysates harvested following the incubation of transfected cells in a transferrin bath for 1, 2, 4, 8, or 12 min. Transferrin remaining on the plasma membrane was removed from intact cells by acid stripping prior to solubilization, and levels of internalized transferrin were then determined by immunoblot. In these studies, transfusion with syntaphilin was associated with both a significant slowing of the kinetics of transferrin internalization and a decrease in the total amount internalized (Fig. 4A). Conversely, no significant change in transferrin internalization dynamics was found in cells transfected with the syntaphilin-ΔCC mutant, which is unable to bind to dynamin.

To examine the effect of syntaphilin expression on a single cell rather than a population of cells, we then studied the effect of syntaphilin and dynamin-1 transfection on transferrin internalization in COS-7 cells using immunocytochemistry (Fig. 4B). Cells were imaged using confocal microscopy in 1-μm slices in the Z-plane; analysis was performed using a slice intermediate to the nucleus and the uppermost section of the cell, which allowed for reliable discrimination between the plasma membrane and cytosolic compartment. In untransfected cells, transferrin internalization occurred efficiently with most of the transferrin signal appearing in vesicles in the cell cytoplasm (Fig. 4B, column 1). Co-transfection of cells with syntaphilin and dynamin-1 resulted in a nearly complete arrest of this process; in these cells, few vesicular structures are seen, with most of the transferrin signal detectable at the cell surface (Fig. 4B, column 2). Examination of multiple cells revealed a second population of co-transfected cells expressing 2–3-fold higher levels of dynamin-1 (Fig. 4B, column 3). These cells are marked by the presence of numerous transferrin-staining vesicles in their cytoplasmic space, suggesting that the inhibitory effect of syntaphilin on transferrin internalization is dependent upon the stoichiometry of syntaphilin and dynamin-1 expression and that the high levels of dynamin-1 afford a cellular rescue of the receptor endocytosis process. In keeping with the hypothesis that syntaphilin-mediated inhibition of transferrin internalization requires its binding to dynamin-1, transferrin

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**Fig. 3.** Syntaphilin inhibits the binding of dynamin-1 to amphiphysin. A, co-immunoprecipitation of amphiphysin with anti-dynamin-1 but not anti-syntaphilin antibody in SDS extracts of rat brain synaptosomes following chemical cross-linking with DSP. Cross-linked synaptosomal extracts were immunoprecipitated with nonimmune rabbit or mouse IgGs or antibodies to syntaphilin (philin) or dynamin-1 (dyn-1), as indicated. Immunoblot for dynamin-1, amphiphysin (amph), syntaptotagmin 1 (Syt-1A), SNAP-25, and syntaphilin were performed sequentially. Input lanes contain 20% of the extract used for each IP. B, syntaphilin expression in HEK 293 cells inhibits the binding of dynamin-1 to amphiphysin. Lysates from HEK 293 cells transfected with dynamin-1 (P1, P2, S1) or co-transfected with dynamin-1 and syntaphilin (P2, S2) were used for binding to bacterially expressed GST-amphiphysin (φ1 μg) immobilized on glutathione-Sepharose beads. Binding reactions are shown in three lanes: I, input to the binding reaction; P, precipitated pellet (bound to GST-amphiphysin); S, supernatant remaining after the precipitation. Input lanes contain 20% of the lysates used for each binding reaction. Equal amounts of pellet and supernatant fractions (20%) were loaded. The remaining supernatant from the S2 fraction was used for co-immunoprecipitation with antibody against the T7-His-tag. The I, P, and S fractions were sequentially immunoblotted for dynamin-1 (T7-His-syntaphilin (His-philin), and GST-amphiphysin. C, quantification of dynamin-1 binding to GST-amphiphysin in lysates from cells transfected with dynamin-1 or co-transfected with dynamin-1 and syntaphilin or dynamin-1 and syntaphilin-ΔCC. Relative levels of dynamin-1 binding were determined as the product of dynamin-1 found in pellet fractions following binding to GST-amphiphysin and total dynamin-1 levels from pellet and supernatant fractions using lysates from four separate transfection experiments. Bar, mean ± S.D.; *, p < 0.01.

Dynamin-1 and syntaxin-1A are independent events and that syntaphilin binding to dynamin-1 occurs exclusively of the interaction of dynamin-1 with amphiphysin.

To test this hypothesis, we performed a series of binding studies using a GST fusion protein of amphiphysin and lysates from HEK 293 cells co-transfected with dynamin-1 and syntaphilin or transfected with dynamin-1 alone (Fig. 3B). Using the dynamin-1 transfection lysates, we found binding of dynamin-1 to amphiphysin (P1). In studies using lysates from the co-transfected cells, the binding of dynamin-1 to amphiphysin was highly attenuated (P2), with an increase in the amount of dynamin-1 remaining in the supernatant (S2). Immunoprecipitation of the supernatant collected from the binding study with anti-syntaphilin antibody suggests that much of the unbound dynamin-1 is in complex with syntaphilin (IP). To test whether this inhibition is dependent upon the binding of dynamin-1 by syntaphilin, we repeated amphiphysin binding studies using lysates from cells transfected as above or with dynamin-1 and a syntaphilin mutant lacking the dynamin-1 binding domain (ΔCC). Quantification of dynamin-1 binding to amphiphysin showed a 90% decrease in dynamin-1 binding in extracts from cells co-transfected with syntaphilin with no significant change in dynamin-1 binding in extracts from cells co-transfected with the ΔCC mutant (Fig. 3C). Combined with our findings from immunoprecipitation studies in cross-linked brain synaptosomes, these data support the hypothesis that syntaphilin binding to dynamin-1 inhibits the interaction of dynamin-1 with amphiphysin.
internalization in cells co-transfected with dynamin-1 and the syntaphilin-ΔCC mutant (Fig. 4B, column 4) was unchanged.

Using slice images gathered from numerous independent experiments, we quantified levels of transferrin internalization in various cell (co)transfection groups as measured by the percent of total transferrin signal internal and not adherent to the plasma membrane (Fig. 4C). Untransfected cells showed high levels of transferrin internalization (81 ± 23%). These levels remained high in cells transfected with a syntaxin mutant lacking the transmembrane domain (stx-ΔTM), confirming that the assay is a valuable method for studying dynamin-mediated endocytosis independent of vesicle fusion (stx-ΔTM is a potent inhibitor of SNARE-mediated exocytosis) (14). COS-7 cells co-transfected with dynamin-1 and syntaphilin were separated into two populations based on intensity of dynamin-1 immunofluorescence. In cells expressing syntaphilin and low levels of dynamin-1, the level of internalized transferrin signal was 28 ± 22%. Again, high levels of dynamin-1 expression were associated with an effective rescue (78 ± 12%) from syntaphilin-mediated inhibition of transferrin internalization. This rescue appears to be specific, as transfection with dynamin-1 alone had no effect on transferrin internalization levels (78 ± 19%). Finally, no effect on transferrin internalization was observed in cells co-transfected with dynamin-1 and the syntaphilin-ΔCC mutant (82 ± 12%) consistent with a mechanistic requirement for a direct interaction between the two proteins.

**DISCUSSION**

Using a multidisciplinary approach, we have characterized syntaphilin, first described as a syntaxin-binding partner that inhibits SNARE-mediated synaptic vesicle fusion, as an inhibitor of dynamin-mediated vesicle endocytosis. The coiled-coil domain of syntaphilin interacts directly with the coiled-coil domains of the syntaxin-1A carboxyl-terminal and the dynamin-1 pleckstrin homology domain forming independent complexes with the two proteins in vivo. Complex formation between syntaphilin and syntaxin-1A or dynamin-1 in vivo occurs exclusively of their interaction with SNAP-25 and amphiphysin, respectively, consistent with results from recombinant protein competition studies that demonstrate an inhibitory effect of syntaphilin on binding of syntaxin-1A to SNAP-25 (7) and dynamin-1 to amphiphysin. Finally, syntaphilin expression in COS-7 cells is associated with the arrest of dynamin-mediated endocytosis, an effect that is abolished by a compensatory overexpression of dynamin or by expression of a syntaphilin mutant lacking the dynamin-binding domain.

Numerous studies have provided evidence for a presynaptic mechanism of synaptic plasticity in mature neurons as embodied by variability in the efficacy of neurotransmitter release (4, 15). Here we characterize a neuronal protein possessing the biochemical and functional properties to be a molecular modulator of presynaptic strength. We have described previously syntaphilin as a syntaxin-1A-binding protein capable of inhibiting SNARE complex formation and synaptic vesicle exocytosis in cultured hippocampal neurons (7). In those studies, we found that syntaphilin expression in hippocampal neuron micro-island cultures diminished the frequency, but not the amplitude, of miniature postsynaptic excitatory currents, implicating a presynaptic action for syntaphilin as a negative modulator of vesicle release from the active zone. We have now demonstrated that syntaphilin also binds to dynamin-1 and inhibits the interaction of dynamin with amphiphysin. The cooperative role of dynamin and amphiphysin in synaptic ves-
icle endocytosis has been well characterized (9, 16–18), although it is unclear whether complete endocytosis of vesicles by a dynamin-mediated mechanism represents faster or slower recovery from a state of higher or lower release (19). Regardless, our findings suggest that, in addition to an inhibitory effect on SNARE complex formation, syntaphilin exerts a negative influence on dynamin-mediated endocytosis, as measured by transferrin internalization in transfected COS-7 cells.

Among the proteins identified so far as potential dynamin partners, the majority are predicted to bind directly or indirectly through SH3 domains, a common characteristic of endocytic protein interactions, such as amphiphysin, synaptojanin, endophilin, intersectin, and sydnapin 1 (20). Dynamin may bind directly to the membrane via its PH domain to anionic phospholipids, which in turn would stimulate its GTPase activity. The PH domain of dynamin has been shown to bind to the G protein βγ, and this binding could prevent dynamin self-assembly and domain interactions within the dynamin tetramer, consequently inhibiting the intrinsic GTPase activity of dynamin (21). As demonstrated by our in vitro binding studies, the inhibition of the amphiphysin-dynamin interaction is mediated through syntaphilin binding to the dynamin PHD. These results are in line with a previous report that deletion of the PHD of dynamin-1 diminishes binding of dynamin-1 to amphiphysin (13), perhaps suggesting that the PHD is necessary to maintain or stabilize the interaction of the dynamin-1 PR with the amphiphysin SH3 domain. Our findings are consistent with the hypothesis that syntaphilin binds to the PHD of dynamin and thus interferes with its interaction with amphiphysin, suggesting a potential role for the PH domain in regulating interactions of dynamin with its partners, except its well-known role in membrane association.

Overexpressed dynamin-1 in co-transfected COS cells has a different distribution than its normal distribution in neurons. The PHD has been characterized as being required for membrane association of dynamin-1. It could be that binding of syntaphilin to this region contributes to this change in its localization. Alternatively, amphiphysin binding to dynamin, which is thought to be necessary for proper targeting of dynamin-1, is also blocked by syntaphilin. Both mechanisms could contribute to a change in the subcellular targeting and localization of dynamin-1 in transfected COS cells. Whereas the results of our previous and present studies have led us to propose that syntaphilin possesses both the biochemical and functional properties to act as an inhibitor of synaptic activity, it is possible that syntaphilin possesses a different function in its endogenous environment. It is conceivable, for example, that syntaphilin could function as a chaperone for SNARE and dynamin-amphiphysin complex formation. In this case, syntaphilin overexpression could lead to a disruption of the normal stoichiometry of the three proteins and result in an inhibition of syntaxin-1A and dynamin-1 function (22). Conversely, by binding to syntaxin-1A and dynamin-1, syntaphilin could mediate a shift in vesicle dynamics at the synapse from SNARE- and dynamin-dependent vesicle release and recycling to a state approximating a model of "kiss-and-run" or "kiss-and-stay" neurotransmission (19, 23–25). These possibilities would suggest that syntaphilin might have a positive, rather than negative, modulatory effect on synaptic strength or vesicle release and recycling kinetics. It is also possible that syntaphilin could have a more diverse role as a modulator of nonsynaptic vesicle trafficking events. Double-label immunohistochemistry in rat brain slices and immunocytochemistry in dissociated hippocampal neurons in culture (26) localize syntaphilin to synaptic and extra-synaptic areas in both axons and dendrites, raising the possibility that syntaphilin could also modulate post-synaptic fusion and endocytotic events such as neurotransmitter receptor insertion and internalization (27, 28).

Our current findings challenge the conclusions drawn from our previous work. That is, does syntaphilin inhibit neurotransmitter release in cultured hippocampal cells by inhibiting SNARE complex formation and synaptic vesicle exocytosis (7) or by inhibiting dynamin-mediated synaptic vesicle depletion secondary to deficient vesicle recycling? This dual possibility constitutes both the most exciting element of our findings about syntaphilin and the greatest challenge to future study of its function in neurons. Technologically, the use of lipophilic dyes in fluorescent confocal microscopy, in conjunction with the more traditional methods of electrophysiology, have allowed for the independent study of mechanisms underlying synaptic vesicle exocytosis and endocytosis in living cells. It would be interesting to see whether levels of syntaphilin expression at a given synapse are associated with different levels of synaptic strength. Likewise, it remains necessary to describe the mechanism by which differential targeting of syntaphilin to particular synapses occurs. In neurons, control of syntaphilin expression and targeting to particular synapses could be one mechanism underlying the presynaptic modulation of synaptic activity.

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