Neuron, Vol. 25, 191–201, January, 2000, Copyright ©2000 by Cell Press

Syntaphilin: A Syntaxin-1 Clamp that Controls SNARE Assembly

Guifang Lao,* Volker Scheuss,† Claudia M. Gerwin,* Qingning Su,* Sumiko Mochida,‡ Jens Rettig,† and Zu-Hang Sheng*§
*Synaptic Function Unit
National Institute of Neurological Disorders and Stroke
National Institutes of Health
Bethesda, Maryland 20892-4154
†Department of Membrane Biophysics
Max-Planck Institute for Biophysical Chemistry
37077 Göttingen
Federal Republic of Germany
‡Department of Physiology
Tokyo Medical University
Tokyo 160-8402
Japan

Summary

Syntaxin-1 is a key component of the synaptic vesicle docking/fusion machinery that forms the SNARE complex with VAMP/synaptobrevin and SNAP-25. Identifying proteins that modulate SNARE complex formation is critical for understanding the molecular mechanisms underlying neurotransmitter release and its modulation. We have cloned and characterized a protein called syntaphilin that is selectively expressed in brain. Syntaphilin competes with SNAP-25 for binding to syntaxin-1 and inhibits SNARE complex formation by absorbing free syntaxin-1. Transient overexpression of syntaphilin in cultured hippocampal neurons significantly reduces neurotransmitter release. Furthermore, introduction of syntaphilin into presynaptic superior cervical ganglion neurons in culture inhibits synaptic transmission. These findings suggest that syntaphilin may function as a molecular clamp that controls free syntaxin-1 availability for the assembly of the SNARE complex, and thereby regulates synaptic vesicle exocytosis.

Introduction

Synaptic vesicle docking and fusion at release sites requires the association of proteins on both vesicle and plasma membranes (reviewed by Rothman, 1994; Bajjalieh and Scheller, 1995; Südhof, 1995; Hilfiker et al., 1999). Syntaxin interacts with the synaptic vesicle–associated protein synaptobrevin/VAMP and the plasma membrane–associated protein SNAP-25 (synaptosome-associated protein of 25 kDa), constituting the SNARE complex, the biochemical intermediate essential for vesicular transport and/or fusion processes (Trimble et al., 1988; Oyler et al., 1989; Bennett et al., 1992; Yoshida et al., 1992; Söllner et al., 1993; Calakos et al., 1994; Hayashi et al., 1994; Weis and Scheller, 1998). This complex's formation comprises the minimal molecular requirement for membrane fusion in vitro (Weber et al., 1998; Chen et al., 1999). Synaptotagmin, a calcium-binding integral protein of the synaptic vesicle membrane, is thought to sense Ca^{2+} at nerve terminals and trigger neurotransmitter release (Bommert et al., 1993; Geppert et al., 1994; Li et al., 1995).

Several lines of evidence suggest that the process of synaptic vesicle docking/fusion and the SNARE protein–protein interactions are tightly regulated. Morphologically, the majority of synapses in the hippocampus have more than five synaptic vesicles docked at the active zone; however, the average number of vesicles released upon Ca^{2+} influx is less than one (Allen and Stevens, 1994). Formation of the core exocytosis complex has been proposed to provide specificity to membrane fusion events. Recent studies have demonstrated that SNARE family members participating in different trafficking steps can form highly stable complexes (Yang et al., 1999). Thus, the information for fusion specificity may not be directly encoded in the structures of the SNARE core proteins themselves but instead may depend on their regulatory factors, which determine SNARE binding specificity and spatial targeting to different subcellular membranes. Furthermore, membrane fusion and release processes are initiated several hundred microseconds after the action potential arrives at the presynaptic terminal (Berrett and Stevens, 1972; Robitaille et al., 1990). However, biochemical analysis has shown that the minimal fusion core complex (the SNARE complex) is very stable (Söllner et al., 1993; Hayashi et al., 1994). This raises the question of how the Ca^{2+}-triggered membrane fusion can be regulated so rapidly and tightly when the main intermediate is so stable. It seems reasonable to assume that assembly of functional fusion machinery might be tightly controlled by some synaptic proteins that regulate the accessibility of SNARE components by interacting with the individual SNARE proteins. Thus, the identification of regulators or molecular switches involved in the assembly of the fusion core complexes is critical for elucidating the molecular mechanisms underlying neurotransmitter release.

Recent studies have made significant progress in understanding this regulatory mechanism through isolation of the SNARE complex–interacting proteins, including Munc18/n-Sec1/rbSec1, complexins, Doc2, tomosyn, snapin, and septin CDCrel-1 (Pevsner et al., 1994a, 1994b; McMahon et al., 1995; Verhage et al., 1997; Fujita et al., 1998; Beites et al., 1999; Ilardi et al., 1999). However, how these complexes are rearranged during the priming and triggering of exocytosis remains to be determined. In the current study, we have searched for syntaxin regulatory proteins with the yeast two-hybrid selection, which favors the identification of transiently and weakly binding/regulatory proteins. A brain-enriched protein called syntaphilin was isolated that competes with SNAP-25 for binding to syntaxin-1 and inhibits SNARE complex assembly by absorbing free syntaxin-1

§To whom correspondence should be addressed (e-mail: zsheng@codon.nih.gov).

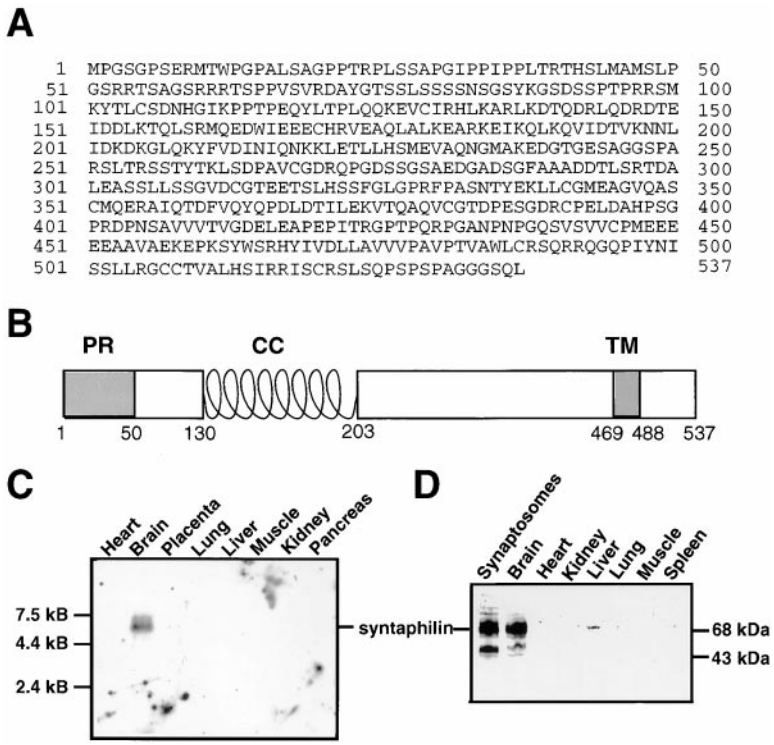

Figure 1. Structure and Distribution of Syntaphilin

(A) Predicted amino acid sequence of human syntaphilin.

(B) Domain structure of syntaphilin. Human syntaphilin is a 537 amino acid protein that contains an N-terminal proline-rich domain (PR), a CC, and a C-terminal TM.

(C) The tissue distribution of syntaphilin mRNA. Poly(A)$^+$ (2 μg) RNA from eight different human tissues (Clontech) was probed with human syntaphilin cDNA. Syntaphilin mRNA (5.5 kb) was detected in brain tissue.

(D) The tissue distribution of syntaphilin protein. Rat tissue homogenates (50 μg protein/lane) and solubilized synaptosome preparation (20 μg/lane), as indicated, were immunoblotted with the anti-syntaphilin antibody. Bands were visualized with ECL.

at nerve terminals. Transient overexpression of syntaphilin in cultured hippocampal neurons or introduction of syntaphilin coiled-coil domain (CC) into presynaptic superior cervical ganglion neurons (SCGNs) in culture significantly reduces neurotransmitter release. Our results suggest that syntaphilin acts as a syntaxin clamp in regulating assembly of the SNARE complexes during membrane fusion events.

Results

Molecular Identification of Syntaphilin Using the Yeast Two-Hybrid System

To identify proteins important for regulating synaptic vesicle exocytosis by binding to components of the SNARE complex, we have used the C-terminal half (181–288) of syntaxin-1A (Bennett et al., 1992; Yoshida et al., 1992) as a molecular bait to screen a human brain cDNA library via the yeast two-hybrid selection (Fields and Song, 1989). Screening $\sim 2 \times 10^6$ colonies led to the isolation of more than 100 clones that specifically activated *His3* and *lacZ* reporter genes. A total of 40 clones, which were strongly positive for β-galactosidase activity, were selected and retransformed into fresh yeast cells with syntaxin bait or various control baits. The specificity of the bait and prey interactions was then confirmed by transactivation assays of β-galactosidase induction and histidine autotrophy. Utilizing these techniques, we isolated six classes of complementary DNAs encoding α-SNAP (10 clones), β-SNAP (13 clones), SNAP-25 (3 clones), SNAP-29 (2 clones), syntaxin (5 clones), and two overlapping uncharacterized cDNAs (clone 11) containing an open reading frame (ORF) with a stop codon and the entire 3′ untranslated region, but no obvious N-terminal residues. Gene databank searches identified a human brain-specific cDNA (KIAA0374) that was isolated through a cloning procedure for isolation of multiple brain-specific long cDNAs (Nagase et al., 1997). The predicted amino acid sequence of clone 11 isolated from our yeast two-hybrid selection is identical to clone KIAA0374 except for the lack of 141 amino acid residues at the N terminus. The full-length cDNA, which is 5.5 kb in length, was obtained from the Kazusa DNA Research Institute (Kisarazu, Japan). Because of its specific interaction with syntaxin-1A and selective localization at synapses (see below), we named this protein syntaphilin.

The cDNA of human syntaphilin had several in-frame stop codons upstream of the start methionine and an ORF encoding 537 amino acids with a calculated molecular weight of 58 kDa (Figure 1A). The start codon of the ORF was consistent with Kozak consensus sequences (Kozak, 1987). The sequence of syntaphilin shows no significant homology with any known protein. Analysis of the syntaphilin sequence demonstrates that it is composed of a C-terminal hydrophobic segment characteristic of a transmembrane domain (TM), predicting a membrane-bound protein. The N-terminal half contains a 73 amino acid region with high potential ($p = 1$) to form a CC that may be involved in mediating synaptic protein–protein interactions (Weimbs et al., 1997) (Figure 1B). Syntaphilin is a serine-rich protein with a sequence that contains 12% serine and numerous consensus sites for protein phosphorylation by PKA, PKG, PKC, and CaMKII. Syntaphilin also contains 13 repeats of proline-rich motifs (PPXXPP, PXXP, or PXP) that specifically act as low-affinity binding domains for Src homology 3 (SH3) (Xu et al., 1997; Nguyen et al., 1998). SH3 modules have

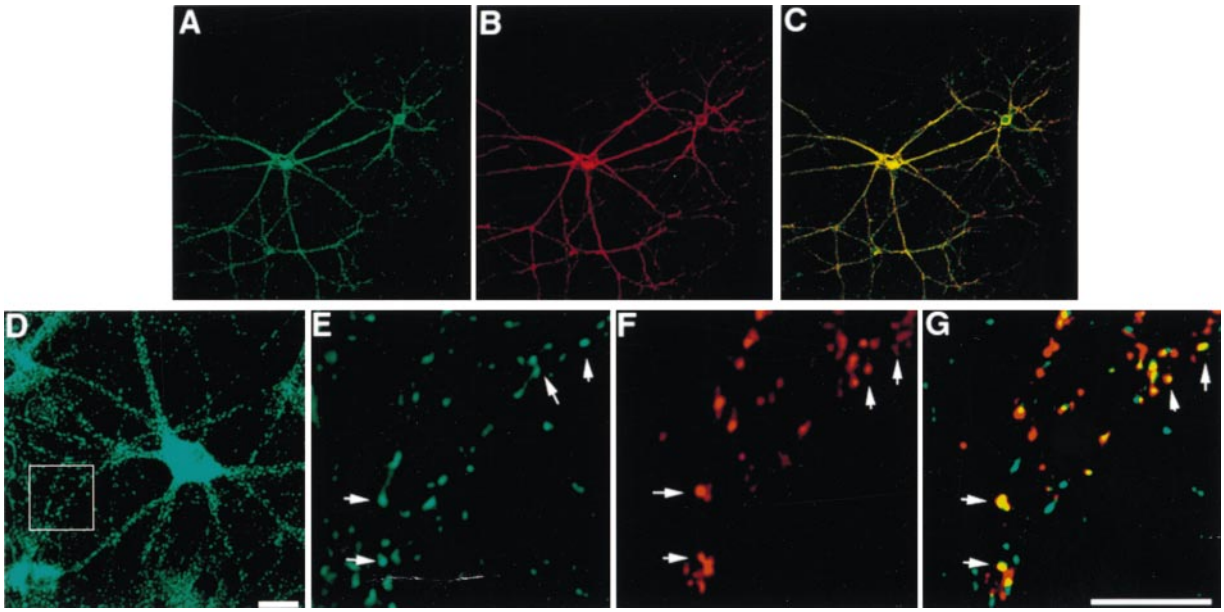

Figure 2. Syntaphilin Is Expressed in Cultured Hippocampal Neurons

(A, B, and C) Low-density hippocampal cultures were double stained with the antibodies to syntaphilin (green) and syntaxin-1 (red). Syntaphilin and syntaxin-1 are to a large extent colocalized along the processes.

(D) The hippocampal cells were stained with anti-syntaphilin antibody and visualized with FITC-coupled secondary antibody. Syntaphilin is present in the cell soma and is clustered on the neuronal processes.

(E, F, and G) The hippocampal neurons were colabeled with antibodies against syntaphilin (green) and synaptophysin (red). Double punctate staining (G) on the neuronal processes suggests that syntaphilin is partially colocalized with presynaptic marker synaptophysin in synapses (arrowheads).

Scale bars, 10 μm.

been shown to mediate protein–protein interactions in diverse signaling cascades (Cohen et al., 1995; Pawson and Scott, 1997). The sequence features of syntaphilin suggest that its role may be further regulated through signal transduction pathways at nerve terminals.

Brain-Enriched Expression of Syntaphilin mRNA and Protein

The tissue distribution of syntaphilin was analyzed by Northern blot of mRNAs and immunoblot of tissue homogenates. Syntaphilin mRNA, 5.5 kb in length, was prominently expressed in brain, while its expression was not detected in other tissues tested (Figure 1C). Our Northern blot data is consistent with the previous report on the KIAA0374 clone, which showed its brain-specific expression in an RT-PCR-based amplification of mRNAs (Nagase et al., 1997). Immunoblot analysis using a polyclonal antibody raised against a syntaphilin fusion protein showed that a 68 kDa band was readily detected in rat brain homogenate and synaptosome preparations and barely detectable in other tissues (Figure 1D), in agreement with the mRNA expression data. A minor 50 kDa band was also detected with our antibody that may represent an alternate syntaphilin gene product or a stable degradation product. Our transfection experiments in HEK 293 T cells with a cDNA of syntaphilin coding sequence confirmed that only a 68 kDa band was detected (see below), which is larger than the predicted mass based on the ORF of syntaphilin (58 kDa), suggesting a posttranslational modification of syntaphilin. By immunoblotting various homogenates, syntaphilin was found to be widely present in anatomically and functionally distinct areas of rat brain including cortex, hippocampus, olfactory bulb, striatum, midbrain, and pons (data not shown).

Subcellular Localization of Syntaphilin

The subcellular distribution of syntaphilin in neurons was examined in low-density hippocampal cultures using a polyclonal anti-syntaphilin antibody. Syntaphilin was expressed in the cell body of neurons and in a punctate pattern along the processes of the neurons (Figures 2A and 2D). To demonstrate that syntaphilin is expressed in the same regions in neurons as its binding partner syntaxin-1, we performed double-labeling experiments for the two proteins. As shown in Figures 2A–2C, syntaphilin and syntaxin-1A are to a large extent colocalized along the entire axon. Since syntaxin-1 and SNAP-25 were reported to have a widespread distribution and not to be restricted to the nerve terminal (Garcia et al., 1995; McMahon et al., 1995), we sought to investigate whether syntaphilin is localized at synapses by double labeling for syntaphilin and the synaptic vesicle marker protein synaptophysin. The results showed that syntaphilin mostly, but not always, correlated with the synaptophysin staining (Figures 2D–2G), indicating that syntaphilin may be present at a restricted set of synapses. The staining of neurons was blocked by preincubating syntaphilin antibody with a syntaphilin fusion protein, confirming the specificity of the antibody (data not shown).

To examine the subcellular distribution of syntaphilin

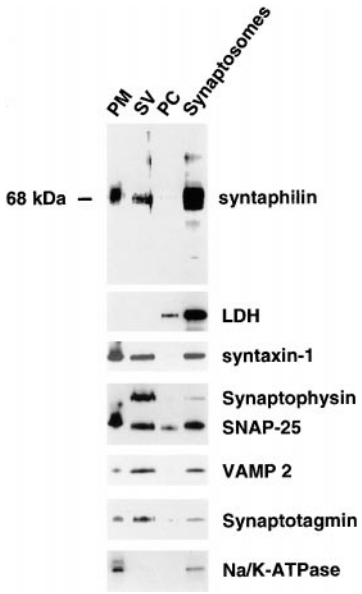

Figure 3. Distribution of Syntaphilin in Subcellular Fractions of Rat Brain Synaptosomes

Crude synaptosomes were sedimented from rat brain homogenate by differential centrifugation and separated into fractions enriched in presynaptic cytosol (PC), synaptic vesicles (SV), and synaptosome plasma membrane (PM). Equal amounts (8 μg) of synaptosome fractions and 50 μg crude synaptosomes were analyzed by SDS-PAGE and sequentially immunoblotted with antibodies as indicated on the same blot membrane. The subcellular location of syntaphilin was determined by comparing to the markers for synaptic vesicle (VAMP, synaptophysin, and synaptotagmin), plasma membrane (K/Na-ATPase, syntaxin, and SNAP-25), and cytosol (LDH).

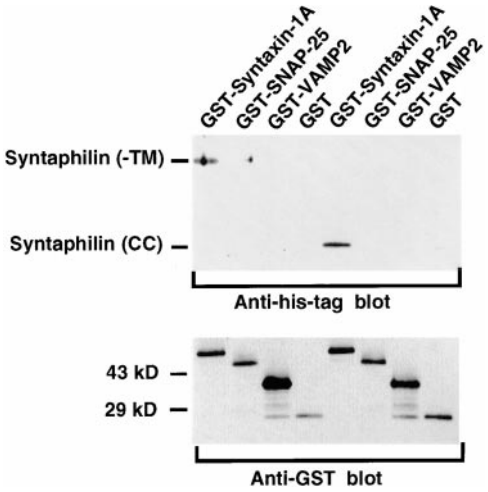

Figure 4. Specific Interaction of Recombinant Syntaphilin with Syntaxin In Vitro

Bacterial expressed GST or GST fusion proteins (~1 μg each) were immobilized on glutathione-Sepharose and then incubated with either His-tagged CC or −TM of syntaphilin. Unbound reactants were removed by washing, and bound protein complexes were eluted from the matrix, separated by SDS-PAGE, and immunoblotted with anti-His-tag antibody. Membranes were then stripped and reprobed with an anti-GST antibody.

in more detail, we used a subcellular fractionation assay from synaptosomal preparations. Rat cerebral synaptosomes were fractionated into cytosol, synaptic vesicle, and synaptic plasma membrane fractions and then analyzed by sequential immunoblotting with various antibodies against syntaxin-1, SNAP-25, VAMP2, synaptotagmin, synaptophysin, Na^+/K^+-ATPase, and lactate dehydrogenase (LDH) as indicated. Syntaphilin was associated with membrane fractions and absent from the cytosolic fraction (Figure 3), which is consistent with its structural prediction of a C-terminal hydrophobic transmembrane segment. Syntaphilin was present primarily in the plasma membrane fractions and to a lesser extent in the synaptic vesicle fraction, a distribution profile similar to that of both syntaxin and SNAP-25 (Figure 3). In contrast, immunoreactivity corresponding to VAMP2, synaptophysin, and synaptotagmin, markers of synaptic vesicles, was detected predominantly in the synaptic vesicle fraction. Neither Na^+/K^+-ATPase, a marker of the plasma membrane, nor LDH, a marker of the cytosolic fraction, was detected in the synaptic vesicle fraction, indicating the relative purity of these subcellular fractions. The enrichment of syntaphilin in the membrane fractions and the presence of a hydrophobic membrane anchor domain suggest that syntaphilin is a membrane-bound protein. To confirm this point, we extracted the brain homogenate with HEPES, 1 M NaCl, extreme pH (pH 3 and 11), and 1% Triton X-100. While syntaphilin is not extracted well in HEPES, 1 M NaCl, or in extreme pH, it is solubilized effectively by 1% Triton X-100 (data not shown). The inability of high salt or extreme pH to extract syntaphilin from membranes further supports the conclusion that syntaphilin behaves as an integral membrane protein.

Specific Association of Syntaphilin with Syntaxin-1A

Since the yeast two-hybrid system may identify low-affinity interactions that may not normally occur either in vitro or in vivo, we sought to confirm the selective and direct interaction between syntaphilin and syntaxin-1A using in vitro binding assays with recombinant proteins. While both His-tagged CC and transmembrane domain deleted segment (−TM) of syntaphilin bound to GST-syntaxin-1A, no binding was detectable to GST alone or other components of the SNARE complex, including SNAP-25 and VAMP2 (Figure 4).

Next, we sought to further confirm the syntaphilin-syntaxin-1A interaction in a mammalian expression system. A cDNA encoding a His-tagged full-length syntaphilin was cotransfected into HEK 293 T cells with the cDNA encoding syntaxin-1A. The association of syntaphilin with syntaxin-1A was then confirmed by immunoprecipitation with either anti-syntaxin-1 or anti-syntaphilin antibodies (Figures 5A–5B). Furthermore, we examined whether syntaphilin is a syntaxin-1 or syntaxin-1-SNAP-25 complex–associated protein in the rat synaptosome preparation. Syntaphilin was coimmunoprecipitated by anti-syntaxin-1 antibody but not by anti-SNAP-25 or normal control IgG (Figure 5C). Thus, the results from in vitro binding assays, immunoprecipitation studies, and yeast two-hybrid selection indicate that syntaphilin directly interacts with syntaxin-1A and further suggest that they may interact in neurons and be involved in synaptic vesicle exocytosis.

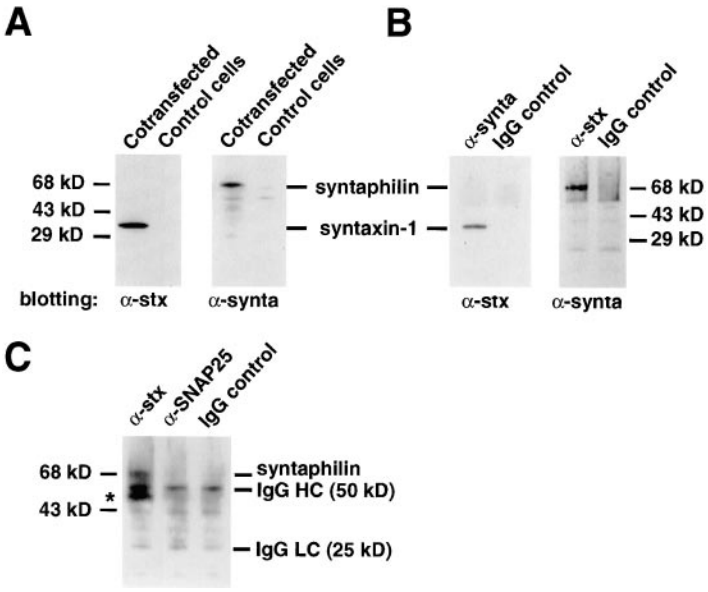

Figure 5. Coimmunoprecipitation of Syntaxin-1A with Syntaphilin from Transfected HEK 293 T Cells and Solubilized Rat Brain Synaptosomes

(A) HEK 293 T cells were cotransfected with syntaphilin (synta) and syntaxin-1A (stx) constructs. The coexpression of syntaxin-1A and syntaphilin was confirmed by immunoblotting of transfected or untransfected Triton X-100 cell extracts with antibodies as indicated.

(B) The syntaxin-syntaphilin complex was immunoprecipitated from Triton X-100 cell extracts by using either anti-syntaxin-1 (α-stx) or anti-syntaphilin (α-synta) antibodies, and the isolated immunoprecipitates were probed with either antibodies to syntaphilin or syntaxin, respectively. Normal IgG was used as a control.

(C) The syntaxin-syntaphilin complex was immunoprecipitated from solubilized rat brain synaptosomes by anti-syntaxin-1 (α-stx) antibody, or as control by anti-SNAP-25 and mouse normal IgG, and the isolated immunoprecipitates were probed with anti-syntaphilin. The degradation of syntaphilin after immunoprecipitation, which was also observed in both brain homogenates and synaptosomes (Figure 1D), is marked with an asterisk.

The relative amount of syntaphilin was estimated to be about 26%, 21%, and 83% of that of syntaxin-1, SNAP-25, and VAMP2, respectively, in the detergent extract of rat brain homogenate by Western blotting using each antibody and purified recombinant protein as standards for quantitation. Coomassie-based estimation of the relative ratios for the interaction of recombinant syntaphilin with GST-syntaxin-1A indicated an approximate 1:1 molar ratio (data not shown). Under these conditions, about 16% of total syntaxin-1 and 60% of total syntaphilin in the brain extract was coimmunoprecipitated by anti-syntaphilin and anti-syntaxin-1 antibodies, respectively (data not shown), suggesting that, in neurons, syntaphilin is mostly associated with syntaxin-1, while syntaxin-1 is only partly associated with syntaphilin. This may be due to the fact that syntaphilin is less abundant than syntaxin-1 (26% of syntaxin-1). In addition, we found that even more (about 85%) syntaphilin was coimmnuoprecipitated with syntaxin-1 in the plasma membrane fraction purified from crude synaptosomes via sucrose gradient centrifugation, suggesting a relative enrichment of syntaphilin-syntaxin complex at nerve terminals.

Syntaphilin and SNAP-25 Compete for Binding to Syntaxin-1

To gain insight into the cellular functions of syntaphilin, we investigated the biochemical consequence of syntaphilin interaction with syntaxin. Since both SNAP-25 and syntaphilin bind to the C-terminal half of syntaxin, we wondered whether syntaphilin and SNAP-25 could bind simultaneously to syntaxin-1 or whether their interactions with syntaxin-1 are mutually exclusive. To answer this question, we performed a series of competition experiments. First, we repeated in vitro binding assays with recombinant proteins. GST-syntaxin-1A was bound to glutathione-Sepharose beads and incubated with a constant concentration of SNAP-25 and increasing concentrations of syntaphilin. After extensive washing of the beads, we assayed for both syntaphilin and SNAP-25 bound to GST-syntaxin-1A by Western blot. The binding signal intensity of SNAP-25 diminished progressively while that of syntaphilin increased (Figure 6A), indicating that syntaphilin binds to syntaxin-1 in a manner competitive with SNAP-25. Next, we performed isolation of SNARE complexes from solubilized synaptosomes using the affinity beads of GST-SNAP-25, GST-VAMP2, or GST alone as a control, in the absence or presence of 0.4 μM recombinant full-length syntaphilin. Addition of syntaphilin prevented the affinity isolation of native syntaxin-1 on SNAP-25 affinity beads (Figure 6B). Conversely, syntaphilin was unable to prevent both native syntaxin and SNAP-25 or their heterodimers binding to GST-VAMP2 affinity beads. These results are consistent with our coimmunoprecipitation studies with solubilized synaptosomes, which showed that anti-SNAP-25 antibody failed to precipitate syntaphilin (Figure 5C), suggesting a selective interaction of syntaphilin with the free syntaxin at nerve terminals. Hence, the free syntaxin-1 pool within synaptosomes that was available for interaction with SNAP-25 could be absorbed by exogenous syntaphilin, further suggesting that SNAP-25 and syntaphilin compete for these free syntaxin molecules.

The Functional Effect of Syntaphilin on Synaptic Transmission

We next investigated the physiological consequences of the observed interaction of syntaphilin with syntaxin on synaptic transmission at intact synapses. For this purpose, we transiently overexpressed syntaphilin in hippocampal neurons grown in microisland culture (Bekkers and Stevens, 1991). Using the Semliki Forest virus (SFV) expression system (Liljestrom and Garoff, 1991; Owe-Larsson et al., 1999), 50%–70% of the cells were

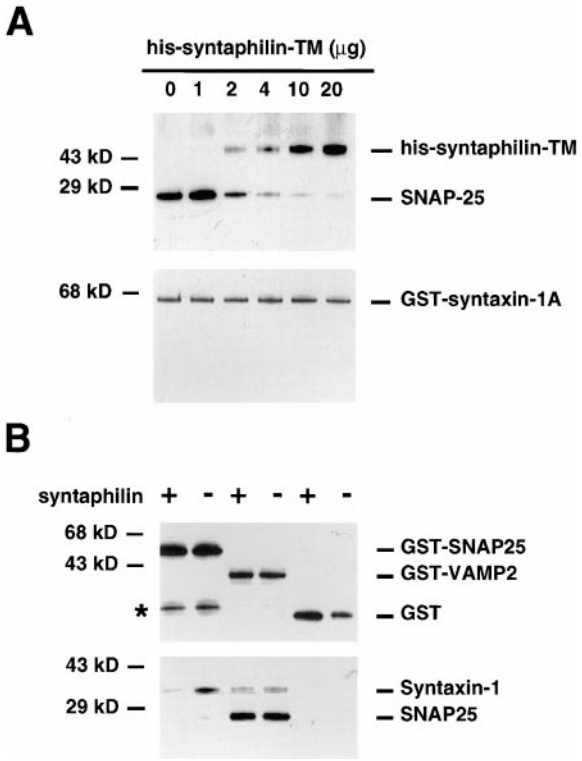

Figure 6. Syntaphilin Competes with SNAP-25 for Binding to Syntaxin-1

(A) Binding competition between SNAP-25 and syntaphilin. Immobilized GST-syntaxin-1A (100 nM) was incubated with equal concentrations (250 nM) of recombinant SNAP-25 expressed in HEK 293 T cells and increasing concentrations of His-syntaphilin (−TM) as indicated. Bound proteins were visualized by sequentially immunoblotting with both anti-His and anti-GST antibodies.

(B) Affinity isolation of the native SNARE proteins from synaptosomes. Affinity beads with ~1–2 μg of GST-SNAP-25, GST-VAMP2, or GST alone were incubated with 42 μg of solubilized rat synaptosomes in the absence or presence of 0.4 μM of recombinant full-length syntaphilin. The native synaptic proteins bound to affinity beads were then electrophoresed on 10%–20% Tricine/SDS-PAGE and detected by the antibodies to GST (top) or syntaxin-1 and SNAP-25 (bottom). The degradation of GST-SNAP-25 is marked with an asterisk.

infected and started to express syntaphilin after ~4 hr. The subcellular localization of overexpressed syntaphilin was comparable to that of endogenous syntaphilin, indicating a correct targeting. The level of syntaphilin expression with the SFV system was 3- to 10-fold higher than that of endogenous syntaphilin as judged by densitometric analysis of the immunocytochemical signal. Six to twelve hours after infection, isolated neurons were stimulated at a frequency of 0.2 Hz, and the resulting autaptic postsynaptic currents were measured. As shown in Figure 7A, syntaphilin-overexpressing cells displayed a marked reduction in EPSC amplitude compared to control cells (3.74 ± 0.98 nA, n = 10 for syntaphilin-infected cells versus 8.84 ± 1.52 nA, n = 10 for control cells; $p < 0.05$). This reduction is not due to the infection procedure itself, since neurons infected with green fluorescent protein displayed no reduction in mean amplitude (Figure 7A). Autaptic responses in cultured hippocampal neurons are characterized by a

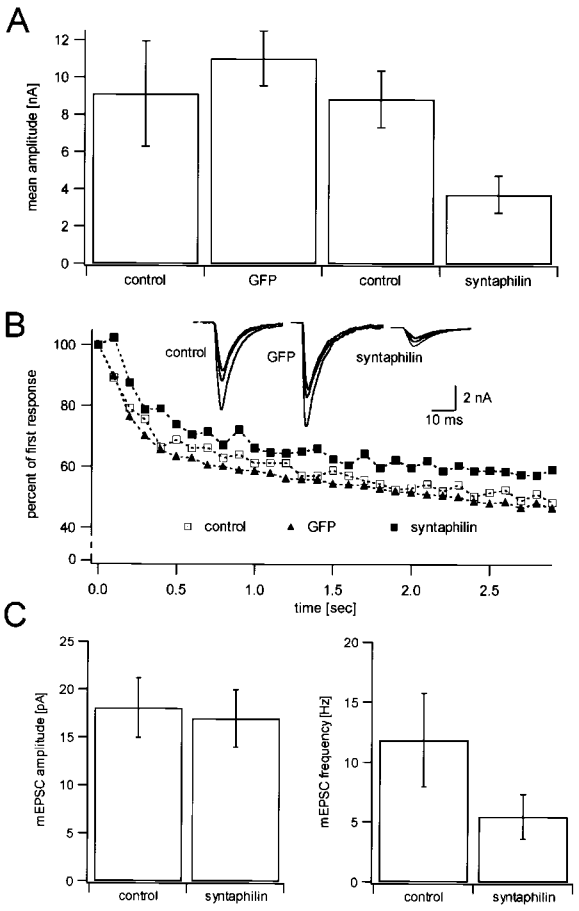

Figure 7. Effect of Syntaphilin Overexpression on Synaptic Transmission in Cultured Hippocampal Neurons

(A) Summary of the mean autaptic EPSC amplitude at 0.2 Hz stimulation in isolated hippocampal neurons. The average EPSC amplitude in GFP-overexpressing neurons was 11.04 ± 1.47 nA (mean ± SEM, n = 10) and not significantly different from the average amplitude in the corresponding control neurons, which was 9.12 ± 2.38 nA (mean ± SEM, n = 10). In contrast, the average EPSC amplitude in syntaphilin-overexpressing neurons was 3.74 ± 0.98 nA (mean ± SEM, n = 10) and significantly different ($p < 0.05$; two-tailed t test) from the average amplitude in the corresponding control neurons, which was 8.48 ± 1.52 nA (mean ± SEM, n = 10).

(B) Averaged EPSC amplitudes normalized to the first response in a train of 30 stimuli at 10 Hz in control neurons (open squares, n = 10), GFP-overexpressing neurons (closed triangles, n = 10) and syntaphilin-overexpressing neurons (closed squares, n = 10). Example traces of the first, tenth, and thirtieth EPSC in a train are shown in the insets. Control neurons displayed an average depression of 50.8% ± 4.2% with a time constant of 671 ± 107 ms (mean ± SEM, n = 10), GFP-containing neurons displayed an average depression of 50.3% ± 3.2 % with a time constant of 595 ± 113 ms (mean ± SEM, n = 10), and syntaphilin-containing neurons displayed an average depression of 58.1% ± 5.7% with a time constant of 619 ± 105 ms (mean ± SEM, n = 10). No significant difference was found between the corresponding parameters.

(C) Summary of the mean miniature EPSC amplitude and frequency in isolated hippocampal neurons. The average mEPSC amplitude in syntaphilin-overexpressing neurons was 17.0 ± 3.2 pA (mean ± SEM, n = 9), and the average mEPSC frequency was 5.45 ± 1.86 Hz (mean ± SEM, n = 10) compared to 18.1 ± 3.3 pA (mean ± SEM, n = 9) and 11.85 ± 3.90 Hz (mean ± SEM, n = 9), respectively, in control neurons. There is no significant difference in the mEPSC amplitude, but the frequency is significantly reduced in syntaphilin-overexpressing neurons ($p < 0.05$, two-tailed t test).

marked activity-dependent depression (Mennerick and Zorumski, 1995; Rosenmund and Stevens, 1996). To examine whether syntaphilin also has an effect on activity-dependent modulation of synaptic transmission, we stimulated single cells with trains of 30 stimuli at 10 Hz. Syntaphilin-overexpressing cells displayed an average steady-state depression of 58.1% ± 5.7% with a time constant of 619 ± 105 ms. These values were not significantly different from uninfected neurons, which had an average depression of 50.8% ± 4.2% with a time constant of 671 ± 107 ms (Figure 7B). In addition, no difference in the average rise and decay time of the postsynaptic response was detected (Figure 7B, inset). In order to learn about the mechanism of syntaphilin action, we next performed measurements of miniature excitatory postsynaptic currents (mEPSCs). As shown in Figure 7C, syntaphilin reduced the frequency of mEPSCs by ~50% (5.45 ± 1.86 Hz, n = 10 for syntaphilin-infected cells versus 11.85 ± 3.90 Hz, n = 9 for control cells; $p < 0.05$) without affecting its amplitude (17.0 ± 3.2 pA, n = 10 for syntaphilin-infected cells versus 18.1 ± 3.3 pA, n = 9 for control cells). Therefore, we conclude that overexpression of syntaphilin leads to a decrease in synaptic transmission, probably by reducing the presynaptic availability of syntaxin, without affecting its dynamic features.

The lack of reduction of mEPSC amplitude implicates a presynaptic action of syntaphilin. However, to unambiguously eliminate a possible postsynaptic effect of syntaphilin, we examined its role in synaptic transmission at the well-characterized cholinergic synapses formed between superior cervical ganglion neurons (SCGN) in culture (Mochida et al., 1994, 1995, 1996; Ilardi et al., 1999). This synapse is an ideal system for these experiments because proteins can be introduced into the relatively large (30–40 μm) presynaptic cell bodies by microinjection, the injected proteins can rapidly diffuse to the nerve terminals forming synapses with adjacent neurons, and the effects on stimulated release of acetylcholine can be accurately monitored by recording the excitatory postsynaptic potentials (EPSPs) evoked by action potentials in the presynaptic neurons. We injected the CC of syntaphilin, the syntaxin-binding domain, into the presynaptic SCGNs to determine whether it would compete with SNAP-25 for binding to syntaxin-1 and consequently disrupt the formation of functional SNARE complex in vivo. After a stable period of control recordings for 20–30 min, 50 μM recombinant syntaphilin-CC was diffused into presynaptic neurons from a suction pipette (at t = 0) for 2–3 min. EPSP amplitude gradually decreased over a period of 30 min (Figures 8A and 8B). The maximum decrease, −36% ± 4.2% (n = 6, mean ± SEM), was observed 30–40 min after starting injection. In contrast, injection of 50 μM heat-denatured syntaphilin-CC produced no significant decrease in EPSP amplitude during 1 hr of recording (−2.5% ± 4.4%, n = 4, at 30 min after injection) (Figure 8B), indicating that the inhibitory effect on synaptic transmission is dependent on the native conformation of syntaphilin. Although the peak of EPSPs was reduced by syntaphilin-CC, the time course was not significantly changed (Figure 8A), indicating that the kinetics of exocytosis were not affected by introduction of syntaphilin. The simplest interpretation of our results is that inhibition of synaptic transmission is due to competitive block of the interaction of syntaxin-SNAP-25 by the excess injected syntaphilin-CC and resultant decrease of the formation of functional SNARE complexes. This interpretation is consistent with the results from our in vitro biochemical studies, which showed that syntaphilin interrupted the interaction of syntaxin-1 with SNAP-25 (Figures 6A and 6B). Altogether, the physiological data from intact synapses formed between either hippocampal neurons or SCGNs in culture is consistent with our assumption based on in vitro biochemical observations, suggesting syntaphilin acts as a syntaxin-1 clamp at nerve terminals, controls formation of the functional SNARE fusion complexes, and consequently modulates the process of synaptic vesicle exocytosis.

Discussion

In this paper, we have described the identification of a protein named syntaphilin that directly interacts with syntaxin-1. Syntaphilin is predominantly expressed in brain and is especially abundant in synaptosome preparations. This protein is associated with membrane fractions and is mostly colocalized with the synaptic vesicle marker synaptophysin, indicating that syntaphilin is present at synapses. More interestingly, syntaphilin competes with SNAP-25 to bind to syntaxin-1 and inhibits SNARE complex formation by absorbing free syntaxin-1 at nerve terminals. Transient overexpression of syntaphilin in cultured hippocampal neurons or introduction of syntaphilin CC into presynaptic superior cervical ganglion neurons (SCGNs) in culture significantly reduces neurotransmitter release. Due to its synaptic localization, its specific interaction with syntaxin-1, and its functional effects on synaptic transmission, syntaphilin is uniquely positioned to regulate the formation of the SNARE complex that is involved in synaptic vesicle exocytosis.

The SNARE complexes assemble into a four-helix bundle formed by the 70 membrane-proximal residues of SNARE proteins. The minimal core consists of two helical domains from SNAP-25 and one from syntaxin arranged in parallel with one helical domain from VAMP (Fasshauer et al., 1998; Sutton et al., 1998). To complete the Ca^{2+}-triggered membrane fusion process, the assembly of the SNARE complexes must be rapidly and tightly controlled under steady-state conditions at nerve terminals. This mechanism requires the existence of presynaptic molecules capable of regulating the availability of free SNARE proteins to form a functional release machinery by binding to individual SNARE proteins, and thus limiting or promoting their interaction with other SNARE components.

Indeed, our results have demonstrated that syntaphilin might function as a SNARE regulator during synaptic vesicle exocytosis. Syntaphilin is highly enriched in neurons, with a subcellular localization profile similar to the pattern of syntaxin-1 and SNAP-25, the binding partner or competitor of syntaphilin, respectively. The localization of syntaphilin suggests that it may play a role in synaptic vesicle fusion. Additionally, we showed that

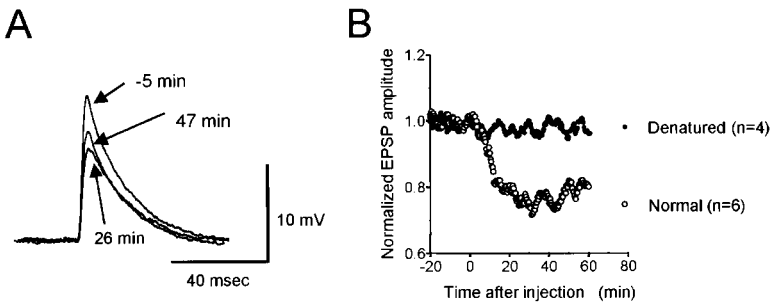

Figure 8. Effects of Syntaphilin on Synaptic Transmission of SCGNs in Culture

(A) The CC of syntaphilin was introduced into the presynaptic neuron by diffusion from a suction pipette beginning when the membrane was disrupted by applying suction at t = 0. The pipette concentration of the protein was 50 μM. Postsynaptic potentials from one representative experiment recorded 5 min before injection and 26 and 47 min after injection are illustrated.

(B) Normalized, averaged excitatory postsynaptic potentials (EPSPs) were plotted from experiments with syntaphilin-CC (open circles) and heat-denatured syntaphilin-CC (closed circles) at a concentration of 50 μM.

syntaphilin specifically competes with SNAP-25 for binding to syntaxin-1. Syntaphilin has a stable CC of 73 residues (p = 1), which is required for the interaction with syntaxin-1. By binding to the H3 CC of syntaxin-1, syntaphilin prevents syntaxin-1 from interacting with SNAP-25 and blocks the assembly of the four-helix bundle that makes up the SNARE complexes at presynaptic release sites. This competition should then result in fewer functional SNARE complexes being formed when syntaphilin is overexpressed, and consequently in a reduction in synaptic transmission. Finally, the sequence features of syntaphilin, including 13 repeats of proline-rich motifs (PPXXPP, PXXP, or PXP) and numerous consensus protein phosphorylation sites (11 sites for PKC, 3 sites for PKA/PKG, and 8 sites for CaMKII), suggest that it may be the target for modulation of synaptic transmission through signal transduction pathways at nerve terminals. One possible model for syntaphilin function is that, by interacting with free syntaxin-1, syntaphilin might serve as a molecular clamp for the assembly of functional SNARE fusion machinery. Stochastic or regulated inactivation of this clamp by either protein phosphorylation or interaction with the SH3 signaling proteins would then increase the availability of syntaxin-1 and promote the assembly SNARE core complexes, thereby enhancing the strength of neurotransmitter release. Further tests of this model, especially the modulation of syntaphilin through signal transduction pathways and genetic studies, are underway.

Our data indicate that syntaphilin is a novel member of the increasing family of regulators of the neurotransmitter release machinery. Recently, several other proteins including Munc18 (rSec1), tomosyn, and complexins were shown to be capable of regulating syntaxin-1 availability to form SNARE complexes. In contrast to Munc18, which competitively inhibits the binding of both SNAP-25 and VAMP to syntaxin-1 in vitro (Pevsner et al., 1994a, 1994b), syntaphilin blocks only SNAP-25 binding to syntaxin-1A and has no effect on the interaction of VAMP with syntaxin-1 (Figure 6B), indicating that syntaphilin and Munc18 regulate the assembly of SNARE via different interaction targets. Tomosyn was found to have the ability to replace Munc18 from syntaxin-1 via the CC competition and subsequently form a new complex with syntaxin, SNAP-25, and synaptotagmin (Fujita et al., 1998). Given that the amount of tomosyn was estimated to be 3% of syntaxin-1 at synapses, and that the molar ratio of tomosyn to syntaxin-1 was ~1:1, up to 97% of syntaxin-1 in nerve terminals is not associated with or regulated via the tomosyn pathway. Complexins weakly bind to free syntaxin but compete with α-SNAP for binding strongly to the SNARE complex (McMahon et al., 1995). Together, these findings suggest that synaptic exocytosis can be modulated by various molecules acting on different SNARE components or protein–protein interactions. Thus, it is likely that the fusion machinery requires multiple regulatory molecules that coexist at synapses and cooperatively control the assembly of SNARE complexes during synaptic vesicle exocytosis.

The mutually exclusive interaction of syntaphilin and SNAP-25 with syntaxin-1 indicates that syntaphilin may act to negatively regulate the formation of the functional SNARE complexes through a stable association with syntaxin-1. We and others have found that the SNARE proteins such as syntaxin-1 and SNAP-25 have a widespread distribution along the entire axon and are not restricted to the nerve terminal (Garcia et al., 1995; McMahon et al., 1995; see also Figure 2E). However, our immunocytochemical data showed that there is ~50%–60% colocalization of synaptophysin-containing varicosities with syntaphilin, which was expressed in a punctate pattern along the processes of the neurons. Compared with the relative abundances of other characterized SNARE regulators such as complexins and tomosyn, which are only 6% and 3% relative to that of syntaxin-1, respectively, in brain (McMahon et al., 1995; Fujita et al., 1998) syntaphilin is present in a ratio of 26% of syntaxin-1 and is enriched at synapses. Thus, syntaphilin might be physiologically sufficient to serve as a SNARE regulator at synapses. For example, the syntaphilin-containing synapses may represent either negatively regulated or even silent synapses, while synapses without or with less syntaphilin may represent ones in an active state. Our functional experiments, which showed a significant reduction in synaptic transmitter release after overexpression of syntaphilin in cultured hippocampal cells or microinjection of syntaphilin into presynaptic SCGN in culture, might support this assumption. However, one cannot exclude the possibility that the localization of syntaphilin and syntaxin, which are not restricted to nerve terminals, may reflect other functions of these molecules, in addition to their participation in the formation of functional SNARE fusion complexes.

Regulation of exocytosis is critical for proper function at the synapse. Our findings on syntaphilin establish this molecule as another possible regulator of the assembly of the functional SNARE complex and a potential target for modulation of transmitter release. Further biochemical, genetic, and physiological characterization of syntaphilin will shed light on the regulation of functional SNARE formation during the exocytotic membrane fusion events and contribute to an understanding of synaptic plasticity.

Experimental Procedures

Isolation of Syntaphilin

The yeast two-hybrid system was used to clone neuronal protein(s) that interact with syntaxin-1A. The C-terminal half (CT, 181–288) of rat syntaxin-1A cDNA was inserted in frame into the pGBT9 bait vector containing the GAL4 DNA-binding domain (Clontech). Yeast two-hybrid screens of a human brain cDNA library in vector pACT1 (Clontech) with the GAL4 activation domain were performed and evaluated according to the protocols described for the MATCHMAKER yeast two-hybrid system (Clontech). Yeast cells (Y190) were sequentially cotransformed with syntaxin-1A CT bait and library prey vectors. Positive clones were selected on plates lacking leucine, tryptophan, and histidine with 50 mM 3-aminotriazole and confirmed by filter assay for β-galactosidase activity.

Fusion Protein Construction, Preparation, and In Vitro Binding

Full-length syntaxin-1A, SNAP-25, and VAMP2 were subcloned into GST-fusion vectors, pGEX-2 or pGEX-4T (Pharmacia). Syntaphilin was subcloned into hexahistidine-tagged fusion protein vector (pET28, Novagen). Fusion proteins were prepared as crude bacterial lysates by mild sonication in PBS (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 1% Triton X-100 [TX-100], plus protease inhibitors). His-syntaphilin proteins were purified by binding to Ni^{2+}-charged nitrilotriacetic acid agarose columns (Qiagen) and eluted with 500 mM imidazole in PBS. The eluates were concentrated with Centriprep-10 filtration units (Amicon) and dialyzed in a 10,000 mw cutoff dialysis cassette (Pierce) against PBS. Approximately 1 μg of GST fusion proteins were bound to glutathione-Sepharose beads (Pharmacia) in TBS buffer (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 0.1% TX-100, plus protease inhibitors), incubated at 4°C for 1 hr with constant agitation, and washed with TBS to remove unbound proteins. Glutathione-Sepharose beads coupled with similar amounts of GST fusion proteins were added to the His-syntaphilin protein and incubated with gentle mixing for 3 hr at 4°C. The beads were washed three times with TBS buffer, and bound proteins were eluted in 15 mM reduced glutathione in 50 mM Tris-HCl (pH 8). Eluates were separated from the beads by centrifugation at 10,000 × g for 1 min and electrophoresed on a 10%–20% SDS-tricine gradient gel. Bound His-syntaphilin was detected by monoclonal anti-T7-Tag antibody (Novagen), an antibody to the His-tagged peptide sequence, and GST fusion proteins were detected by anti-GST antibody (Pharmacia). HRP-conjugated secondary antibodies and ECL (Amersham) were used to visualize the bands.

Transfection of HEK 293 T Cells

HEK 293 T cells were maintained in minimal essential medium (MEM) with 10% fetal bovine serum (Gibco) and 0.5% L-glutamine. Syntaphilin cDNA was subcloned into the EcoRI/XhoI sites of pcDNA3.1-HisA vector (Invitrogen), while cDNA of syntaxin-1A was subcloned into the EcoRI/XhoI sites of pcDNA3.1 vector (Invitrogen). HEK 293 T cells cultured in 100 mm dishes were transfected with 5–10 μg of cDNA by calcium phosphate coprecipitation, according to the transfection kit protocol (Invitrogen). After 48 hr, the cells were harvested with PBS and then solubilized in TBS buffer with 1% TX-100. Cell lysates were centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was used for immunoblotting and coimmunoprecipitation studies.

Synaptosome Preparation

Rat brain synaptosomes were prepared by differential and discontinuous Percoll gradient centrifugation and solubilized as described (Sheng et al., 1996). Synaptosome fractions were isolated as described (Dunkley et al., 1988). Briefly, after Percoll-sucrose gradient centrifugation, the synaptosomes were washed once in medium M (0.32 M sucrose, 1 mM K_2HPO_4, 0.1 mM EDTA [pH 7.5]), resuspended and homogenized in medium L (1 mM K_2HPO_4, 0.1 mM EDTA [pH 8.0]), and incubated at 0°C for 1 hr. The resulting suspension was layered over 5 ml of 1 M sucrose in medium L and centrifuged for 30 min at 96,300 × g in an SW27 rotor. The supernatant was mixed to homogeneity and centrifuged again for 14 hr at 25,000 × g. The supernatant was collected as synaptosol, and the pellets were homogenized in medium L and applied to a gradient of 7 ml 1.2 M, 1.0 M, 0.8 M, 0.6 M, and 0.4 M sucrose in medium L and centrifuged for 90 min at 68,000 × g in an SW27 rotor. Bands at each interface were collected and washed once with medium L in a Ti50 rotor (45 min at 106,500 × g). The synaptosol was dialyzed against medium L and centrifuged at 140,000 × g in a Ti 50 rotor for 1 hr to separate any remaining synaptic vesicles from soluble proteins. All pellets were then resuspended in 20 mM Tris-HCl (pH 7.5). The concentrations of total proteins in each fraction were determined by protein assay with a BSA standard.

Coimmunoprecipitation

Solubilized proteins (100–300 μg) from synaptosome preparations or homogenates of transfected HEK 293 T cells were incubated with either 3 μg of anti-syntaxin-1 (10H5 or HPC-1), anti-SNAP-25 (BR05) monoclonal antibodies, 5 μl of polyclonal anti-syntaphilin serum, or, as controls, 3 μg of mouse and rabbit normal IgG (Zymed) in 0.5 ml TBS with 0.1% TX-100 and protease inhibitors, and incubated on a microtube rotator at 4°C for 1 hr. Protein A-Sepharose CL-4B resin (2.5 mg) (Pharmacia) was added to each sample, and the incubation continued for an additional 3 hr, followed by three washes with TBS/0.1% TX-100. Subsequent interaction assays of immunoprecipitated and immobilized protein complexes are described in the section on in vitro binding assays above. For multiple detection with different antibodies, blots were first stripped in a solution of 62.5 mM Tris-HCl (pH 7.5), 20 mM DTT, and 1% SDS for 30 min at 50°C with agitation and then washed with TBS/0.1% Tween-20 for 2 × 15 min.

Hippocampal Neuron Culture and Electrophysiology

Microisland culture preparation of hippocampal neurons was performed according to a modified version of published procedures (Bekkers and Stevens, 1991). After 10–14 days in culture, cells were infected with 50 μl of an activated Semliki Forest virus containing the cDNAs of either full-length syntaphilin or green fluorescent protein (GFP) following a protocol given in Olkkonen et al. (1993). All measurements were performed 6–12 hr after infection. Only dots containing a single neuron forming excitatory synapses (autapses) were used. Extracellular recording solution contained (in mM): 172 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, and 4 $MgCl_2$ (pH 7.3, 350 mOsm). For mEPSC recordings, 400 nM tetrodotoxin was added. Patch pipettes (2–3 MΩ) were pulled from borosilicate glass (TWF 150, WPI) on a Sutter puller and backfilled with (in mM): 135 KCl, 10 HEPES, 1 EGTA, 4.6 $MgCl_2$, 4 Na-ATP, 15 creatine phosphate, 50 U/ml phosphocreatine kinase, and 0.2 mg/ml neurobiotin (pH 7.3, 315 mOsm). Currents were recorded with an EPC-9 amplifier driven by the Pulse 8.12 software package (HEKA Electronics, Lambrecht, Germany). Series resistance was compensated 60%–80%; only recordings with access resistance below 10 MΩ were included in the analysis. Analysis was performed with self-written software in IGOR Pro (WaveMetrix, Lake Oswego, OR). For mEPSC analysis, only events larger than 8 pA were considered. Data are expressed as mean ± standard error. Statistical significance was tested by two-paired Student's t test. All measured cells were fixed, and immunohistochemical detection of cells overexpressing syntaphilin was performed with a polyclonal anti-syntaphilin antibody following standard procedures.

Synaptic Transmission between SCGNs

SCG cells from 7 day postnatal rats were prepared as described previously (Mochida et al., 1994). After 4–5 weeks in culture, conventional intracellular recordings were made from two neighboring neurons using microelectrodes filled with 1 M potassium acetate (40–70

MΩ). Postsynaptic responses (EPSPs) were recorded from one of the neurons while action potentials were generated in the other neuron by passage of current through an intracellular recording electrode. Neurons were superfused with modified Krebs' solution consisting of 136 mM NaCl, 5.9 mM KCl, 5.1 mM $CaCl_2$, 11 mM glucose, and 3 mM Na-HEPES (pH 7.4). Recombinant syntaphilin-CC was dissolved in 150 mM KAc, 5 mM Mg^{2+}-ATP, and 10 mM HEPES (pH 7.4) and introduced into the presynaptic cell body by diffusion from a suction glass pipette (17–20 MΩ tip resistance). Fast Green FCF (5%, Sigma) was included in the injection peptide solution to confirm their entry into the presynaptic cell body. The injection pipette was removed 2–3 min after starting injection. EPSPs were recorded once every 20 s (0.05 Hz). Electrophysiological data were collected and analyzed using software written by the late Dr. Tauc (CNRS, France). For Figure 8B, the peak amplitudes of EPSPs were measured and averaged. The resultant values were smoothed by an eight-point moving average algorithm and plotted against recording time with $t = 0$ indicating the beginning of the presynaptic injection.

Acknowledgments

We thank the following people for their critical help: X. Zheng for synaptosome fractionation; C. A. Winters, H. Tian, and R. R. Mirotznik for immunocytochemical staining, J. W. Nagle for DNA sequencing; M. Takahashi for antibodies; M. Chheda and J. Coulombe for comments on the manuscript; and C. Rosenmund for advice on the microisland cultures. V. S. and J. R. thank E. Neher for generous support. The authors would like to thank T. Nagase of Kazusa DNA Research Institute, Japan, for providing the full-length KIAA0374 cDNA. This work was supported by the intramural research program of the National Institute of Neurological Disorders and Stroke, the National Institutes of Health (Z.-H. S.), a grant of the Deutsche Forschungsgemeinschaft (RE 1092/3-2 to J. R.), and a grant from the Human Frontier Science Program and Grant-in-Aid for Scientific Research (B) and Scientific Research on Priority Areas (A) (S. M.).

Received July 13, 1999; revised November 3, 1999.

References

Allen, C., and Stevens, C.F. (1994). An evaluation of causes for unreliability of synaptic transmission. Proc. Natl. Acad. Sci. USA *91*, 10380–10383.

Bajjalieh, S.M., and Scheller, R.H. (1995). The biochemistry of neurotransmitter secretion. J. Biol. Chem. *270*, 1971–1974.

Beites, C.L., Xie, H., Bowser, R., and Trimble, W.S. (1999). The septin CDCrel-1 binds syntaxin and inhibits exocytosis. Nat. Neurosci. *2*, 434–439.

Bekkers, J.M., and Stevens, C.F. (1991). Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in culture. Proc. Natl. Acad. Sci. USA *88*, 7834–7838.

Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science *257*, 255–259.

Berrett, E.F., and Stevens, C.F. (1972). The kinetics of transmitter release at the frog neuromuscular junction. J. Physiol. (Lond.) *227*, 691–708.

Bommert, K., Charlton, M.P., DeBello, W.M., Chin, G.J., Betz, H., and Augustine, G.J. (1993). Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. Nature *363*, 163–165.

Calakos, N., Bennett, M.K., Peterson, K., and Scheller, R.H. (1994). Protein–protein interactions contributing to the specificity of intracellular vesicular trafficking. Science *263*, 1146–1149.

Chen, Y.A., Scales, A.J., Patel, S.M., Doung, Y.-C., and Scheller, R.H. (1999). SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. Cell *97*, 165–174.

Cohen, G.B., Ren, R., and Baltimore, D. (1995). Modular binding domains in signal transduction proteins. Cell *80*, 237–248.

Dunkley, P.R., Heath, J.W., Harrison, S.M., Jarvie, P.E., Glenfield, P.J., and Rostas, J.A.P. (1988). A rapid percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res. *441*, 59–71.

Fasshauer, D., Eliason, W.K., Brünger, A.T., and Jahn, R. (1998). Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry *37*, 10354–10362.

Fields, F., and Song, O. (1989). A novel genetic system to detect protein–protein interactions. Nature *340*, 245–246.

Fujita, Y., Shirataki, H., Sakisaka, T., Asakura, T., Ohya, T., Kotani, H., Yokoyama, S., Nishioka, H., Matsuura, Y., Mizoguchi, A., et al. (1998). Tomosyn: a syntaxin-1-binding protein that forms a novel complex in the neurotransmitter release process. Neuron *20*, 905–915.

Garcia, E.P., McPherson, P.S., Chilcote, T.J., Takei, K., and De Camilli, P. (1995). rbSec1A and B colocalize with syntaxin 1 and SNAP-25 throughout the axon, but are not in a stable complex with syntaxin. J. Cell Biol. *129*, 105–120.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Südhof, T.C. (1994). Synaptotagmin I: a major Ca^{2+} sensor for transmitter release at a central synapse. Cell *79*, 717–727.

Hayashi, T., McMahon, H., Yamaski, S., Binz, T., Hata, Y., Südhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J. *13*, 5051–5061.

Hilfiker, S., Greengard, P., and Augustine, G.J. (1999). Coupling calcium to SNARE-mediated synaptic vesicle fusion. Nat. Neurosci. *2*, 104–106.

Ilardi, J.M., Mochida, S., and Sheng, Z.-H. (1999). Snapin: a SNARE-associated protein implicated in synaptic transmission. Nat. Neurosci. *2*, 119–124.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*, 8125–8131.

Li, C., Ullrich, B., Zhang, J.Z., Anderson, R.G., Brose, N., and Südhof, T.C. (1995). Ca^{2+}-dependent and -independent activities of neural and nonneural synaptotagmins. Nature *375*, 594–599.

Liljestrom, P., and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology *9*, 1356–1361.

McMahon, H.T., Missler, M., Li, C., and Südhof, T.C. (1995). Complexins: cytosolic proteins that regulate SNAP receptor function. Cell *83*, 111–119.

Mennerick, S., and Zorumski, C.F. (1995). Paired-pulse modulation of fast excitatory synaptic currents in microcultures of rat hippocampal neurons. J. Physiol. *488*, 85–101.

Mochida, S., Kobayashi, H., Matsuda, Y., Yuda, Y., Muramoto, K., and Nonomura, Y. (1994). Myosin II is involved in transmitter release at synapses formed between rat sympathetic neurons in culture. Neuron *13*, 1131–1142.

Mochida, S., Saisu, H., Kobayashi, H., and Abe, T. (1995). Impairment of syntaxin by botulinum neurotoxin C1 or antibodies inhibits acetylcholine release but not Ca^{2+} channel activity. Neuroscience *65*, 905–915.

Mochida, S., Sheng, Z.-H., Baker, C., Kobayashi, H., and Catterall, W.A. (1996). Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. Neuron *17*, 781–788.

Nagase, T., Ishikawa, K., Nakajima, D., Ohira, M., Seki, N., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N., and Ohara, O. (1997). Prediction of the coding sequences of unidentified human genes. VII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins in vitro. DNA Res. *28*, 141–150.

Nguyen, J.T., Turck, C.W., Cohen, F.E., Zuckermann, R.N., and Lim, W.A. (1998). Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. Science *282*, 2088–2092.

Olkkonen, V.M., Liljestrom, P., Garoff, H., Simons, K., and Dotti, C.G. (1993). Expression of heterologous proteins in cultured rat hippocampal neurons using the Semliki Forest virus vector. J. Neurosci. Res. *35*, 445–451.

Owe-Larsson, B., Berglund, M.M., Kristensson, K., Garoff, H., Larhammar, D., Brodin, L., and Low, P. (1999). Perturbation of the synaptic release machinery in hippocampal neurons by overexpression of SNAP-25 with the Semliki Forest virus vector. Eur. J. Neurosci. *11*, 1981–1987.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J. Cell. Biol. *109*, 3039–3052.

Pawson, T., and Scott, J.D. (1997). Signaling through scaffold, anchoring, and adaptor proteins. Science *278*, 2075–2080.

Pevsner, J., Hsu, S.C., Braun, J.E., Calakos, N., Ting, A.E., Bennett, M.K., and Scheller, R.H. (1994a). Specificity and regulation of a synaptic vesicle docking complex. Neuron *13*, 353–361.

Pevsner, J., Hsu, S.-C., and Scheller, R.H. (1994b). n-Sec1: a neural-specific syntaxin-binding protein. Proc. Natl. Acad. Sci. USA *91*, 1445–1449.

Robitaille, R., Adler, E.M., and Charlton, M.P. (1990). Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. Neuron *5*, 773–779.

Rosenmund, C., and Stevens, C.F. (1996). Definiton of the readily releasable pool of vesicles at hippocampal synapses. Neuron *16*, 1197–1207.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55–63.

Sheng, Z.-H., Rettig, J., Cook, T., and Catterall, W.A. (1996). Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature *379*, 451–454.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993). SNAP receptors implicated in vesicle targeting and fusion. Nature *362*, 318–324.

Südhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein–protein interactions. Nature *375*, 645–653.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature *395*, 347–353.

Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle–associated integral membrane protein. Proc. Natl. Acad. Sci. USA *85*, 4538–4542.

Verhage, M., de Vries, K.J., Røshol, H., Burbach, J.P.H., Gispen, W.H., and Südhof, T.C. (1997). DOC2 proteins in rat brain: complementary distribution and proposed function as vesicular adapter proteins in early stages of secretion. Neuron *18*, 453–461.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. Cell *92*, 759–772.

Weimbs, T., Low, S.H., Chapin, S.J., Mostov, K.E., Bucher, P., and Hofmann, K. (1997). A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. Proc. Natl. Acad. Sci. USA *94*, 3046–3051.

Weis, W.I., and Scheller, R.H. (1998). Membrane fusion. SNARE the rod, coil the complex. Nature *395*, 328–329.

Xu, W., Harrison, S.C., and Eck, M.J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595–602.

Yang, B., Gonzalez, L., Prekeris, R., Steegmaier, M., Advani, R.J., and Scheller, R.H. (1999). SNARE interactions are not selective: implications for membrane fusion specificity. J. Biol. Chem. *274*, 5649–5653.

Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito, T., and Takahashi, M. (1992). HPC-1 is associated with synaptotagmin and ω-conotoxin receptor. J. Biol. Chem. *267*, 24925–24928.

GenBank Accession Numbers

The GenBank accession number for the syntaphilin sequence reported in this paper is AF187733.