SNAP-25/Syntaxin 1A Complex Functionally Modulates Neurotransmitter γ-Aminobutyric Acid Reuptake*‡§

Hua-Ping Fan‡, Feng-Juan Fan‡§, Lan Bao‡, and Gang Pei‡

From the ‡Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and the §Graduate School of Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China

Neurotransmitter γ-aminobutyric acid (GABA) release to the synaptic clefts is mediated by the formation of a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which includes two target SNAREs syntaxin 1A and SNAP-25 and one vesicle SNARE VAMP-2. The target SNAREs syntaxin 1A and SNAP-25 form a heterodimer, the putative intermediate of the SNARE complex. Neurotransmitter GABA clearance from synaptic clefts is carried out by the reuptake function of its transporters to terminate the postsynaptic signaling. Syntaxin 1A directly binds to the neuronal GABA transporter GAT-1 and inhibits its reuptake function. However, whether other SNARE proteins or SNARE complex regulates GABA reuptake remains unknown. Here we demonstrate that SNAP-25 efficiently inhibits GAT-1 reuptake function in the presence of syntaxin 1A. This inhibition depends on SNAP-25/syntaxin 1A complex formation. The H3 domain of syntaxin 1A is identified as the binding sites for both SNAP-25 and GAT-1. SNAP-25 binding to syntaxin 1A greatly potentiates the physical interaction of syntaxin 1A with GAT-1 and significantly enhances the syntaxin 1A-mediated inhibition of GAT-1 reuptake function. Furthermore, nitric oxide, which promotes SNAP-25 binding to syntaxin 1A to form the SNARE complex, also potentiates the interaction of syntaxin 1A with GAT-1 and suppresses GABA reuptake by GAT-1. Thus our findings delineate a further molecular mechanism for the regulation of GABA reuptake by a target SNARE complex and suggest a direct coordination between GABA release and reuptake.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, which is released from the presynaptic terminals through the docking and fusion of synaptic vesicles with the plasma membrane. Membrane fusion and subsequent GABA release are catalyzed by the assembly of a ternary complex from soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (1). The ternary complex is composed of two plasma membrane proteins, including syntaxin 1A and synaptosomal associated protein of 25 kDa (SNAP-25), which are called target SNAREs (t-SNAREs), and one vesicle-associated protein synaptobrevin 2 (VAMP-2), which is called vesicle SNARE (2, 3). The t-SNAREs syntaxin 1A and SNAP-25 form a heterodimer, the putative intermediate of the SNARE complex, and offer target sites for the vesicle SNARE VAMP-2 leading to membrane fusion (4, 5). After membrane fusion, GABA is released from synaptic vesicles to synaptic clefts and binds to postsynaptic GABA receptors and thus transmits the signal to the postsynaptic terminals.

GABA is cleared away rapidly from synaptic clefts to terminate synaptic transmission through the reuptake function of its specific, high affinity, sodium- and chloride-dependent transporters (6), which are located on presynaptic terminals and surrounding glial cells (7). GABA transporters are mainly divided into four subtypes, including GAT-1, GAT-2, GAT-3, and BGT-1, of which GAT-1 is the most abundant neuronal subtype (8). GAT-1, GAT-2, and GAT-3 have 12 transmembrane domains, with both N and C termini facing intracellularly. GAT-2 and GAT-3 display about 52% amino acid identity with GAT-1 (8). It is generally believed that GABA reuptake from the synaptic clefts is an important mechanism in the regulation of GABA activity in synaptic neurotransmission (9). Specific GABA transporter inhibitors are revealed to prolong the decay phase of GABA, type A, receptor-mediated postsynaptic potential (10) and to increase the magnitude of responses mediated by the G protein-coupled GABA, type B, receptor (6, 10, 11).

Neurotransmitter transporters are regulated through a variety of signal transduction mechanisms that maintain appropriate levels of transmitter in the synaptic clefts. Both signaling molecules, including extracellular substrate (12, 13) and intracellular second messengers such as kinases and phosphatases (14), and the proteins directly binding to the neurotransmitter transporters are known to act on the transporters and modulate their function. Recent reports of transporters regulated by directly

resonance energy transfer; PTIO (2-phenyl-4,4,5,5-tetramethylimidazol-1-oxyl-3-oxide); NTG, nitroglycerine; Cy3, indocarbocyanine; Cy5, indodicarbocyanine.
binding proteins come from the investigations that the transporters, such as the inhibitory transmitter glycine transporter (15), the major excitatory transmitter glutamate transporter (16), serotonin transporter (17, 18), and norepinephrine transporter (19), are functionally regulated by the t-SNARE syntaxin 1A. As to the GABA transporters, it is reported that syntaxin 1A interacts with the GABA transporter GAT-1 and results in a decrease of its reuptake function (20). Syntaxin 1A positively regulates GAT-1 surface expression (21) but exerts its inhibitory actions by directly binding to GAT-1 and decreasing its transport rate (22). These investigations demonstrate a direct link between neurotransmitter release and reuptake. However, it remains largely unknown whether other SNARE proteins or the SNARE complex regulate GABA reuptake. Further coordination between neurotransmitter release and reuptake needs to be explored.

Increasing studies from the ion channels indicate that Kv2.1 potassium channel and cystic fibrosis transmembrane regulator chloride channel, which reside on the plasma membrane to control the intracellular or extracellular concentrations of respective ions, are functionally regulated by the t-SNARE complex (23, 24). Because the t-SNARE complex acts on the ion channels, and syntaxin 1A physically interacts with and functionally regulates those neurotransmitter transporters, it is most likely that other SNARE proteins or the SNARE complex may modulate those transporters. In this study, we found that SNAP-25 significantly inhibited GABA transporter GAT-1 reuptake function. The physical and functional interactions between SNAP-25 and GAT-1 depended on syntaxin 1A, and SNAP-25 binding to syntaxin 1A inhibited GAT-1 reuptake function through enhancing the physical interaction of syntaxin 1A with GAT-1. Our results also provided the evidence that the SNARE complex formation promoted the direct binding of syntaxin 1A to GAT-1 and resulted in the inhibition of GABA reuptake by GAT-1, elucidating a coordination of neurotransmitter release and reuptake.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**The full lengths of SNAP-25 and VAMP-2 were cloned into modified pcDNA3 vector in-frame with HA at the N terminus. G43D-GFP plasmid was obtained from Dr. Maureen E. Linder, and G43D was cloned into pcDNA3 vector with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1AΔH3 were gifts from Dr. Anjaparavadap P. Naren and Dr. Kevin L. Kirk and were cloned into the pcDNA3 vector in-frame with FLAG at the N terminus. Syntaxin 1AΔH3 was also cloned into the pcDNA3 vector with CFP at the N terminus. Plasmid DNA of GAT-1, EAAC1, GLT1, and GLAST were provided by Dr. Jian Fei. The full lengths of GAT-1, GAT-2, GAT-3, EAAC1, GLT1, and GLAST were cloned into modified pcDNA3 vector in-frame with HA at the N terminus. GAT-2 and GAT-3 were also cloned into the pcDNA3 vector with YFP at the N terminus. Constructions of RNA interference plasmids were also cloned into the pcDNA3 vector with YFP at the N terminus. GAT-2 and GAT-3, EAAC1, GLT1, and GLAST were cloned into modified pcDNA3 vector in-frame with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1B with G43D was cloned into pcDNA3 vector with HA at the N terminus. G43D-GFP plasmid was obtained from Dr. Mauro E. Linder, and G43D was cloned into modified pcDNA3 vector in-frame with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1B with G43D was cloned into pcDNA3 vector with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1B with G43D was cloned into modified pcDNA3 vector in-frame with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1B with G43D was cloned into modified pcDNA3 vector in-frame with HA at the N terminus. Plasmid DNA of syntaxin 1A and syntaxin 1B with G43D was cloned into modified pcDNA3 vector in-frame with HA at the N terminus.

**Cell Culture and Transfection—**Primary cultured hippocampal neurons were prepared from 1-day-old postnatal Sprague-Dawley rats using the method described previously (26). To obtain purely neuronal cultures, the Dulbecco’s modified Eagle’s medium was replaced at 4–6 h later with Neurobasal-A medium containing B27 serum-free supplement (Invitrogen) for hippocampal neuronal culture. Twenty four hours later, the cultures were treated with 5 μM cytosine arabinoside for 72 h. The neurons were transfected using the rat neuron nucleofector kit for primary rat hippocampal or cortical neurons (Program O-03 or G-13, Amaxa Biosystems). In brief, we resuspended the prepared neurons into rat neuron nucleofector solution at room temperature to a final concentration of 4–5 × 10^6 cells/100 μL. We then mixed 100 μL of cell suspension with 1–3 μg of DNA and transferred the nucleofection sample into a certified cuvette (Amaxa Biosystems). The cuvette was inserted into the cuvette holder, and program O-03 or G-13 was run. After the addition of 500 μL of pre-warmed Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, the cell was transferred into the prepared 24-well plates and incubated in a humidified 5% CO2 incubator at 37 °C. After 2–4 h, we carefully replaced the medium with 750 μL of fresh Neurobasal-A medium containing B27 serum-free supplement (Invitrogen) to remove cellular debris. The transfection efficiency was 50–70%.

PC12 cells and HEK293 cells were obtained from the American Type Culture Collection. PC12 cells were maintained in F12K medium (Invitrogen) supplemented with 2 mM l-glutamine, 5% fetal bovine serum, 10% horse serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen). HEK293 cells were maintained in minimum Eagle’s medium (Invitrogen) supplemented with standard supplements. Transient transfection of HEK293 cells was by using the calcium phosphate method.

**[^3]H|GABA Uptake Assay—**GABA uptake in cultured neurons and transfected PC12 or HEK293 cells was performed as described previously (27). The cells were grown in a monolayer on 24-well plates. The cells were incubated in Krebs-Ringer/HEPES (KRH) medium, pH 7.4, containing 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 25 mM HEPES, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 10 mM glucose at 37 °C for 30 min, and then 10 nM [^3]H|GABA (Amersham Biosciences) and 30 μM unlabeled GABA (Sigma) were added to initiate the uptake. Nonspecific uptake was determined with sodium-free KRH medium in which choline chloride was used instead of NaCl. After incubation for 5–15 min, the uptake was terminated by two ice-cold washes with 500 μL of sodium-free KRH medium, followed by immediate lysis in 200 μL of ice-cold 0.1 M NaOH. 100 μL of lysis was loaded onto glass fiber filters and then analyzed for radioactivity. Finally, the filters containing cellular lysate were processed for scintillation counting (Beckman Instruments). Under these conditions the uptake is linear with time. The protein quantity was measured using the BCA kit (Pierce). The GABA uptake activity was measured as fmol/min/mg protein. Data were from at least three separate experiments, two sam-
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In the assay of GABA uptake with drug treatment, NOC-18 (Calbiochem) or NTG (Sigma) or NOC-18/PTIO (Calbiochem) or NO-711 (Sigma) was added into the uptake reaction buffer.

Cell Surface Biotinylation and Western Blotting—Cell surface biotinylation assay in transfected HEK293 cells or cultured neurons was performed as described (28). NOC-18 was added into culture medium without serum to stimulate the primary cultured hippocampal neurons and then washed away before the cell surface biotinylation labeling. The cells were incubated with a solution containing 1 mg/ml sulfo-NHS biotin (Pierce) in phosphate-buffered saline/CaCl2/MgCl2 for 20 min at 4 °C with gentle shaking. The biotinylation solution was removed by two washes in phosphate-buffered saline/CaCl2/MgCl2 plus 100 mM glycine and quenched in this solution by incubating the cells at 4 °C for 45 min with gentle shaking. Then the cells were lysed in 1 ml of RIPA buffer (100 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 250 μM PMSF) at 4 °C for 60 min. The cell lysates were centrifuged at 20,000 × g at 4 °C for 60 min. The supernatant fractions were incubated with Immunopure Immobilized Monomeric Avidin beads (Pierce) at room temperature for 60 min. After efficient washing, the beads were incubated for 30 min at 50 °C in SDS-PAGE loading buffer. Then the samples were analyzed by standard Western blotting using rabbit anti-GAT-1 antibody (1:1000; Abcam) and mouse anti-syntaxin 1A antibody (1:1000; Synaptic Systems). The bands were quantified with ScionImage software (Scion).

Immunocytochemistry—Immunofluorescence staining of transfected PC12 cells was carried out as described (29, 30). The PC12 cells cotransfected FLAG-syntaxin 1A and/or HA-SNAP-25 with GAT-1-GFP were incubated with mouse anti-FLAG (1:500, Sigma) and/or rabbit anti-HA antibody (1:500, Sigma) overnight at 4 °C, followed by second antibodies conjugated with indocarbocyanine (Cy3) and indodicarbocyanine (Cy5) (1:100; Jackson ImmunoResearch). The cells were then washed and mounted with PermaFluor Mountant Medium (Thermo). GAT-1-GFP was identified through GFP fluorescence detection. NOC-18 was added into the culture medium without serum to stimulate PC12 cells transfected with GAT-1-GFP and were washed away before immunohistochemistry. The cells were incubated with mouse anti-syntaxin 1A (1:500; Synaptic Systems) and rabbit anti-SNAP-25 antibody (1:500; Sigma) overnight at 4 °C and followed by second antibodies conjugated with Cy3 and Cy5. The images were captured with the Leica TCS SP2 confocal microscope. The cells exhibiting distinct “ring” staining were quantified among 100 randomly selected positive cells in each group of experiments.

Coimmunoprecipitation—Transfected HEK293 cells were lysed in buffer (100 mM Tris-Cl, pH 7.4, 0.8% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 250 μM PMSF). The supernatants were treated with protein G-agarose beads (Amersham Biosciences) followed by incubation with the immunoprecipitated antibodies (mouse anti-syntaxin 1A, mouse anti-SNAP-25, rabbit anti-GAT-1, and mouse anti-HA) at 4 °C for 2–4 h. Hippocampal neurons were lysed in buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 250 μM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate), and the supernatants were incubated with antibodies overnight at 4 °C before Protein G-agarose beads were added. The brains were removed from 1-day-old postnatal Sprague-Dawley rats. Brain samples were homogenized in ice-cold buffer (4 mM Tris-Cl, pH 7.4, 1 mM EDTA, 0.32 M sucrose, 10 mM glucose, 250 μM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) in a glass-Teflon homogenizer. Homogenates were centrifuged, and the supernatants were spun at 4 °C. The pellets were suspended in the lysis buffer for neurons described above at 4 °C for 1 h and then were centrifuged. The supernatants were incubated with antibodies (rabbit anti-GAT-1/2/3; Chemicon) overnight at 4 °C before Protein G-agarose beads were added. Proteins in the immunoprecipitates were analyzed by standard Western blotting.

Fluorescence Resonance Energy Transfer (FRET) Measurements—FRET measurements were performed essentially as described (31). Image acquisition and determination of FRET efficiency by acceptor photobleaching were obtained using the Leica TCS SP2 confocal microscope and analytical software. Briefly, emission spectra from the cells expressing CFP-syntaxin 1A or CFP-syntaxin 1AΔH3 and YFP-GAT-1 or YFP-GAT-2 or YFP-GAT-3 were obtained with the λ mode, using the 405 nm line of laser. For measurement of FRET efficiency by this method, Leica software application for acceptor photobleaching was applied. The selected cell surface areas were photobleached of YFP-GAT-1 or YFP-GAT-2 or YFP-GAT-3 with 514 nm line of laser. Reduction of the YFP signal after photobleaching for CFP-syntaxin 1A and YFP-GAT-1 cotransfected PC12 cells was on average 78 ± 4.8% (n = 50). FRET was resolved as an increase in the CFP-syntaxin 1A (donor) signal after photobleaching of YFP-GAT-1 (acceptor). Relative FRET efficiency was calculated as (1 − (CFP(1 pre-bleach)/CFP(1 post-bleach))) × 100%. For control purposes, an area of the cell surface without photobleaching was also analyzed for FRET. The cells transfected with HA-SNAP-25 plasmid were confirmed by detection of Cy5 fluorescence followed by incubation with primary antibody against HA and a second antibody conjugated with Cy5 (Jackson ImmunoResearch), whereas the cells transfected with GFP/SNAP-25 siRNA were identified through GFP fluorescence detection.

Statistical Analysis—The quantification was based on at least three independent experiments. The results were presented as mean ± S.E. Statistics differences were determined by Student’s t test for two group comparisons.

RESULTS

SNAP-25/Syntaxin 1A Are Intrinsically Involved in the Inhibition of GAT-1 Uptake Function—It has been shown that syntaxin 1A inhibits the uptake function of GABA transporter GAT-1 in hippocampal neurons (20, 22). As shown in Fig. 1A, 10 μM NO-711, a selective GAT-1 blocker (32), eliminated 88.9 ± 7.5% of GABA uptake in primary cultured hippocampal neurons, confirming the previous report (7) that the GABA uptake is mainly contributed by GAT-1 in hippocampal neurons. To investigate whether other SNARE proteins or SNARE
complex can modulate GAT-1 uptake function, we first examined the effects of down-regulating SNARE protein expression on GAT-1 transport activity in primary cultured hippocampal neurons. The suppression of syntaxin 1 or SNAP-25 expression increased the GABA uptake in hippocampal neurons by transfection with their specific siRNAs, but suppressing VAMP-2 expression did not affect the GABA uptake. Moreover, suppressing syntaxin 1A and SNAP-25 expression simultaneously further increased GABA uptake but suppressing the expression of three SNARE proteins did not enhance this increase (Fig. 1B).

Western blotting analysis showed that RNA interference specifically and effectively mediated the silencing of endogenous syntaxin 1A or SNAP-25 or VAMP-2 (Fig. 1A). A cotransfection of syntaxin 1A with GAT-1 inhibited the GABA uptake, and this inhibition was promoted by increasing syntaxin 1A expression (Fig. 2A). The cotransfection of syntaxin 1A and SNAP-25 with GAT-1 significantly enhanced the syntaxin 1A-mediated inhibition of GABA uptake (Fig. 2A and B), and this enhanced inhibition was further promoted by increasing SNAP-25 expression (Fig. 2B), whereas the cells cotransfected SNAP-25 alone with GAT-1 showed no decrease of GABA uptake (Fig. 2A, the ratio of transfected GAT-1 and FLAG-syntaxin 1A plasmids is 1:0). However, the enhancement of SNAP-25 on syntaxin 1A-mediated inhibition of GAT-1 uptake function depended on the ratio of syntaxin 1A and SNAP-25 expression. When the ratio of cotransfected FLAG-syntaxin 1A and HA-SNAP-25 plasmids was 0.3:1 or 1:3, SNAP-25 significantly enhanced the inhibition of GABA uptake by syntaxin 1A (inhibition from 23.6 ± 6.3% to 47 ± 3.9% or from 50.9 ± 6.8% to 74.8 ± 4.5%, respectively) (Fig. 2A and B). These findings indicate that SNAP-25 inhibits GAT-1 uptake function in the presence of syntaxin 1A, and SNAP-25 enhances the inhibition of GAT-1 uptake function mediated by syntaxin 1A.

Because the inhibition of GAT-1 uptake function is mediated by syntaxin 1A, to further identify whether the inhibition by syntaxin 1A is special for the uptake function of GABA transporter GAT-1 subtype (599 amino acids), we examined the effects of syntaxin 1A on the uptake function of GAT-2 (602 amino acids) or GAT-3 subtype (627 amino acids). As shown in Fig. 2C, neither GAT-2 nor GAT-3 uptake function was affected by syntaxin 1A in cotransfected HEK293 cells.
Physical and Functional Interactions between SNAP-25 and GAT-1 Depend on Syntaxin 1A—To explore the underlying mechanism of the effect of SNAP-25 on syntaxin 1A-mediated inhibition of GAT-1 uptake function, we examined the physical interactions among them. It is reported that syntaxin 1A binds to GAT-1 directly (20, 22) and syntaxin 1A interacts with SNAP-25 during the SNARE complex formation (3–5). Our coimmunoprecipitation experiment in primary cultured hippocampal neurons showed that endogenous syntaxin 1A and SNAP-25 were coimmunoprecipitated with GAT-1 (Fig. 3A). In rat brain tissues and cotransfected HEK293 cells, syntaxin 1A interacted with GAT-1 but not with GAT-2 or GAT-3 (supplemental Fig. 1, A and B), consistent with specific inhibition of GAT-1 uptake function by syntaxin 1A. As reported previously, G43D is a SNAP-25 point mutant that has a mutation of the glycine 43 residue to aspartic acid and is revealed not to interact with syntaxin 1A (34), and syntaxin 1AΔH3 (syn 1AΔH3) is a mutant of syntaxin 1A that lacks the H3 domain (194–266 residues) and is reported not to interact with GAT-1 (22) or SNAP-25 (35, 36). Our data confirmed that SNAP-25 but not G43D was coimmunoprecipitated with syntaxin 1A, and both SNAP-25 and GAT-1 interacted with syntaxin 1A, but neither of them bound to syntaxin 1AΔH3 (Fig. 3B). Both mutants were introduced in the experiments using the HEK293 cells cotrans-
ected SNAP-25 and/or syntaxin 1A with GAT-1 to examine the physical interaction between SNAP-25 and GAT-1. We found that only in the presence of syntaxin 1A was GAT-1 coimmunoprecipitated with SNAP-25 (Fig. 3C). Neither the interaction between GAT-1 and SNAP-25 in presence of syntaxin 1A nor the interaction between GAT-1 and G43D in presence of syntaxin 1A was detected in the cotransfected HEK293 cells (Fig. 3C). These data indicate that the physical interaction between SNAP-25 and GAT-1 depends on syntaxin 1A.

To further identify whether the functional relationship among GAT-1, syntaxin 1A, and SNAP-25 is directly related to their physical interactions, different combinations of their plasmids and mutants were cotransfected in HEK293 cells and the GABA uptake was detected. We found that SNAP-25 enhanced the syntaxin 1A-mediated inhibition of GAT-1 uptake function but not G43D. Our data also showed that VAMP-2 did not affect the syntaxin 1A- and SNAP-25-mediated inhibition of GABA uptake. SNAP-25 or VAMP alone had no effect on GABA uptake by GAT-1 (Fig. 3D). Thus, these findings indicate that the physical interaction among GAT-1, syntaxin 1A, and SNAP-25 is necessary for the SNAP-25-enhanced syntaxin 1A-mediated inhibition of GAT-1 uptake function.

**SNAP-25 Increases the Surface Expression of GAT-1 and Syntaxin 1A**—It is reported that syntaxin 1A up-regulates the cell surface expression of GAT-1 and decreases GAT-1 transport rate (21, 22). To identify whether SNAP-25 regulates the surface expression of GAT-1 and then results in the enhanced syntaxin 1A-mediated inhibition of GAT-1 uptake function, we detected the surface expression of GAT-1 in HEK293 cells cotransfected syntaxin 1A and/or SNAP-25 with GAT-1 by using surface biotinylation assay. The amount of GAT-1 on the cell surface was increased by cotransfected with syntaxin 1A, and this increase was enhanced by further cotransfection with SNAP-25 (~130%, compared with cotransfected with syntaxin 1A). Meanwhile, the surface amount of syntaxin 1A was also increased by further cotransfection with SNAP-25 (~145%, compared with cotransfected with syntaxin 1A) (Fig. 4A). Furthermore, the effect of SNAP-25 on the surface expression of GAT-1 and syntaxin 1A was investigated using immunocytochemistry experiments in GAT-1-GFP transfected PC12 cells, for spherical PC12 cells morphologically show more distinctly subcellular localization than cultured hippocampal neurons. As illustrated in Fig. 4B, in PC12 cells the transfected GAT-1-GFP was largely dispersed in the cytoplasm, and only ~30% of cells exhibited the surface-associated distinct ring staining (Fig. 4B, panel a). Following cotransfection with syntaxin 1A, the surface-associated labeling of GAT-1-GFP was increased, with ~50% of cells exhibiting distinct ring staining (Fig. 4B, panel b). After cotransfection with both syntaxin 1A and SNAP-25, the surface-associated labeling of GAT-1-GFP was further increased, with ~75% of cells exhibiting distinct ring staining (Fig. 4B, panel e). SNAP-25 transfection also elevated the surface-associated labeling of syntaxin 1A, with ~80% of cells exhibiting distinct ring staining (Fig. 4B, panel f), and about 40% of the cells showed distinct surface-associated labeling without transfection with SNAP-25 (Fig. 4B, panel c). These results indicate that SNAP-25 increases the surface expression of GAT-1 and syntaxin 1A, suggesting that the interaction of GAT-1 with syntaxin 1A on the cell surface might be up-regulated by SNAP-25.

**SNAP-25 Potentiates Surface-associated Syntaxin 1A Binding to GAT-1**—The underlying mechanism of the SNAP-25-enhanced syntaxin 1A-mediated inhibition of GAT-1 uptake function was further investigated by using FRET as a noninvasive imaging method to characterize the interaction of GAT-1 with syntaxin 1A regulated by SNAP-25. FRET is a process in which an excited donor fluorophore transfers energy to a lower energy acceptor fluorophore via a short range (~10 nm) dipole-dipole interaction, and thus FRET efficiency detection is usually used to reflect the physical interaction between two directly binding proteins. It has been reported that the syntaxin 1A-mediated inhibition of GAT-1 function depends on syntaxin 1A
binding to GAT-1 on the cell surface (22). Because our findings showed that SNAP-25 increased the surface expression of syntaxin 1A and GAT-1, we presumed that SNAP-25 binding to syntaxin 1A modulated the interaction of GAT-1 with syntaxin 1A on the cell surface. Here the FRET efficiency between CFP-syntaxin 1A and YFP-GAT-1 was detected by the acceptor photobleaching FRET technique (31) in transfected PC12 cells. It has been reported that the expression, targeting, and function of CFP-syntaxin 1A and YFP-GAT-1 show no significant difference from the wild-type syntaxin 1A and GAT-1 (37, 38). We also detected the GABA uptake in cotransfected PC12 cells and found that the uptake function of YFP-GAT-1 was inhibited by CFP-syntaxin 1A (data not shown), confirming that the addition of fluoroprotein tags to the wild-type syntaxin 1A and GAT-1 does not alter their functional interaction. In FRET, the nonradiative transfer of donor energy to the acceptor quenches the emission of light from the donor. Thus FRET detection can be accomplished by comparing the emission of light from the donor in the same sample before and after destroying the acceptor by photobleaching. If FRET is present, a resultant increase in donor emission will occur on photobleaching of the acceptor (39). We initially examined whether the emission of light by the donor (CFP-syntaxin 1A) became more intense after photobleaching of the acceptor (YFP-GAT-1) when two proteins were coexpressed in PC12 cells. A representative example was provided by a Leica confocal microscope with the mixed emission spectra of the CFP-syntaxin 1A donor and YFP-GAT-1 acceptor fluorophores (excitation, 405 nm line of laser), and the images were taken from the photobleached and nonphotobleached regions of the cell before and after localized photobleaching (Fig. 5A). The selected regions were photobleached of YFP-GAT-1 with 514 nm line of laser. A selective increase in the peak emission intensity corresponding to the region of CFP-syntaxin 1A emission was observed in the photobleached but not nonphotobleached region of the cell (Fig. 5A). These results were consistent with a FRET signal originating from the transfer of resonant energy from CFP to YFP and the dequenching of this energy transfer upon photobleaching of the acceptor fluorophore. A representative set of the unmixed images (CFP-syntaxin 1A and YFP-GAT-1) was illustrated from the transfected PC12 cells before and after photobleaching of the acceptor with 514 nm line of laser (Fig. 5B). The CFP-syntaxin 1A images showed an increase in the donor emission (pseudocolored intensity images) after photobleaching, and this increase occurred only in the region of the cell exposed to the photobleaching (Fig. 5B). The averaged relative FRET efficiencies between surface-associated YFP-GAT-1 and CFP-syntaxin 1A is significantly increased by overexpressing SNAP-25 but decreased by suppressing SNAP-25 expression through its specific siRNA. The numbers above the columns indicate changes in the peak emission intensity. Data are from three independent experiments. *, p < 0.05, and **, p < 0.01 as compared with the control cells cotransfected with CFP and YFP or the cells indicated in the figure.

**FIGURE 5.** SNAP-25 potentiates surface-associated syntaxin 1A binding to GAT-1. A, representative example of mixed emission spectra of CFP-syntaxin 1A donor and YFP-GAT-1 acceptor fluorophores (excitation, 405-nm laser line) are shown before (red line) and after (blue line) photobleaching (with the 514-nm laser line) in cotransfected PC12 cells. The red line is a set of unmixed YFP-GAT-1 and CFP-syntaxin 1A (CFP-Syn 1A) images of PC12 cells taken before and after acceptor photobleaching. The region of photobleaching is indicated by the white outlined box. The enlarged pseudocolored images at the bottom show the intensity of CFP emission in the photobleached and nonphotobleached regions of the cell surface taken before and after bleaching. The surface-associated intensity of donor CFP-syntaxin 1A emission in PC12 cells increases after acceptor YFP-GAT-1 photobleaching. Scale bar indicates 5 μm. C, Averaged FRET efficiencies (%) for coexpressed YFP-GAT-1 (YFP-GAT-1) and CFP-syntaxin 1A (CFP-Syn) under the conditions of up-regulating or down-regulating SNAP-25 expression. FRET efficiency between surface-associated YFP-GAT-1 and CFP-syntaxin 1A is significantly increased by overexpressing SNAP-25 but decreased by suppressing SNAP-25 expression through its specific siRNA. The numbers above the columns indicate the cells taken for experiments. Data are from three independent experiments. *, p < 0.05, and **, p < 0.01 as compared with the control cells cotransfected with CFP and YFP or the cells indicated in the figure.
SNAP-25/Syntaxin 1A Complex Inhibits GAT-1 Reuptake Function

We further identified whether the SNARE proteins were functionally involved in the NO-induced inhibition of GAT-1 uptake function. As shown in Fig. 6B, both NOC-18- and NTG-induced inhibitions of GABA uptake in neurons were rescued by suppressing the expression of syntaxin 1A or SNAP-25 but not VAMP-2 through the transfection with their specific siRNAs, respectively. Moreover, suppressing syntaxin 1A and SNAP-25 expression simultaneously further rescued these inhibitions but suppressing the expression of three SNARE proteins did not enhance this rescue. The syntaxin 1A and SNAP-25 involvement in the inhibition of GABA uptake induced by NOC-18 was also confirmed in GAT-1-GFP transfected PC12 cells with overexpression or specific RNA interference.

FIGURE 6. SNAP-25/syntaxin 1A complex mediates NO-induced inhibition of GAT-1 uptake function. A, [3H]GABA uptake in primary cultured hippocampal neurons treated with different concentrations of NOC-18 and/or PTIO for different times. *, p < 0.05, and **, p < 0.01 as compared with the control neurons without treatment. B, [3H]GABA uptake in primary cultured hippocampal neurons treated with 10 μM NOC-18 or 500 μM NTG for 10 min. Specific siRNA for syntaxin 1A, SNAP-25, and VAMP-2 were transfected to down-regulate their expression. *, p < 0.05, and **, p < 0.01 as compared with the cells indicated in the figure. C, [3H]GABA uptake in GAT-1-GFP-transfected PC12 cells treated with 10 μM NOC-18 for 10 min. Specific RNA interference or overexpression of FLAG-syntaxin 1A and HA-SNAP-25 was used to down-regulate or up-regulate the expression of syntaxin 1A and/or SNAP-25. *, p < 0.05, and **, p < 0.01 as compared with the cells indicated in the figure. D, [3H]GABA uptake in HEK293 cells cotransfected FLAG-syntaxin 1A and/or HA-SNAP-25 with GAT-1. Ten micromoles of NOC-18 were used to treat the transfected HEK293 cells for different times. The ratios of transfected plasmids are indicated. Representative immunoblot (IB) shows the expression status of GAT-1, syntaxin 1A, and SNAP-25 in the cells with different transfected combinations. **, p < 0.01 as compared with the cells cotransfected FLAG-syntaxin 1A and HA-SNAP-25 with GAT-1 without NOC-18 treatment. Data in A–D are from three independent experiments, two wells per condition per experiment.

(10.3 ± 2.1%) (Fig. 5C). However, our data showed that the FRET efficiency between YFP-GAT-1 and CFP-syntaxin 1AΔH3 (5.94 ± 1.6%) was decreased to the level of negative control, indicating that the FRET efficiencies that we detected between YFP-GAT-1 and CFP-syntaxin 1A represent the specific interaction of GAT-1 with syntaxin 1A. On the other hand, the FRET efficiencies between CFP-syntaxin 1A and YFP-GAT-2 or YFP-GAT-3 were the same level as the negative control, further confirming that neither GAT-2 nor GAT-3 binds to syntaxin 1A (supplemental Fig. 1C). These data suggest that SNAP-25 potentiates surface-associated syntaxin 1A binding to GAT-1, resulting in enhanced syntaxin 1A-mediated inhibition of GAT-1 uptake function.
SNARE Complex Formation Promotes Surface-associated Syntaxin 1A Binding to GAT-1—Because the above studies demonstrated the functional relationship between the SNARE complex and GAT-1, in which syntaxin 1A and SNAP-25 are involved, we further detected dynamic distribution of GAT-1, syntaxin 1A, and SNAP-25 during the SNARE complex formation. In primary cultured hippocampal neurons, surface biotinylation assay showed that cell surface expression of GAT-1 (~132%, compared with nontreatment) or syntaxin 1A (~143%, compared with nontreatment) was increased by NOC-18 treatment (Fig. 7A). Furthermore, we transfected PC12 cells with GAT-1-GFP and examined the localization of GAT-1-GFP, endogenous syntaxin 1A, and SNAP-25. In GAT-1-GFP-transfected PC12 cells, GAT-1-GFP, endogenous syntaxin 1A, and SNAP-25 were largely dispersed in the cytoplasm (Fig. 7B, panels a–d), with 30–40% of cells exhibiting distinct ring staining (Fig. 7C). Following treatment with 10 μM NOC-18 for 10 min, the surface-associated labeling of GAT-1-GFP, endogenous syntaxin 1A, and SNAP-25 was greatly elevated (Fig. 7B, panels e–h), with 70–92% of cells exhibiting distinct ring staining (Fig. 7C). These data indicate that the SNARE complex formation promotes the translocation of GAT-1, syntaxin 1A, and SNAP-25 to the cell surface, providing the possibility for their functional interaction.

The mechanism of surface-associated SNARE proteins on the inhibition of GAT-1 uptake function was then investigated by FRET to characterize the interaction of surface-associated GAT-1 with syntaxin 1A affected by the SNARE complex formation. As shown in Fig. 8, FRET efficiency between surface-associated YFP-GAT-1 and CFP-syntaxin 1A was significantly increased by promoting the SNARE complex formation through treatment with NOC-18 (21.7 ± 2.3%, compared with the nontreatment 14.3 ± 1.8%). The increase was enhanced by overexpressing SNAP-25 (29.4 ± 1.9%) or was reduced by suppressing SNAP-25 expression through specific siRNA (14.5 ± 2.0%). These data indicate that SNAP-25 participates in the potentiation of surface-associated syntaxin 1A binding to GAT-1 during SNARE complex formation. All the above results suggest that the SNARE complex formation enhances the surface expression of SNAP-25/syntaxin 1A and GAT-1, and SNAP-25 binding to syntaxin 1A potentiates the physical interaction of surface-associated syntaxin 1A with GAT-1, resulting in significant inhibition of GAT-1 uptake function.

DISCUSSION

This study demonstrates that SNAP-25 binding to syntaxin 1A to form a target SNARE complex inhibits neurotransmitter GABA transporter GAT-1 uptake function. Overexpression of SNAP-25 inhibited the GABA uptake by GAT-1, and suppressing its expression increased GAT-1 uptake function in the presence of syntaxin 1A. The H3 domain of syntaxin 1A was required for the physical and functional interactions between SNAP-25 and GAT-1. SNAP-25 binding to syntaxin 1A greatly potentiated the physical interaction of syntaxin 1A with GAT-1.
and significantly enhanced the syntaxin 1A-mediated inhibition of GAT-1 uptake function. Furthermore, nitric oxide-induced SNARE complex formation resulted in the inhibition of neurotransmitter GABA uptake, in which the SNAP-25/syntaxin 1A complex formation promoted direct binding of syntaxin 1A to GAT-1. Our findings provide strong evidence that the SNAP-25 binding to syntaxin 1A during the SNARE complex formation functionally modulates neurotransmitter GABA reuptake.

It has been shown that syntaxin 1A inhibits GAT-1 reuptake function (20). Here we further demonstrate that SNAP-25 binding to syntaxin 1A to form target SNARE complex does not attenuate the association between syntaxin 1A and GAT-1 but instead potentiates their interaction, resulting in the enhanced inhibition of GAT-1 reuptake function. In addition, VAMP-2 binding to the target SNARE complex does not affect the inhibition. Our results suggest that under physiological conditions the SNARE complex is able to modulate GAT-1 reuptake function and the target SNARE complex serves as a key player.

The various forms of regulation affect transporter function in the following two ways: altering the number of transporters on the cell surface or the transport process. It has been reported that syntaxin 1A increases the cell surface expression of GAT-1 but decreases GAT-1 transport rate (21, 22). Our results indicate that SNAP-25 binding to syntaxin 1A further increases GABA transporter GAT-1 expression on the cell surface. The mechanism is not clear, but it is surmised that more GAT-1 may be brought to the cell surface from synaptic vesicle membranes during the membrane fusion, for SNAP-25 binding to syntaxin 1A to form t-SNARE complex promotes membrane fusion. Moreover, our data show that SNAP-25 binding to syntaxin 1A promotes the surface expression of syntaxin 1A and potentiates the physical interaction of syntaxin 1A with GAT-1 on the cell surface, resulting in inhibition of GABA uptake. Thus, SNAP-25-enhanced syntaxin 1A-mediated inhibition of the GAT-1 transport rate might result from more syntaxin 1A or SNAP-25/syntaxin 1A complex binding to GAT-1 on the cell surface.

Accumulating investigations demonstrate that the target SNARE syntaxin 1A physically interacts with and functionally regulates those multitransmembrane proteins such as neurotransmitter transporters (15, 16, 22) and ion channels (44, 45). It seems that syntaxin 1A broadly interacts with the membrane proteins. To further confirm the selectivity of interaction between syntaxin 1A and its binding membrane proteins, we demonstrate the specific interaction of the GABA transporter GAT-1 with syntaxin 1A and the glutamate transporter EAAC1 with syntaxin 1A by using communoprecipitation assay and FRET detection (supplemental Fig. 1 and supplemental Fig. 2A). In contrast, neither GABA transporter GAT-2 nor GAT-3 subtype (supplemental Fig. 1) nor glutamate transporter GLT1 nor GLAST subtype (supplemental Fig. 2A) interacts with syntaxin 1A. Moreover, the interaction of syntaxin 1A with G protein-coupled receptor δ-opioid receptor or β2-adrenergic receptor is not detected (supplemental Fig. 2B). Thus, the interaction between syntaxin 1A and multitransmembrane proteins could be selective.

Syntaxin 1A is revealed to interact with the inhibitory neurotransmitter glycine transporters GLYT1 and GLYT2 (15) and the major excitatory neurotransmitter glutamate transporter EAAC1 (16), resulting in the inhibition of their reuptake function. This study provides the possibility that the SNARE complex may functionally modulate those transporters. Indeed, the properties of two ion channels, Kv2.1 potassium channel and cystic fibrosis transmembrane regulator chloride channel, are reported to be directly affected by the target SNARE complex (23, 24), as mediated by their direct interaction with the H3 domain of syntaxin 1A (45, 46). Moreover, accumulating evidence also reveals that syntaxin 1A interacts with other membrane proteins and regulates their functions, and its H3 domain is the only reported binding site (45–47). Our results also indicate that the physical interaction between SNAP-25 and GAT-1 is mediated by the H3 domain of syntaxin 1A. Therefore, we can speculate that the function of those syntaxin 1A-binding proteins might be modulated by the SNARE complex, but it remains to be further investigated.

Previous studies demonstrate that the stimulation with nitric oxide, which promotes the SNARE complex formation (40) and induces neurotransmitter release (48, 49), inhibits the neurotransmitter GABA reuptake (41). Here we demonstrate that the nitric oxide-induced inhibition of GABA reuptake is rescued by attenuating the formation of the SNARE complex by suppressing the SNARE protein expression in primary cultured hippocampal neurons and PC12 cells. Thus attenuation of the
SNARE complex contributes to the rescued inhibition of GABA reuptake, further indicating that the SNARE complex mediates the coupling of neurotransmitter GABA release with the inhibition of GABA reuptake.

Postsynaptic neurotransmission is highly dependent on the levels of neurotransmitters in the synaptic clefts. Thus the inhibition of GABA reuptake by the SNARE complex during neurotransmitter release is pivotal for facilely achieving the level of neurotransmission, and a better understanding of this process is a priority but also to increase surface expression of the transporter to the cell surface. The physiological significance of increased GABA transporter surface expression by the target SNARE complex remains unknown. One possibility is that the complex is used not only to keep the transporter functionally suppressed until a time when GABA release from the synaptic vesicles is a priority but also to increase surface expression of the transporter. Thus the reuptake can be resumed quickly until the disassembly of the complex after GABA release. Therefore, neurotransmitter release and reuptake are coordinated together through the assembly and disassembly of the SNARE complex to maintain normal neurotransmission. Maladjustment of the coordination might result in abnormal neurotransmission, and a better understanding of the underlying mechanism may provide a great help to the related neurological disorders of the central nervous system.

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