A Second SNARE Role for Exocytic SNAP25 in Endosome Fusion

Yoshikatsu Aikawa,* Kara L. Lynch, Kristin L. Boswell, and Thomas F.J. Martin*

Department of Biochemistry, University of Wisconsin, Madison, WI 53706

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins play key roles in membrane fusion, but their sorting to specific membranes is poorly understood. Moreover, individual SNARE proteins can function in multiple membrane fusion events dependent upon their trafficking itinerary. Synaptosome-associated protein of 25 kDa (SNAP25) is a plasma membrane Q (containing glutamate)-SNARE essential for Ca^{2+}-dependent secretory vesicle–plasma membrane fusion in neuroendocrine cells. However, a substantial intracellular pool of SNAP25 is maintained by endocytosis. To assess the role of endosomal SNAP25, we expressed botulinum neurotoxin E (BoNT E) light chain in PC12 cells, which specifically cleaves SNAP25. BoNT E expression altered the intracellular distribution of SNAP25, shifting it from a perinuclear recycling endosome to sorting endosomes, which indicates that SNAP25 is required for its own endocytic trafficking. The trafficking of syntaxin 13 and endocytosed cargo was similarly disrupted by BoNT E expression as was an endosomal SNARE complex comprised of SNAP25/syntaxin 13/vesicle-associated membrane protein 2. The small-interfering RNA-mediated down-regulation of SNAP25 exerted effects similar to those of BoNT E expression. Our results indicate that SNAP25 has a second function as an endosomal Q-SNARE in trafficking from the sorting endosome to the recycling endosome and that BoNT E has effects linked to disruption of the endosome recycling pathway.

INTRODUCTION

The distribution and restriction of proteins to appropriate membrane compartments is essential for eukaryotic cell function. Compartments in the secretory pathway are dynamically maintained by a balance of protein sorting and export via vesicle formation and import of proteins by vesicle fusion. The basic mechanisms of vesicle formation and consumption are conserved with organelle-specific molecular components being drawn from the families of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), Rab and Sec 1 proteins (Bock *et al.*, 2001; Zerial and McBride, 2001).

The ~35 members of the coiled-coil SNARE protein family are classified as Q- (containing glutamate) or R (containing arginine)-SNAREs based on the residue present in a central hydrophilic layer of the coiled-coil domains (Fasshauer *et al.*, 1998; Weimbs *et al.*, 1998). Cellular membrane compartments contain a dynamic array of Q- or R-SNARE proteins characteristic of the compartment (Chen and Scheller, 2001). The specificity of donor–acceptor membrane fusion resides in part with the *trans* pairing of specific Q- and R-SNAREs into four-helix bundles that generally consist of a 3Q–1R complex (McNew *et al.*, 2000). Assembly of the four-helix bundle draws the membranes into apposition and promotes fusion (Weber *et al.*, 1998). Because SNARE proteins recycle after fusion, they are present in membrane intermediates in the recycling pathway, and individual SNARE proteins can participate in multiple fusion events by engaging distinct SNARE protein partners (Nichols and Pelham, 1998; Hay, 2001). It is important to map the trafficking itinerary of a given SNARE protein to determine potential steps at which it may function in fusion.

Synaptosome-associated protein of 25 kDa (SNAP25) is predominantly localized in the plasma membrane in neural and neuroendocrine cells where it participates in Ca^{2+}-dependent fusion of secretory vesicles with the plasma membrane by contributing two Q motifs to a complex with the plasma membrane Q-SNARE syntaxin 1 and the vesicular R-SNARE vesicle-associated membrane protein (VAMP) 2 (Sutton *et al.*, 1998; Jahn and Sudhof, 1999; Chen and Scheller, 2001). However, SNAP25 is also found in intracellular membranes (Duc and Catsicas, 1995; Marxen *et al.*, 1997; Morgans and Brandstatter, 2000; Tao-Cheng *et al.*, 2000; Aikawa *et al.*, 2006). We found that intracellular SNAP25 in PC12 cells localized to the recycling endosome (RE) and *trans*-Golgi network (TGN) compartments, which was dynamically maintained through endocytosis by a dynamin-independent, ADP-ribosylation factor 6 (ARF6)-regulated pathway (Aikawa *et al.*, 2006). Although syntaxin 1A, the plasma membrane Q-SNARE partner for SNAP25, was not endocytosed with SNAP25, SNAP25-containing primary endocytic vesicles merged into a sorting endosome (SE) compartment with the endosomal Q-SNARE syntaxin 13. SNAP25 was subsequently trafficked to the RE-TGN compartment from which it recycled back to the plasma membrane (Aikawa *et al.*, 2006).

This itinerary of SNAP25 through the endosome recycling pathway raised the question as to whether this SNARE protein has a second functional role in endosome fusion. The endocytic trafficking pathway consists of a series of hetero-

geneous and dynamic organelles that have been characterized through studies of recycling membrane proteins such as the transferrin (Tf) receptor (Gruenberg, 2001; Maxfield and McGraw, 2004). Tf is internalized by clathrin-dependent endocytosis into primary endocytic vesicles that merge into a peripheral cellular compartment of SEs. Tf receptor recycling back to the plasma membrane occurs either directly from the SE compartment or from an RE compartment located in the perinuclear centriolar region of the cell (Daro *et al.*, 1996; Sheff *et al.*, 1999; Gruenberg, 2001; Maxfield and McGraw, 2004). The mechanisms that govern trafficking between and fusion of endosomal compartments are incompletely understood. A SNARE complex consisting of syntaxin 7, syntaxin 8, vti1b, and VAMP 8 was characterized as mediating the homotypic fusion of late endosomes, and syntaxin 13 has been implicated in the homotypic fusion of early endosomes (McBride *et al.*, 1999; Antonin *et al.*, 2002). However, the roles of numerous other SNARE proteins distributed on endosomes (Chen and Scheller, 2001; Hay, 2001), and the specific fusion events they mediate in endosomal trafficking, remain to be characterized.

To assess the role of SNAP25 in endosome trafficking in PC12 cells, we expressed the light chain (LC) of botulinum neurotoxin E (BoNT E), a highly selective protease for SNAP25 family proteins (Blasi *et al.*, 1993; Schiavo *et al.*, 1993). We found that cleavage of SNAP25 arrested endosomal SNAP25 and syntaxin 13 trafficking from the SE to the RE compartment. This was accompanied by the inhibition of cargo (e.g., Tf) transit to the RE and by the destabilization of SNAP25/syntaxin 13/VAMP 2 complexes. A similar inhibition of endosome trafficking was observed in PC12 cells lacking SNAP25. Our results identify a second role for SNAP25 as a Q-SNARE at a trafficking step in the endosomal recycling pathway, and they imply that the effects of BoNT E on neural and neuroendocrine cells are not restricted to regulated vesicle exocytosis at the plasma membrane.

MATERIALS AND METHODS

Cell Culture and Transfections

PC12 cells were cultured and transfected with DNA constructs as described previously (Aikawa and Martin, 2003). Cells were plated onto polylysine-coated coverslips. In some experiments where indicated, PC12 cells were induced to differentiate with 50 ng/ml nerve growth factor (NGF) treatment for the indicated times. Hippocampal rat neurons were prepared from embryonic (E)18 rats and cultured in Neurobasal/B27 medium.

Plasmids

Expression vectors encoding GFP-Rab-11 were generously provided by T. Balla (National Institutes of Health, Bethesda, MD), human Tf receptor by F. R. Maxfield (Weill Medical College of Cornell University, New York, NY), green fluorescent protein (GFP)-Rab5, GFP-Rab5Q79L, and GFP-Rab5S34N by S. Ferguson (Robarts Research Institute, London, Ontario, Canada), myc-syntaxin 13 by H. Hirling (Laboratorie de Neurobiologie Cellulaire, Lausanne, Switzerland), and cDNAs encoding rat and human SNAP23 by P. A. Roche (National Institutes of Health). The N-terminal enhanced green fluorescent protein (EGFP) construct encoding wild-type mouse SNAP25b, rat and human SNAP23, and rat syntaxin 13 were prepared by PCR amplification of open reading frames and cloning into pEGFP-C1 (Clontech, Mountain View, CA). The following oligonucleotides were used as primers: 5′-GATCTCGAGACATGTATCGG-3′ and 5′-CGCGAAT-TCATTTAGAAGCAA-3′ to make the EGFP expression vector encoding rat syntaxin 13. The C-terminal EGFP and enhanced cyan fluorescent protein constructs encoding rat VAMP 2 were prepared by PCR amplification of the open reading frame and ligation into pEGFP-N1 (Clontech). Site-directed mutagenesis of the SNAP25 b gene to make the D179K mutant was also performed using PCR. Small hairpin RNA (shRNA) vectors for down-regulation of SNAP25 were constructed in the Expression Arrest pSM2 vector (Open Biosystems, Huntsville, AL) with 22mers that target nucleotides 335–356 (SNAP25 shRNA 1) and 473–494 (SNAP25 shRNA 2) of SNAP25b using designs based on the method of Paddison *et al.* (2004). All constructs were checked by sequencing.

Immunofluorescence and Endocytosis Assays

The uptake of 30 μg/ml Alexa568-conjugated Tf (Molecular Probes, Carlsbad, CA) was conducted in PC12 cells expressing human Tf receptor at 37°C in calcium-free Locke's solution for the indicated times. Cells were moved to an ice bath, and unbound Tf was removed by extensive washing with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde for 30 min at room temperature. The uptake of Alexa 647-cholera toxin B (CTxB) (Molecular Probes) and all immunofluorescence were conducted as described previously (Aikawa and Martin, 2003). The primary antibodies used were monoclonal anti-SNAP25 (Sternberger Monoclonals, Lutherville, MD), monoclonal anti-hemagglutinin (HA) (BAbCO, Richmond, CA), monoclonal anti-myc 9E10 (Covance, Princeton, NJ), and monoclonal anti-TGN-38 (K. Howell, University of Colorado, Denver, CO).

Immunoprecipitation and Western Blotting

Transfected cells were washed with PBS before harvesting in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and Boehringer complete protease inhibitor cocktail). Lysates were clarified by sedimentation, and supernatants were adsorbed with protein G-Sepharose beads before conducting 3-h incubations with protein G-Sepharose bead-immobilized SNAP25 monoclonal antibody. Beads were washed five times with lysis buffer and eluted in sample buffer containing 0.2 M dithiothreitol for analysis by Western blotting. Primary antibodies used for Western blotting were Rab 3A monoclonal (BD Transduction Laboratories, Lexington, KY); GFP monoclonal JL-8 (BD Biosciences, San Jose, CA), SNAP25 monoclonal (Sternberger Monoclonals), monoclonal anti-HA (BAbCO), and monoclonal anti-myc 9E10 (Covance).

Confocal Microscopy and Image Analysis

Cells were examined using an MRC-600 (Bio-Rad, Hercules, CA) or C1 (Nikon, Tokyo, Japan) laser scanning confocal microscope with a 63× oil immersion objective. For the determination of fluorescence intensities of SNAP25, syntaxin 13, and Tf in the perinuclear region, images were thresholded, and regions of interest were quantitated using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). Fifty to 150 cells were examined per condition, and significance of differences was assessed by Student's *t* test.

Quantification of Neurite Outgrowth

Transfected PC12 cells were fixed after 2 d of NGF treatment. Transfected (GFP-positive) cells were measured and grouped into different categories based on neurite length. Classes were defined as cells with processes of 0–0.5, 0.5–1, and 1–3 times cell body diameter. Three independent series of transfections were carried out and analyzed. Significance of differences was assessed by Student's *t* test.

RESULTS

Botulinum Neurotoxin E Inhibits the Endosomal Trafficking of SNAP25

SNAP25 dynamically recycles between the plasma membrane and a RE-TGN compartment in PC12 cells (Aikawa *et al.*, 2006). Although SNAP25 has a well characterized role as a SNARE protein in regulated secretory vesicle exocytosis at the plasma membrane (Banerjee *et al.*, 1996; Chen *et al.*, 1999; Jahn and Sudhof, 1999), a function for intracellular SNAP25 has not been identified. In vitro studies suggested that SNAP25 might function in homotypic early endosome fusion (Sun *et al.*, 2003), but this has not been assessed in vivo. To investigate an intracellular role for SNAP25, we expressed the light chain of BoNT E (BoNT E LC) in PC12 cells. This neurotoxin protease cleaves a 26 amino acid C-terminal fragment from SNAP25 and inactivates the protein for exocytic SNARE complex formation (Hayashi *et al.*, 1994; Banerjee *et al.*, 1996; Chen *et al.*, 1999). Under our expression conditions, BoNT E LC efficiently cleaved SNAP25 as indicated by the detection of a SNAP25 (1-180) fragment in the portion of cells that were transfected (Figure 1A, bottom row, asterisk) and by the complete conversion of GFP-SNAP25 coexpressed with BoNT E LC to the GFP-SNAP25 (1-180) fragment (Figure 1A, top row, asterisk).

BoNT E LC expression markedly altered the cellular distribution of SNAP25. In the absence of BoNT E LC expression (Figure 1C, top row), GFP-SNAP25 localized to the
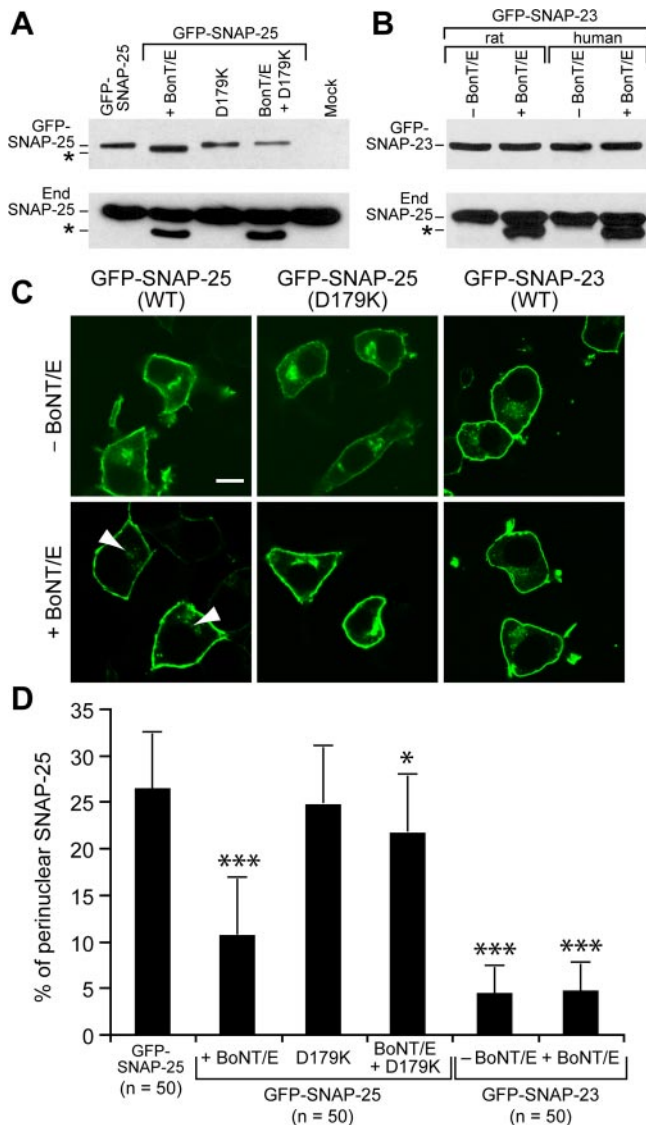
Figure 1. BoNT E expression inhibits the transit of SNAP25 to the recycling endosome. (A) Representative Western blot shows the effect of BoNT E LC expression on endogenous SNAP25 (marked End, bottom) and on GFP-SNAP25 or GFP-SNAP25 D179K (top). (B) Representative Western blot shows the effect of BoNT E LC expression on endogenous SNAP25 (bottom) or rat or human GFP-SNAP23 (top) detected with monoclonal GFP (top) or SNAP25 (bottom) antibodies. Asterisks designate the cleaved product SNAP25 (1-180). GFP-D179K SNAP25, rat SNAP-23 and human SNAP-23 were not cleaved by BoNT E. (C) PC12 cells were transfected with plasmids encoding GFP-SNAP25, GFP-SNAP25 D179K, or GFP-SNAP23 with or without BoNT E LC as indicated. The distribution of GFP-SNAP25 in a perinuclear RE pool was altered by coexpression of BoNT E (arrowheads). (D) The fluorescence intensity of GFP-SNAP25 or GFP-SNAP23 was quantitated in 50 randomly selected cells, and the percentage of perinuclear to total fluorescence was plotted as mean values ± SD; *** and * indicate $p < 0.001$ and $p < 0.05$, respectively, for comparison with control by t test. The perinuclear distribution of SNAP25 was restored in cells expressing GFP-SNAP25 D179K.

plasma membrane (~75%) as well as to a perinuclear region (~25%) that corresponds to the RE and TGN (Aikawa *et al.*, 2006). Expression of BoNT E LC shifted the intracellular distribution of GFP-SNAP25 to peripherally distributed endosomes and strongly reduced GFP-SNAP25 in the perinu-

clear RE-TGN compartment (Figure 1C, bottom row, arrowheads) by 62% (Figure 1D).

To assess whether the intracellular redistribution of SNAP25 was specifically dependent on its cleavage by BoNT E, we determined the cellular distribution of the BoNT E-resistant D179K mutant of GFP-SNAP25. Confirming our previous studies (Zhang *et al.*, 2002), cleavage of GFP-SNAP25 D179K by BoNT E LC was not detected (Figure 1A). Importantly, the perinuclear RE distribution of GFP-SNAP25 D179K was unaltered by coexpression of BoNT E LC (Figure 1C, middle, and D). Additional studies showed that endogenous SNAP25 detected by immunocytochemistry exhibited a similar redistribution as GFP-SNAP25 in BoNT E-expressing cells (our unpublished data). Overall, the results indicate that the cleavage of SNAP25 by BoNT E LC per se is responsible for the altered endosomal trafficking of SNAP25.

We next addressed whether SNAP23, a closely related homologue of SNAP25, was involved in the BoNT E-induced redistribution of SNAP25. SNAP23 exhibits ~58% sequence identity with SNAP25 (Radvichandran *et al.*, 1996; Wang *et al.*, 1997), and rat SNAP23 contains a conserved BoNT E scissile bond (Macaulay *et al.*, 1997; Vaidyanathan *et al.*, 1999). Because of this similarity, SNAP23 might function redundantly with SNAP25 in rat PC12 cells, and its cleavage by BoNT E could be involved in the altered trafficking of SNAP25. Because SNAP23 is found at very low levels in PC12 cells (Grant *et al.*, 1999), we expressed GFP-SNAP23. However, we found that neither rat nor human SNAP23 was cleaved in BoNT E LC-expressing PC12 cells (Figure 1B). Lack of cleavage of rat SNAP23 is consistent with the reported need to use high BoNT E concentrations for cleavage in vitro, whereas human SNAP23 is known not to be a substrate for BoNT E (Macaulay *et al.*, 1997; Vaidyanathan *et al.*, 1999). GFP-SNAP23 localized to the plasma membrane and to a perinuclear vesicular compartment in PC12 cells, but the perinuclear distribution of SNAP23, constituting ~3.5% of the protein, was completely unaffected by BoNT E LC expression (Figure 1C, right, and D). These results eliminated the possibility that the BoNT E-induced redistribution of intracellular SNAP25 was mediated through cleavage of SNAP23. The results indicate that SNAP23 does not function redundantly with SNAP25 in the endocytic pathway of PC12 cells but rather that SNAP23 and SNAP25 traffic independently.

BoNT E Destabilizes an Endosomal SNARE Complex and Disrupts Trafficking to the RE

The preceding studies indicated that BoNT E LC expression promotes the redistribution of SNAP25 from a perinuclear RE compartment into dispersed peripheral endosomes. To characterize the peripheral endosomes containing SNAP25 in BoNT E-expressing cells, we conducted colocalization studies for Rab5, which is involved in primary endocytic vesicle and SE fusion, and for Rab11, which is involved in RE trafficking, as well as for the TGN marker TGN-38. Endogenous SNAP25 exhibited substantial overlap with Rab5- and Rab11-positive endosomes as well as with TGN-38 (Figure 2, A and B). BoNT E LC expression led to a redistribution of Rab5- and Rab11-positive compartments to a peripheral location without altering the distribution of TGN-38 (Figure 2, A and B). SNAP25 maintained considerable colocalization with the Rab5- and Rab11-positive endosomes but not with TGN-38–containing compartments in the BoNT E LC-expressing cells (Figure 2, A and B, arrowheads). Thus, cleavage by BoNT E causes SNAP25 to remain in peripheral endosomes that likely comprise the SE compartment. This suggests that BoNT E cleavage inhibits
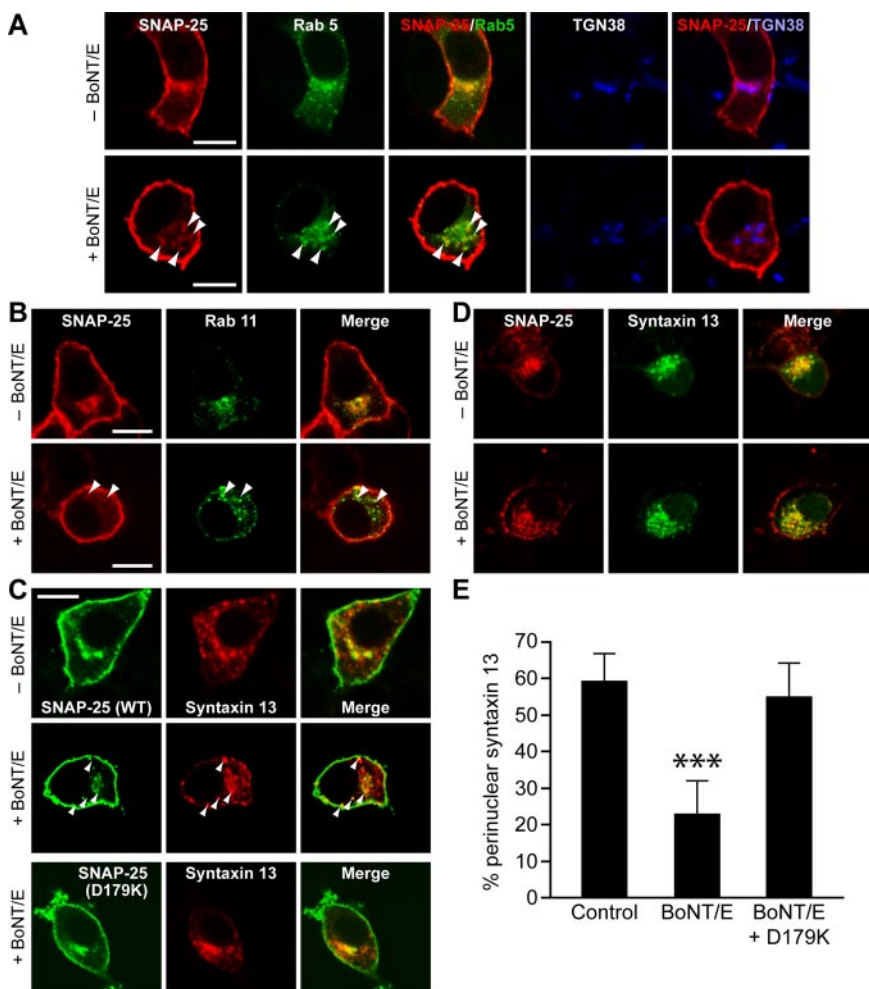
Figure 2. BoNT E expression inhibits the transit of syntaxin 13 to the VAMP 2-positive recycling endosome. (A and B) PC12 cells transfected with plasmids encoding GFP-Rab5, GFP-Rab11, or BoNT E LC as indicated were stained with rabbit SNAP25 and mouse TGN38 antibodies. Arrowheads indicate examples of overlap of SNAP25 (red) with Rab5 (green) or Rab11 (green) in cells expressing BoNT E. (C) PC12 cells transfected with plasmids encoding myc-syntaxin 13, GFP-SNAP25, or GFP-SNAP25 D179K and BoNT E LC as indicated were stained with monoclonal myc antibody. Arrowheads indicate examples of colocalization of syntaxin 13 (red) with SNAP25 (green). The perinuclear distribution of syntaxin 13 disrupted by BoNT E LC was restored by expression of SNAP25 D179K. (D) Hippocampal neurons transfected with plasmids encoding syntaxin 13-GFP (green) and BoNT E LC as indicated were stained with monoclonal SNAP25 antibody (red). (E) The immunofluorescence intensity of syntaxin 13 in images similar to those in C was quantitated in 50 randomly selected cells, and the percentage of perinuclear to total fluorescence was plotted as mean values $\pm$ SD; *** corresponds to $p < 0.001$.

trafficking of SNAP25 from the SE to the perinuclear RE compartment.

To further address this issue, we examined the distribution of syntaxin 13, a SNARE protein that localizes to peripheral SEs and to the perinuclear RE. Previous studies showed that SNAP25 and syntaxin 13 coimmunoprecipitate in a complex from cellular extracts (Prekeris *et al.*, 1998; Hirling *et al.*, 2000; Aikawa *et al.*, 2006). BoNT E LC expression led to a redistribution of myc-syntaxin 13 from the perinuclear RE compartment to SEs, which also contained SNAP25 (Figure 2C, top and middle rows, arrowheads). Expression of the BoNT E-resistant SNAP25 D179K protein restored the distribution of myc-syntaxin 13 to a perinuclear RE distribution (Figure 2C, bottom row). The ~60% perinuclear distribution of myc-syntaxin 13 in control cells was reduced to ~20% in cells expressing BoNT E LC, but it was restored to ~55% in cells coexpressing BoNT E LC and SNAP25 D179K (Figure 2E). Similar results were observed in the cell bodies of primary hippocampal neurons (Figure 2D), indicating a general role for SNAP25 in trafficking to the RE. These results demonstrate that the endosomal trafficking of syntaxin 13 is dependent upon SNAP25. Together, the results indicate that intact SNAP25 is required for the convergence of Rab5-, Rab11-, and syntaxin 13-containing SEs into the perinuclear RE compartment.

BoNT E cleavage of SNAP25 destabilizes exocytic SNARE complexes consisting of SNAP25, syntaxin 1A, and VAMP 2 (Hayashi *et al.*, 1994). We determined whether BoNT E cleav-

age has a similar effect on endosomal SNARE complexes. An endosomal SNARE complex consisting of SNAP25, syntaxin 13, and VAMP 2 has been isolated previously (Prekeris *et al.*, 1998; Hirling *et al.*, 2000). We found that expressed myc-syntaxin 13 could be isolated with immobilized SNAP25 antibody from PC12 cell detergent extracts, indicating an association of the two proteins (Figure 3A). BoNT E LC expression led to a strong decrease in myc-syntaxin 13 associated with SNAP25, indicating that the C terminus of SNAP25 was essential for this association (Figure 3A). We also determined the effect of BoNT E LC expression on the association of GFP-VAMP 2 with myc-syntaxin 13 (Figure 3B). GFP-VAMP 2 was coimmunoisolated with myc-syntaxin 13 in control cells, but this complex was strongly reduced in BoNT E LC-expressing cells (Figure 3B). Coexpression of SNAP25 D179K, and to a much lesser extent rat SNAP23, restored the association of GFP-VAMP 2 with myc-syntaxin 13 in BoNT E LC-expressing cells (Figure 3B). The results indicate that the association of syntaxin 13 with VAMP 2 and SNAP25 is dependent upon intact SNAP25. Thus, BoNT E cleavage destabilizes an endosomal SNARE complex that contains SNAP25.

SNAP25-containing primary endocytic vesicles merge with syntaxin 13 in SEs before the delivery of SNAP25 to the RE (Aikawa *et al.*, 2006; Figure 8). To determine the possible site at which SNAP25/syntaxin 13-containing endosomes encounter VAMP 2 for formation of an endosomal SNARE complex, we determined the distribution of VAMP 2 (Figure 3C). VAMP 2 in PC12 cells has previously been reported to localize to the RE
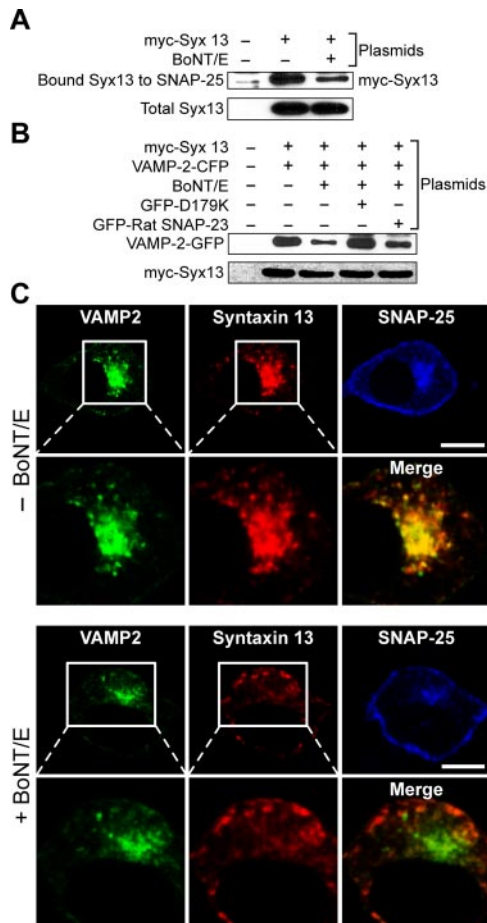
Figure 3. BoNT E expression destabilizes an endosomal SNARE complex. (A) PC12 cells were transfected with the indicated plasmids and SNAP25 immunoprecipitates were prepared from detergent lysates for analysis by Western blotting for myc-syntaxin 13. myc-Syntaxin 13 in SNAP25 immunoprecipitates decreased in cells expressing BoNT E LC. (B) PC12 cells were transfected with the indicated plasmids and myc-syntaxin 13 immunoprecipitates were prepared from detergent lysates for analysis by Western blotting for VAMP 2-GFP. VAMP 2-GFP in myc-syntaxin 13 immunoprecipitates was decreased in BoNT E LC-expressing cells but was restored in cells coexpressing GFP-SNAP25 D179K. (C) PC12 cells transfected with plasmids encoding VAMP 2-GFP, myc-syntaxin 13, and BoNT E LC as indicated were fixed for staining with myc and SNAP25 antibodies. VAMP 2 (green) colocalized with syntaxin 13 (red) and SNAP-25 (cyan) in the perinuclear RE in control cells (top), whereas syntaxin 13 was dispersed to a peripheral location away from perinuclear VAMP 2 in BoNT E LC-expressing cells (bottom).

(de Wit *et al.*, 1999; Martinez-Arca *et al.*, 2003). Consistent with this, we found that VAMP 2 exhibited substantial colocalization with syntaxin 13 in a perinuclear compartment in control cells (Figure 3C, top). Whereas BoNT E LC expression markedly shifted the distribution of syntaxin 13 to peripheral SEs, it had no effect on the localization of VAMP 2 in the RE (Figure 3C, bottom). These results indicate that SNAP25 transit to the RE is not blocked in BoNT E LC-expressing cells due to alterations in the morphology of the RE. Instead, the results suggest that SNAP25/syntaxin 13-containing SEs encounter VAMP 2 upon entry into the RE. SNAP25 cleavage by BoNT E blocks SE convergence into the RE (Figure 2, A and C) and inhibits endosomal SNARE complex formation (Figure 3, A and B). Together, the results suggest that BoNT E blocks the trafficking

of SNAP25 in the SE compartment to the perinuclear RE compartment by destabilizing endosomal SNARE complexes.

SNAP25 Is Required for General Endosome Trafficking to the RE

The preceding studies indicated a requirement for SNAP25 in its own endosomal transit from SEs to the RE. Because previous studies (Aikawa *et al.*, 2006) showed that SNAP25-containing primary endocytic vesicles merge with those derived from the clathrin-dependent pathway at an earlier stage, we determined whether the endosomal trafficking of Tf was also dependent on SNAP25 function. We incubated control and BoNT E LC-expressing cells with Alexa 568-conjugated Tf for 5, 15, and 30 min. Although SNAP25-containing primary endocytic vesicles do not colocalize with Tf-labeled primary endocytic vesicles (Aikawa *et al.*, 2006), expressed syntaxin 13-GFP exhibited substantial colocalization with Tf in 5-min incubations (Figure 4A, arrowheads) as reported previously (Prekeris *et al.*, 1998). In 15- and 30-min incubations, Tf entered the perinuclear RE in control cells as reported previously (Daro *et al.*, 1996; Sheff *et al.*, 1999), which contained syntaxin 13-GFP (Figure 4A, arrowheads). However, in BoNT E LC-expressing cells, Tf was not transported into the perinuclear RE but remained associated with peripherally distributed SEs containing syntaxin 13-GFP (Figure 4A). BoNT E LC expression reduced Tf transit into the perinuclear RE by ~40% (Figure 4B). Similar Tf uptake studies in cells coexpressing GFP-SNAP25 and BoNT E LC showed that the peripherally distributed SEs into which Tf was delivered in 15- and 30-min incubations also contained GFP-SNAP25 (Figure 4C, arrowheads). These results reveal that Tf trafficking to the RE is blocked by SNAP25 cleavage. The inhibition of endosomal trafficking by BoNT E seems to be at a step beyond the merger of Tf-, SNAP25-, and syntaxin 13 in the SE and before transit to the RE. This implies that BoNT E may generally inhibit cargo traffic to the RE compartment.

We examined the endocytic trafficking of CTxB, which binds plasma membrane GM1 glycosphingolipids and is trafficked into the Golgi by a clathrin-independent endocytic pathway (Nichols *et al.*, 2001). In cells that expressed the constitutive Rab5^{Q79L} mutant, Alexa 643-conjugated CTxB accumulated in the expanded SE vacuole in a 10-min incubation, which indicates that CTxB internalization in PC12 cells is mediated via Rab5-positive endosomes (Figure 4D, top, arrowheads). In control cells, the transit of Alexa 643-conjugated CTxB to the perinuclear RE and Golgi was observed after 15-min incubations (Figure 4D, bottom row, arrowheads). In contrast, in cells expressing BoNT E LC, Alexa 643-conjugated CTxB was retained in peripheral SEs that contained SNAP25 (Figure 4E, top row). Accumulation of CTxB in the perinuclear region was reduced by 45% in BoNT E LC-expressing cells (Figure 4F). Expression of the BoNT E-resistant SNAP25 D179K mutant, however, restored the trafficking of Alexa 643-conjugated-CTxB to the perinuclear RE-TGN compartment (Figure 4E, bottom row). These results complement those obtained for Tf uptake and indicate that SNAP25 functions generally in the endocytic pathway at a point beyond the convergence of multiple endocytic pathways in the SE compartment. SNAP25 function seems to be required for transit of cargo from SEs to the perinuclear RE compartment.

SNAP25 Down-Regulation Similarly Disrupts Endosomal Trafficking

The preceding studies indicate that BoNT E cleavage of SNAP25 inhibits endosome trafficking from the SE-to-RE compartments. Inhibition of trafficking might result from a loss-of-function of SNAP25 such as the inability to participate in the formation of SNARE complexes involving syntaxin 13 on SEs
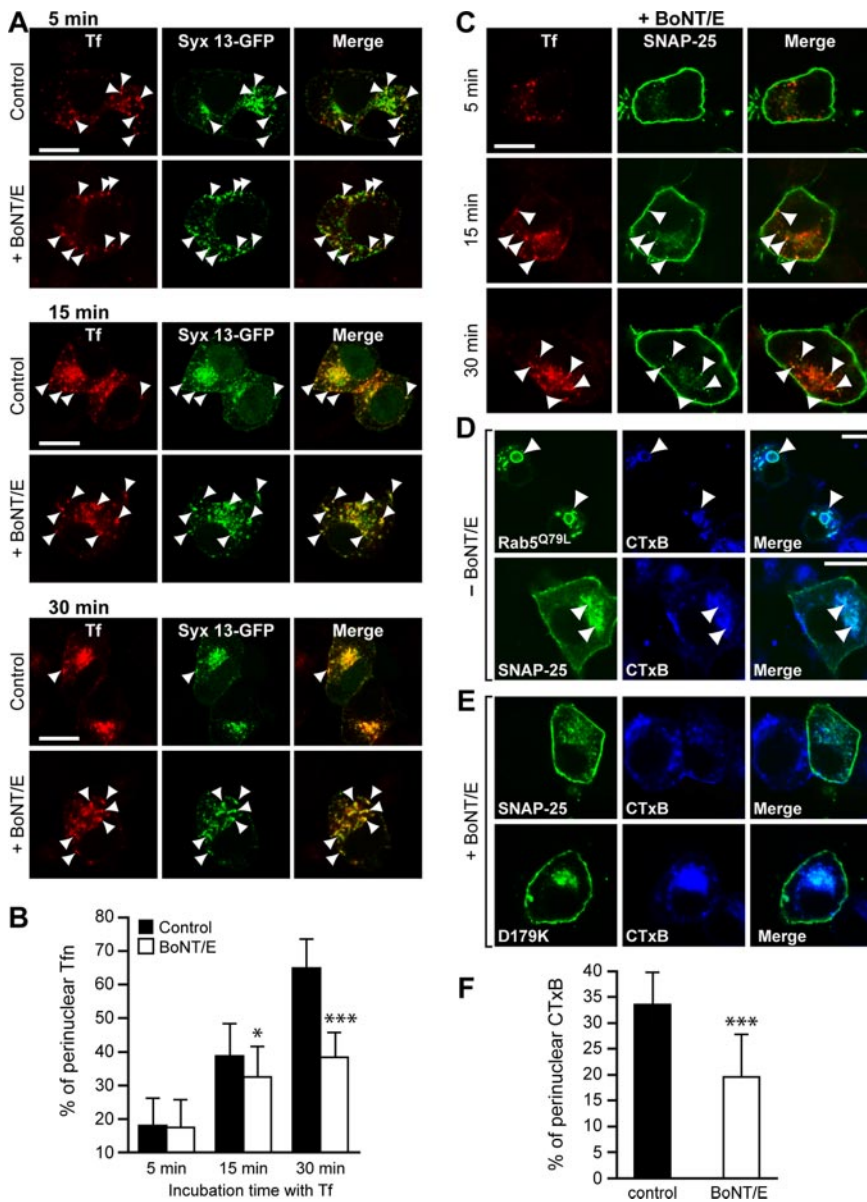
Figure 4. BoNT E inhibits the transit of Tf and CTxB to the recycling endosome. (A) PC12 cells expressing syntaxin 13-GFP, human Tf receptor, and BoNT E LC where indicated were incubated with Alexa 568-conjugated Tf for 5, 15, and 30 min. Extensive colocalization of syntaxin 13-GFP (green) with Tf (red) was observed (arrowheads). In cells expressing BoNT E LC, Tf and syntaxin 13-GFP were retained in peripheral endosomes. (B) The fluorescence intensity of Alexa568-conjugated Tf was quantitated in 50 randomly selected cells, and the percentage in the perinuclear RE to total fluorescence was plotted as mean values ± SD; *** denotes $p < 0.001$ and *$p < 0.05$ for comparison with control by t test. (C) PC12 cells expressing GFP-SNAP25, human Tf receptor, and BoNT E LC were incubated with Alexa568-conjugated Tf. Arrowheads indicate examples of colocalization of Tf (red) with GFP-SNAP25 (green). (D and E) PC12 cells expressing GFP-Rab5^{Q79L} or GFP-SNAP25 or GFP-SNAP25 D179K were incubated with Alexa643-conjugated CTxB on ice for 30 min, washed, and incubated at 37°C for 15 min to internalize the CTxB. CTxB localizes to the vacuolar SE compartment in Rab5^{Q79L}-expressing cells (D, top). CTxB uptake into a perinuclear RE/TGN compartment is inhibited in BoNT E LC-expressing cells (E, top) and is restored in cells expressing GFP-SNAP25 D179K (E, bottom). Arrowheads indicate examples of colocalization of SNAP25-GFP (green) and CTxB (cyan). (E) The fluorescence intensity of CTxB was quantitated in 50 randomly selected cells, and the percentage of perinuclear to total fluorescence was plotted as mean values ± SD, with *** denoting $p < 0.001$ for BoNT E-expressing versus control cells.

and VAMP 2 on the RE. Alternatively, the BoNT E LC cleavage fragments of SNAP25 might act in some manner as dominant inhibitors of endosome trafficking. To assess these alternative explanations and to independently determine whether SNAP25 is required for endosome trafficking, we tested the impact of down-regulating SNAP25 in PC12 cells. One of several siRNA-expressing plasmids (shRNA 2) was found to largely eliminate the expression of GFP-SNAP25 from a cotransfected plasmid and to down-regulate endogenous SNAP25 in proportion to transfection efficiency (Figure 5, A and B). Cells transfected with the shRNA 2 plasmid exhibited no immunoreactive SNAP25 and syntaxin 13 was dispersed from an RE to peripheral SEs (Figure 5C) similar to the effect of BoNT E expression (Figure 3C). The effective down-regulation of SNAP25 protein levels was accompanied by the inhibition of Tf uptake into the perinuclear RE compartment (Figure 5D). Instead, Tf remained associated with the peripheral SEs containing syntaxin 13 (Figure 5D). The inhibition of Tf and syntaxin 13 trafficking to the RE by shRNA 2 was highly significant (Figure 5, E and F). These

results indicate that abolishing SNAP25 expression has an impact on endosomal trafficking similar to that of BoNT E expression. Thus, it is the loss of SNAP25 function rather than the dominant inhibitory effects of cleavage fragments that mediates BoNT E inhibition of endosome trafficking. Overall, the results strongly support the conclusion that SNAP25 is required for SE-to-RE trafficking.

SNAP25 Is Not Required for Rab5-Dependent Homotypic Early Endosome Fusion

Recent in vitro studies suggested that SNAP25 was required for homotypic early endosome fusion in HeLa cell membrane fractions (Sun *et al.*, 2003). However, SNAP25 was not found to colocalize with primary endocytic vesicles loaded with Tf in 5-min incubations in PC12 cells (Aikawa *et al.*, 2006). Moreover, BoNT A, a neurotoxin protease that also cleaves SNAP25, did not inhibit homotypic early endosome fusion in membrane preparations from PC12 cells (Holroyd *et al.*, 1999). Thus, it was unclear whether SNAP25 was required for homotypic early endosome fusion events in
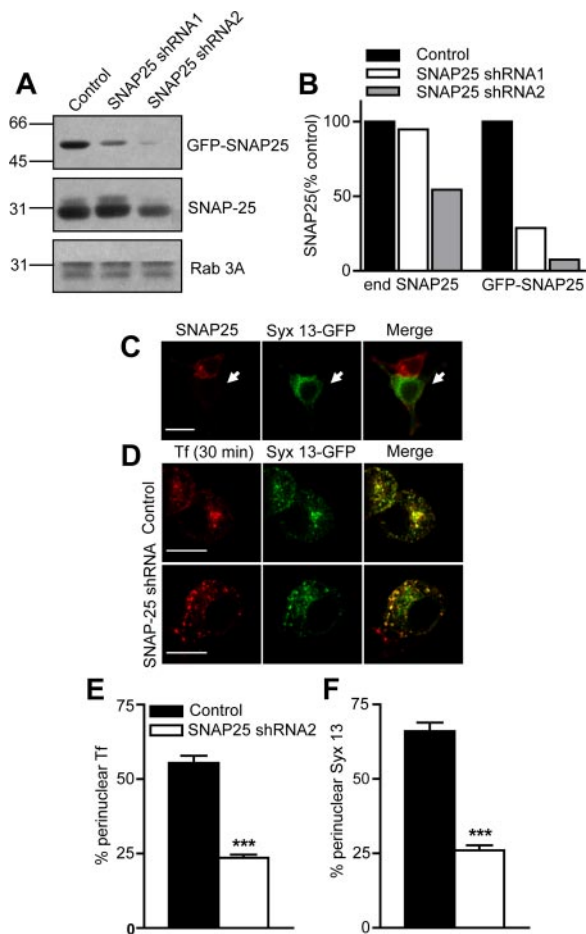
Figure 5. Endosome trafficking is inhibited in SNAP25-deficient PC12 cells. (A) PC12 cells transfected with GFP-SNAP25 and control or SNAP25 shRNA plasmids 1 or 2 were subjected to Western blotting with GFP, SNAP25, or Rab 3A antibodies as indicated. SNAP25 shRNA 2 expression extensively down-regulated GFP-SNAP25 and down-regulated endogenous SNAP25 levels in proportion to transfection efficiencies without affecting Rab 3A levels. (B) Densitometry was used to quantitate relative levels of endogenous (end) SNAP25 and GFP-SNAP25 in A. (C) PC12 cells transfected with SNAP25 shRNA 2 plus plasmid encoding syntaxin 13-GFP were stained with SNAP25 antibody. Syntaxin 13-GFP was dispersed into peripheral endosomes in cells lacking SNAP25. (D) PC12 cells were transfected with control or SNAP25 shRNA 2 plasmid and with plasmids encoding syntaxin 13-GFP (green) and transferrin receptor. Cells were imaged after a 30-min incubation with Alexa568-conjugated Tf (red). Bars, 10 μm. (E) The fluorescence intensity of Tf was quantitated in 50 randomly selected cells (similar to D), and the percentage of perinuclear to total fluorescence was plotted as mean values $\pm$ SD, with *** denoting $p < 0.001$ for cells transfected with SNAP25 shRNA 2 versus control plasmid. (F) The fluorescence intensity of syntaxin 13-GFP was quantitated in 50 randomly selected cells (similar to D), and the percentage of perinuclear to total fluorescence was plotted as mean values $\pm$ SD, with *** denoting $p < 0.001$ for cells transfected with SNAP25 shRNA 2 versus control plasmid.

PC12 cells in vivo. To address this, we determined whether BoNT E inhibits the homotypic early endosome fusion that is promoted by expression of the constitutive Rab5^{Q79L} mutant, which results in the formation of enlarged SE vacuoles (Stenmark *et al.*, 1994; Roberts *et al.*, 1999). Although BoNT E LC effectively inhibited the interaction of syntaxin 13 with VAMP 2 in Rab5^{Q79L}-expressing cells (Figure 6A), it did not prevent the formation of syntaxin 13-containing SE vacuoles

induced by Rab5^{Q79L} expression (Figure 6B). These results indicate that intact SNAP25 is not required for Rab5-dependent homotypic early endosome fusion.

A second approach for assessing the role of SNAP25 in endosomal trafficking would be to prevent its internalization from the plasma membrane or its trafficking to the SE compartment. To do so, we took advantage of our finding that ARF6 regulates SNAP25 endocytosis (Aikawa *et al.*, 2006). Overexpression of the constitutive-active ARF6^{Q67L} mutant promotes extensive SNAP25 internalization into a vacuolar compartment but inhibits SNAP25 transit out of this compartment to syntaxin 13-containing SEs (Aikawa *et al.*, 2006). In the current studies, we found that the vacuolar accumulation of SNAP25 induced by ARF6^{Q67L} expression was not affected by BoNT E LC coexpression (Figure 6C, arrowheads), which indicates that ARF6^{Q67L} arrests SNAP25 trafficking at an early stage after internalization. Expression of ARF6^{Q67L} was found to prevent Tf accumulation in the RE compartment (Figure 6E, arrowheads), which is similar to the effect of BoNT E LC expression (Figure 4A). However, ARF6^{Q67L} expression failed to affect Rab5^{Q79L}-induced SE vacuole formation (Figure 6D, middle row). These results also indicate that SNAP25 is not required for Rab5-dependent homotypic early endosome fusion.

Previous studies showed that the dominant-negative ARF6^{T27N} mutant interfered with SNAP25 internalization from the plasma membrane (Aikawa *et al.*, 2006), and this was confirmed in the current work (Figure 6D, bottom row). Expression of the ARF6^{T27N} mutant also inhibited Tf accumulation in the RE compartment (Figure 6E, bottom row) consistent with its inhibition of SNAP25 internalization. However, ARF6^{T27N} expression did not affect Rab5^{Q79L}-induced SE vacuole formation (Figure 6D, bottom row). Collectively, the results indicate that SNAP25 is not required for Rab5-dependent homotypic early endosome fusion and are consistent with the conclusion that SNAP25 is required for a later step in the endosome recycling pathway.

Recycling SNAP25 Is Required for Neurite Outgrowth

In addition to the well characterized role of SNAP25 in regulated vesicle exocytosis, SNAP25 has been shown to be essential for neurite outgrowth in PC12 cells (Osen-Sand *et al.*, 1993, 1996). The basis for a SNAP25 requirement in neurite outgrowth remains unclear. Although it was suggested that SNAP25 might mediate constitutive vesicle exocytosis and membrane addition to the plasma membrane (Tang, 2001), we found no effect of BoNT E LC expression on the constitutive trafficking of a VSV-G-GFP fusion protein to the plasma membrane in PC12 cells (our unpublished data). Our results showing that SNAP25 is essential for a step in the endosome recycling pathway suggested the alternative explanation that neurite outgrowth may use membrane recycled through the SNAP25-dependent endosome recycling pathway. To address this possibility, we determined the effect of altering endosomal SNAP25 levels on NGF-induced neurite outgrowth in PC12 cells.

We confirmed a requirement for SNAP25 in NGF-induced neurite outgrowth by measuring the total length of neuritic processes in BoNT E LC-expressing compared with control cells. Neurite lengths were measured and grouped into three categories: 1) very short (<0.5 cell body diameter), 2) intermediate length (0.5–1 cell body diameter), and 3) long (>1 cell body diameter). BoNT E LC expression was found to decrease the number of cells with long neurites (27 versus 56% for control, $p < 0.001$) and to increase the number of cells with short neurites (58 versus 28% for control, $p < 0.01$) (Figure 7A). The results were similar to those reported pre-
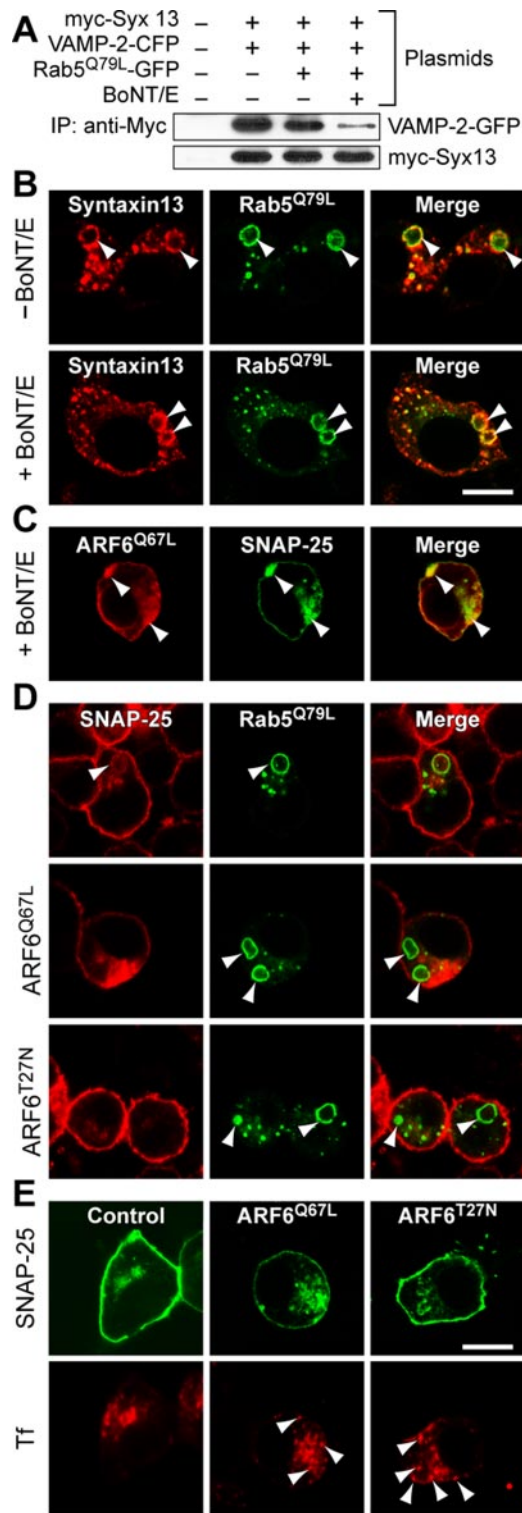
Figure 6. SNAP25 is not required for Rab5-dependent homotypic early endosome fusion. (A) Myc antibody immunoprecipitates were prepared from detergent lysates of cells transfected with indicated plasmids and analyzed by Western blotting for VAMP 2-GFP. Coimmunoprecipitation of VAMP-2-GFP with myc-syntaxin 13 was decreased by BoNT E LC expression even in Rab5^{Q79L}-expressing cells. (B) PC12 cells transfected with plasmids encoding myc-syntaxin 13, GFP-Rab5^{Q79L} without (top) or with (bottom) BoNT E LC (bottom) were stained with monoclonal myc antibody. Syntaxin 13 (red) localized to Rab5^{Q79L}-promoted SE vacuoles (green) in control and BoNT E

viously using BoNT E (Martinez-Arca *et al.*, 2000) or BoNT A (Osen-Sand *et al.*, 1996; Grosse *et al.*, 1999; Morihara *et al.*, 1999) to inactivate SNAP25.

To alter the endosomal distribution of SNAP25, we conducted similar studies in cells expressing wild-type ARF6, ARF6^{Q67L}, or ARF6^{T27N}. Expression of wild-type ARF6, which colocalized with SNAP25 in NGF-treated cells in the cell body and in neuritic growth cones (Figure 7C, top row, arrowheads), had no effect on neurite outgrowth (our unpublished data). In contrast, the expression of ARF6^{T27N}, which inhibits SNAP25 internalization, resulted in fewer cells with long neurites and more cells with short neurites (Figure 7B). Similar, but stronger effects, on neurite outgrowth were observed in cells expressing ARF6^{Q67L} (Figure 7B), which colocalized with SNAP25 in endosomal compartments and in cytoplasmic vesicles in growth cones (Figure 7C, middle row) that lacked syntaxin 13 (our unpublished data). High levels of ARF6^{Q67L} expression traps SNAP25 in an endosomal vacuole before its merge with syntaxin 13-containing SEs (Aikawa *et al.*, 2006). Cells that expressed high levels of ARF6^{Q67L} exhibited SNAP25-containing endosomal vacuoles that were devoid of syntaxin 13 (our unpublished data) and exhibited very short neurites (Figure 7C, bottom row). These results indicate that blocking the normal endosomal trafficking of SNAP25 by expressing ARF6 mutants has a strong inhibitory effect on NGF-induced neurite outgrowth. The results are consistent with the proposal that neurite outgrowth relies upon a recycling pool of membrane that is dependent upon the function of SNAP25 in endosome trafficking.

DISCUSSION

SNAP25 Functions in Endosome Fusion

The key conclusion of this study is that SNAP25, a SNARE protein required for regulated secretory vesicle exocytosis at the plasma membrane in neural and neuroendocrine cells, has a second SNARE role in the endosome recycling pathway. The site at which SNAP25 is required for endosome fusion corresponds to the trafficking of endocytosed cargo from SEs to the RE (Figure 8). The finding that SNAP25 functions in the endosome recycling pathway provides a rationale for understanding the dynamic trafficking itinerary of SNAP25 (Aikawa *et al.*, 2006) and clarifies the multiple cellular effects of botulinum neurotoxins that target SNAP25 for proteolysis.

SNAP25 is internalized by a dynamin-independent, ARF6-regulated endocytic pathway that maintains the intracellular pool of SNAP25 (Aikawa *et al.*, 2006). SNAP25-containing primary endocytic vesicles merge with those derived from clathrin-dependent endocytosis to enter an early endosomal compartment referred to as the SE (Figure 8). In the SE, SNAP25 colocalizes with syntaxin 13, which functions in trafficking

LC-expressing cells (arrowheads). (C) PC12 cells transfected with plasmids encoding ARF6^{Q67L} and BoNT E LC were stained with SNAP25 antibody. SNAP25 localization (green) to ARF6^{Q67L}-promoted vacuoles (red) was not prevented in cells expressing BoNT E LC. (D) PC12 cells transfected with plasmids encoding GFP-Rab5^{Q79L} (green) with either control or ARF6^{Q67L}- or ARF6^{T27N}-encoding plasmids were fixed and stained with monoclonal SNAP25 antibody (red). Arrowheads indicate the Rab5^{Q79L}-induced SE vacuole. (E) PC12 cells transfected with plasmids encoding GFP-SNAP25 (green), human transferrin receptor and ARF6^{Q67L} or ARF6^{T27N} as indicated were incubated with Alexa 568-conjugated Tf (red) for 30 min. Alexa 568-Tf accumulated in the perinuclear RE in control cells but not in cells expressing ARF6^{Q67L} or ARF6^{T27N} (arrowheads).
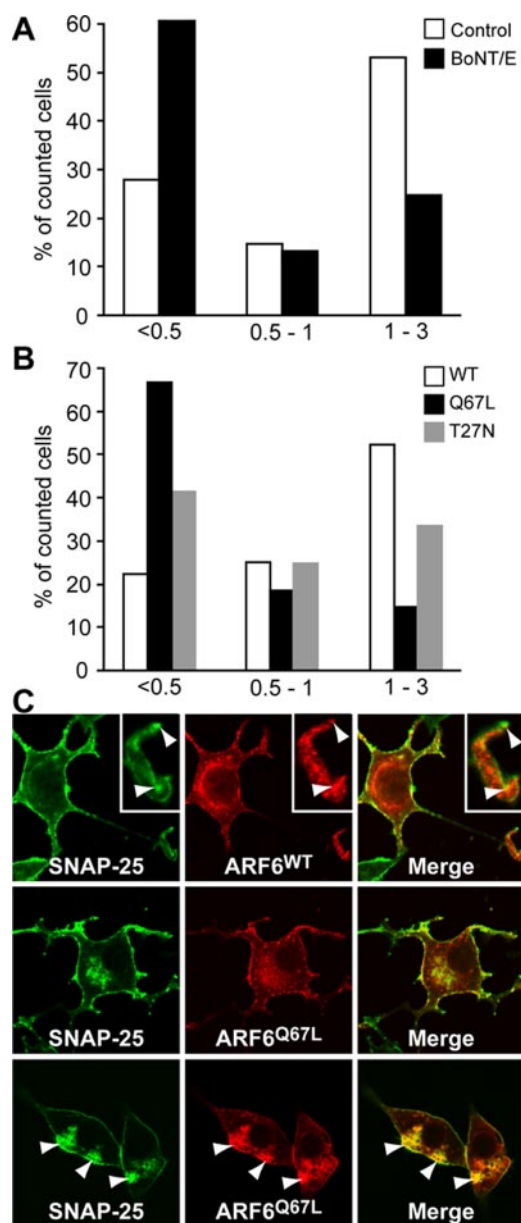
Figure 7. Role of endosome recycling pathway in NGF-induced neurite extension. (A) PC12 cells expressing GFP (white) or GFP plus BoNT E LC (black) were treated with NGF for 2 d to induce neurite outgrowth. Neurite length in transfected cells was measured and grouped into the indicated categories. (B) PC12 cells expressing ARF6[WT] (white), ARF6[Q67L] (black), or ARF6[T27N] (gray) were treated with NGF for 2 d to induce neurite outgrowth. (C) PC12 cells expressing ARF6[WT] or ARF6[Q67L] were treated with NGF for 2 d to induce neurite outgrowth. SNAP25 (green) and ARF6 (red) were localized by immunofluorescence. Arrowheads indicate examples of ARF6 colocalization with SNAP25. Cells expressing high levels of ARF6[Q67L] (bottom row) exhibited vacuoles containing SNAP25 and very short neurites.

within the endosome recycling pathway (Prekeris *et al.*, 1998; Hirling *et al.*, 2000). The exact relationship between SEs and the RE compartment in cells has not been established. It is thought that the tubulovesicular SE compartment sorts vacuolar cargo to late endosomes, whereas tubular portions deliver recycling membrane cargo to the RE (Maxfield and McGraw, 2004). It is unclear whether tubular SE elements give rise to the RE com-
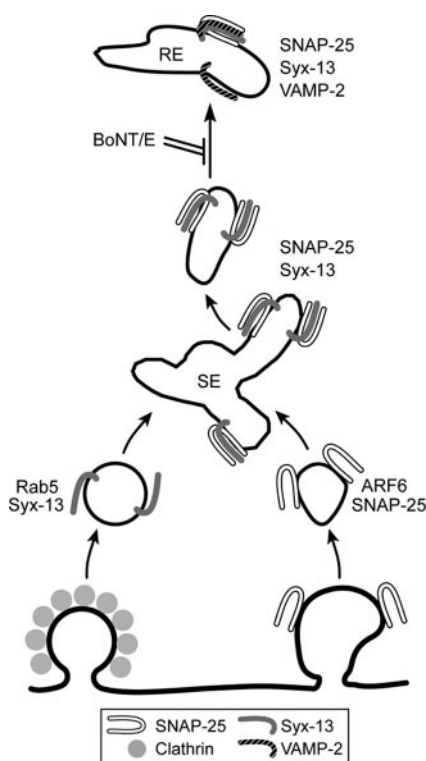
Figure 8. Summary of the trafficking itinerary of SNAP25. SNAP25 is internalized into tubulovesicular endosomes from the plasma membrane by a dynamin-independent, ARF6-regulated pathway (right), which is distinct from the clathrin-dependent pathway of endocytosis (left) (Aikawa *et al.*, 2006). Expression of ARF6[T27N] blocks SNAP25 internalization, whereas ARF6[Q67L] expression enhances SNAP25 internalization but blocks merger of SNAP25 endosomes into SEs. SNAP25 endosomes lack syntaxin 1 but merge with syntaxin 13 in the SE (Aikawa *et al.*, 2006). Tubular elements derived from the SE either coalesce to form the RE compartment or fuse with a preexisting RE compartment. The trafficking of cargo from peripheral SEs to the perinuclear RE requires intact SNAP25 and is inhibited by BoNT E. Syntaxin 13/SNAP25-containing SE elements encounter VAMP 2 in the RE to form SNARE complexes consisting of SNAP25/syntaxin 13/VAMP 2.

partment or transit to and fuse with a preexisting RE compartment (Sheff *et al.*, 2002). Our results are compatible with either possibility but indicate that SNAP25 function is required for this trafficking step in the endosomal recycling pathway (Figure 8). BoNT E LC expression inhibited the transit of SNAP25/syntaxin 13-containing SEs and internalized cargo (Tf and CTxB) to the perinuclear RE compartment. VAMP 2, which is predominantly located in the RE compartment, coisolates with SNAP25/syntaxin 13-containing SNARE complexes (Prekeris *et al.*, 1998; Hirling *et al.*, 2000; Sun *et al.*, 2003; Aikawa *et al.*, 2006). BoNT E LC expression disrupted this endosomal SNARE complex. Such complexes isolated from PC12 cell detergent extracts may represent accumulated *cis*-SNARE complexes in the RE that arise from *trans*-complexes that mediate fusion of SEs into the RE (Figure 8). That BoNT E LC expression resulted in the destabilization of these SNARE complexes is consistent with an essential role for SNAP25 at this trafficking step for fusion events involving SNAP25/syntaxin 13 on SEs and VAMP 2 in the RE (Figure 8).

The involvement of SNAP25 in intracellular membrane fusion events in the endosomal pathway had not previously been established in vivo. Previous in vitro studies in HeLa

cell homogenates showed that BoNT E inhibited early endosome fusion (Sun *et al.*, 2003; Yan *et al.*, 2004). Homotypic early endosome fusion requires Rab5 and the Rab5 effectors EEA1 and Rabaptin-5, which interact with syntaxin 13 (McBride *et al.*, 1999). To determine whether SNAP25 is essential for Rab5-dependent homotypic early endosome fusion in PC12 cells, we expressed the Rab5^{Q79L} mutant, which induces the formation of an enlarged SE vacuole (Stenmark *et al.*, 1994; Roberts *et al.*, 1999). In PC12 cells, this enlarged SE compartment contained SNAP25 and syntaxin 13 as expected. However, coexpression of BoNT E LC failed to alter the Rab5^{Q79L}-induced enlargement of the SE compartment, indicating that SNAP25 is not essential for Rab5-dependent homotypic early endosome fusion. The results are consistent with a primary role for SNAP25 at a later step in the endosome recycling pathway.

Multiple Roles for SNAP25 as a SNARE Protein

SNAP25 is an essential participant in at least two membrane fusion steps in neuroendocrine cells: in regulated secretory vesicle exocytosis and in SE transit to the RE in the endosome recycling pathway. This is consistent with the isolation of SNAP25 in two distinct SNARE complexes containing syntaxin 1A or syntaxin 13 with VAMP 2 (Prekeris *et al.*, 1998; Sutton *et al.*, 1998). These results provide additional evidence for the concept that SNARE proteins are multifunctional and can engage in several combinatorial interactions that mediate distinct membrane-specific fusion reactions (Nichols and Pelham, 1998; Hay, 2001).

Sorting steps during membrane budding reactions allow distinct combinations of SNARE proteins to distribute into separate membrane domains where they participate in fusion reactions characteristic of the individual donor and acceptor membranes. SNAP25 endocytosis segregates it from syntaxin 1A but SNAP25-containing primary endocytic vesicles merge with syntaxin 13-containing endosomes in the SE compartment (Aikawa *et al.*, 2006). Whereas homotypic SE fusion seems to involve syntaxin 13 (McBride *et al.*, 1999), the present as well as previous work (Holroyd *et al.*, 1999 but see Sun *et al.*, 2003) indicates that homotypic SE fusion does not involve SNAP25. Instead, SNAP25 is involved in later possibly heterotypic fusion steps in the recycling pathway where SNAP25/syntaxin 13-containing tubular elements from SEs encounter VAMP 2 in the RE compartment. Syntaxin 13 also seems to function in the recycling from the RE back to the plasma membrane, whereas SNAP25 does not (Prekeris *et al.*, 1998). Overall, the sorting and trafficking itinerary of an individual SNARE protein would confer its ability to adopt multiple roles in several distinct donor–acceptor membrane interactions.

The siRNA-mediated down-regulation of SNAP25 inhibited endosomal trafficking in a manner similar to BoNT E LC expression. These results indicate that the loss-of-function in SNAP25 results in endosome trafficking deficits. Is it possible that the disruption of endosomal trafficking in BoNT E LC-expressing or SNAP25-deficient cells is secondary to the inhibition of Ca^{2+}-dependent vesicle exocytosis? This is unlikely for a number of reasons. First, we observed endosome trafficking deficits in cells that were not stimulated to exhibit regulated vesicle exocytosis. Constitutive vesicle exocytosis does not seem to involve SNAP25. Second, the expression of tetanus toxin LC, which inhibits regulated vesicle exocytosis, did not result in altered endosome trafficking (our unpublished data). Third, PC12 cells that are incapable of regulated exocytosis due to synaptotagmin depletion do not exhibit altered endosomal SE to RE trafficking (K. Lynch and T. Martin, unpublished findings). These findings indicate that the inhibition of regulated vesicle exocytosis per se does not secondarily affect endosomal trafficking to the RE.

Consequences for Understanding Botulinum Neurotoxin Effects

BoNTs E and A are highly specific proteases for SNAP25 that inhibit Ca^{2+}-triggered vesicle exocytosis in neurons and neuroendocrine cells (Blasi *et al.*, 1993; Schiavo *et al.*, 1993). However, treatment by these toxins has other effects, which include the inhibition of neurite outgrowth (Osen-Sand *et al.*, 1996; Grosse *et al.*, 1999; Morihara *et al.*, 1999; Martinez-Arca *et al.*, 2000) and receptor trafficking (Lan *et al.*, 2001; Washbourne *et al.*, 2004). These effects of neurotoxins had been interpreted to indicate a role for SNAP25 in constitutive vesicle exocytosis at the plasma membrane (Tang, 2001), but we were unable to detect any inhibitory effects of BoNT E LC expression on constitutive VSV-G trafficking to the plasma membrane. Our finding that SNAP25 function in the endosome recycling pathway is inhibited by BoNT E cleavage provides an alternative interpretation that some effects of BoNT E or BoNT A result from the inhibition of membrane recycling. We found that BoNT E LC expression, which inhibits neurite outgrowth, disrupted the endosomal trafficking of both SNAP25 and syntaxin 13 (but not VAMP 2). Conversely, the overexpression of syntaxin 13 or SNAP25, but not other SNARE proteins, was reported to enhance neurite outgrowth in PC12 cells (Tang, 2001).

To assess the role of endosomal SNAP25 in neurite outgrowth, we altered the internalization of SNAP25 or its transit into the SE by expressing ARF6 mutants (Aikawa *et al.*, 2006). Blocking SNAP25 internalization or its transit to the SE strongly decreased neurite outgrowth in PC12 cells similar to a recent study on retinal neurons (Albertinazzi *et al.*, 2003). Although the results are consistent with a proposed key role for endosomal SNAP25 in facilitating membrane recycling for neurite outgrowth, it is also likely that ARF6 mutants act on a variety of effectors that contribute to this outcome. ARF6 proteins regulate recycling in the endocytic pathway to deliver membrane to regions of plasma membrane remodeling (Radhakrishna and Donaldson, 1997; D'Souza-Schorey *et al.*, 1998; Prigent *et al.*, 2003). Overexpression of the Sec10 subunit of the exocyst complex, recently reported to be an effector of ARF6 (Prigent *et al.*, 2003), was found to block neurite outgrowth in PC12 cells (Vega and Hsu, 2001). These studies delineate a role for ARF6 in the polarized membrane insertion of recycling surface components internalized via the endocytic pathway and indicate a central role for recycling membrane in neurite outgrowth.

Although our studies indicated that BoNT E inhibits trafficking to the RE compartment in hippocampal neurons, the consequences of this for neuronal trafficking need further investigation. Development of the nervous system, including axon and dendrite extension, is normal in the SNAP25$^{-/-}$ mouse (Washbourne *et al.*, 2002). It may be the case that SNAP23 functions redundantly with SNAP25 in neurons to compensate for some functions in the SNAP25$^{-/-}$ mouse, although this was not found to be the case for PC12 cells where SNAP23 exhibited a distinct intracellular trafficking itinerary.

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