E08-04-0344 Holz

Supplementary Figure 1. Co-expression of BoNT E-resistant SNAP25 constructs does not inhibit BoNT E-induced proteolysis of wild type SNAP25.

A. HEK293T cells were co-transfected with SNAP25(WT), citSNAP25(D179K) with or without BoNT E light chain. Cells were analyzed by SDS-PAGE and immunoblotted with antibodies to SNAP25. Each condition was assayed in duplicate. The presence of citSNAP25 (D179K) caused no decrease in sensitivity of wild-type SNAP25 to BoNT E. B. Soluble SN2(D179K) does not inhibit BoNT E-induced proteolysis of endogenous SNAP25 in permeabilized chromaffin cells. Chromaffin cells were permeabilized and then incubated with various concentration of recombinant BoNT E in the presence or absence of 100 μM recombinant citSN2. Cells were analyzed by SDS-PAGE and immunoblotted with antibodies to GFP (upper) and SNAP25 (lower). The lower bands in the anti-SNAP25 panel are SNAP25 cleavage products. The ability of BoNT E to cleave endogenous SNAP25 was not altered by the presence of citSN2.

Supplementary Figure 2. HEK293T cell plasma membrane FRET images in TIRFM. Donor-stimulated emission was measured with TIRFM in the plasma membrane of HEK293T cells co-expressing CssYs and syntaxin. CssYs was excited at 442 nm, and emissions at 465-500 nm (cerulean alone) and 580-605 nm (citrine and cerulean) were detected on a CCD camera with a beamsplitter. The images were processed as described in Experimental Procedures into cerulean emission (A), citrine emission (B), and the pixel-by-pixel ratio of citrine/cerulean emission (donor-stimulated emission, C). An epifluorescence image of the field is presented in D. (E) The relative frequency of citrine/cerulean ratios at each pixel in the upper right cell.

Supplementary Figure 3. Chromaffin cell plasma membrane FRET images in TIRFM. Donor-stimulated emission was measured with TIRFM in the plasma membrane of chromaffin cells expressing CssYs with or without syntaxin. Both cells were co-transfected with BoNT E. Methods were identical to those in supplementary Figure 2. The images were processed into cerulean emission (A, D), citrine emission (B, E), and the pixel-by-pixel ratio of citrine/cerulean emission (donor-stimulated emission, C, F).

Supplementary Figure 4. FRET in intact HEK cells measured with confocal microscopy. HEK293T cells were transfected with plasmids encoding CssY or CssYs with or without syntaxin. FRET was measured by the increase in donor emission upon acceptor bleaching. On average, neither CssY nor CssYs showed significant FRET. Co-expression of syntaxin had little effect on FRET with CssY (4% increase), but profoundly enhanced FRET with CssYs (20% increase).

SUPPLEMENTAL MATERIAL

Construction of fluorescently-labeled probes.

CitSNAP25(D179K), cerSNAP25(D179K): A sense primer containing an *EcoRI* site consensus sequence (GG AAT TCC ATG GCC GAG GAC GCA GAC) and anti-sense primer containing a *BamHI* site (CGG GAT CC TTA ACC ACT TCC CAG CAT) were used to amplify full length SNAP25. This construct was subcloned into citrine-C1 or cerulean-C1.

CssYs [cer- SNAP25 (1-100)-SNAP25 (81-141)-cit-SNAP25(142-206)]: A sense primer containing a *KpnI* site (GAC GGT ACC ATG CTA GGA AAA TTC TGC)consensus sequence and anti-sense primer containing a *BamHI* site (CGG GAT CC TTA ACC ACT TCC CAG CAT) were used to amplify SNAP25 (81-141)-cit-SNAP25(142-206). This construct was subcloned into cer-SNAP25(1-100).

CssYs180 [cer- SNAP25 (1-100)-SNAP25 (81-141)-cit-SNAP25(142-180)]: A sense primer containing a *KpnI* site (GAC GGT ACC ATG CTA GGA AAA TTC TGC)consensus sequence and anti-sense primer containing a *BamHI* site (GGT GGA TCC CCT CTT AAT CTG GCG ATT CTG) were used to amplify SNAP25 (81-141)-cit-SNAP25(142-180) from sYs (list below). This construct was subcloned into cer-SNAP25(1-100).

CssY [cer-SNAP25 (1-100)-SNAP25 (81-141)-cit]: A sense primer containing a *KpnI* site (GAC GGT ACC ATG CTA GGA AAA TTC TGC)consensus sequence and anti-sense primer containing a *BamHI* site (GGT GGA TCC CTT GTA CAG CTC GTC CAT GCC) were used to amplify SNAP25 (81-141)-cit-SNAP25(142-206). This construct was subcloned into cer-SNAP25(1-100).

Cs [CerSN1; cerSNAP25 (1-100)]: A sense primer containing an *EcoRI* site consensus sequence (GG AAT TCC ATG GCC GAG GAC GCA GAC) and anti-sense primer containing a *KpnI* site

(CGC GGT ACC AGCATC ACT GGA TTT AAG) were used to amplify residues 1-100 of SNAP25. This construct was subcloned into cerulean-C1.

sYs [CitSN2; SNAP25 (81-141)-cit-SNAP25(142-206)]: A sense primer containing an *NheI* site (CCG CTA GCG ATG CTA GGA AAA TTC TGC GGG)consensus sequence and anti-sense primer containing an *AgeI* site (GACCGGTAGGGCATCGTTTGTTACCCT) were used to amplify residues 81-141 of SNAP25. This construct was subcloned into cit-SNAP25(142-206).

MycSNAP25 (81-206): A sense primer containing an *EcoRI* site (CGA ATT CGG CTA GGA AAA TTC TGC GGG) consensus sequence and anti-sense primer containing a *KpnI* site (CGC GGT ACC TTA ACC ACT TCC CAG CAT) were used to amplify residues 81-206 of SNAP25. This construct was subcloned into pCMV-myc vector.

Myc-sYs-SNAP25 [myc-SNAP25(1-141)-cit-SNAP25(142-206, D179K)]: A sense primer containing an *NheI* site (CGG CTA GCG ATG GCA TCA ATG CAG AAG CTG ATC) consensus sequence and anti-sense primer containing an *AgeI* site (GAC CGG TAG GGC ATC GTT TGT TAC CCT GCG GAT) were used to amplify residues 1-141 of myc-SNAP25. This construct was subcloned into cit-SNAP25(142-206) construct.

HAcerSNAP25 (D179K): A sense primer containing an *SalI* site (CGG TCG ACC ATG GTG AGC AAG GGC GAG GAG CTG) consensus sequence and anti-sense primer containing a *KpnI* site (CGC GGT ACC TTA ACC ACT TCC CAG CAT) were used to amplify cerulean-SNAP25(D179K). This construct was subcloned into pCMV-HA vector.

SNAP-25(D179K) rescues secretion by substituting for cleaved, endogenous SNAP25.

We verified that proteins with the D179K mutation do not interfere with the cleavage of endogenous SNAP25 in two ways (sFigure 1). Panel A. Wild-type SNAP25 (lanes 1-8) was co-expressed in HEK293T cells with (lanes 3-6) or without (lanes 1,2,7,8) citSNAP25(D179K), and

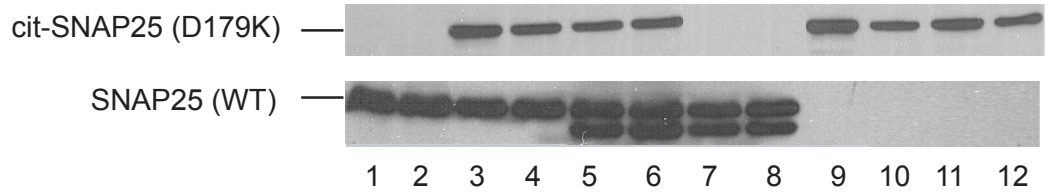
with (lanes 5-8) or without (lanes 1-4) BoNT E light chain. Lanes 9-12 had citSNAP25(D179K) without wild-type SNAP25, with (lanes 11,12) or without (lanes 9,10) BoNT E. After 24 hours, the cells were harvested and the proteins separated by SDS-PAGE and examined by immunoblotting. Wild-type SNAP25 was equally sensitive to BoNT whether or not citSNAP25(D179K) was present (panel A). In panel B, cultured bovine chromaffin cells were permeabilized, and then exposed to various concentrations of recombinant BoNT E with or without soluble recombinant citSN2(D179K) for 8 min. Again, cells were harvested, and the proteins separated by SDS-PAGE and blotted. Equivalent amounts of endogenous SNAP25 were cleaved at all concentrations of BoNT E in the presence and absence of citSN2(D179K). In neither assay did the presence of the D179K-bearing protein interfere with cleavage of toxin-sensitive SNAP25. Recombinant citSN2(D179K) caused no decrease in the sensitivity of endogenous SNAP25 to BoNT E.

FRET in plasma membrane-bound SNAP25 constructs detected in living cells by total internal fluorescence microscopy (TIRFM). Total internal fluorescence microscopy was used to measure FRET selectively in the plasma membrane in living cells sFigure 2. HEK293T cells expressing CssY or CssYs, alone or in combination with syntaxin or syntaxin and VAMP, were excited at 442 nm and emission was detected at 465-500 nm (cerulean emission alone) and 580-605 nm (citrine and cerulean emissions). The citrine signal was calculated by subtracting the spillover from cerulean. FRET was measured in the images by the pixel-to-pixel ratio of the citrine/cerulean emissions. Images from cells co-expressing CssYs and syntaxin and a histogram of the relative frequency of citrine/cerulean ratios at each pixel in the upper right cell are shown in sFigure 2E. Similar experiments were performed with chromaffin cells (sFigure 3).

FRET in intact cells measured with confocal microscopy. We investigated whether co-expression of syntaxin with CssY and CssYs could cause changes in FRET in living cells as detected by confocal microscopy. We used increased donor fluorescence upon acceptor bleaching as a measure of FRET. FRET efficiency was calculated as the resulting increase in cerulean emission as a percentage of the post-bleach cerulean emission. The technique was established using a soluble FRET standard, CFP-L16-cit (Mattheyses et al., 2004). As expected, transiently-expressed CFP-L16-cit had a 30 % FRET efficiency (data not shown). Co-transfection of unlinked CFP and cit gave 0 % FRET efficiency. The results from individual cells expressing CssY or CssYs with or without syntaxin were plotted in sFigure 3. On average, neither CssY nor CssYs showed significant FRET in living HEK293 cells. Similar to the FRET results in isolated membranes (Figure 2) and with TIRF (Figure 5), co-expression of syntaxin had little effect on bleaching FRET seen with CssY, but profoundly enhanced the FRET of CssYs.

Supplementary Fig. 1

A.

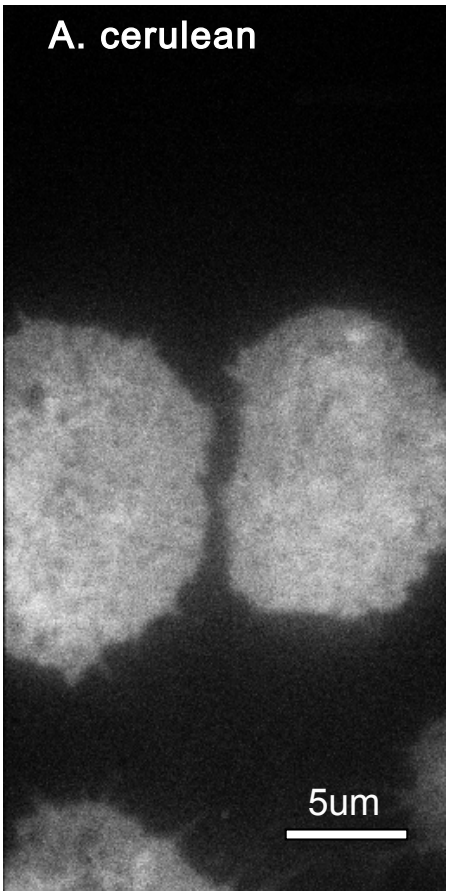


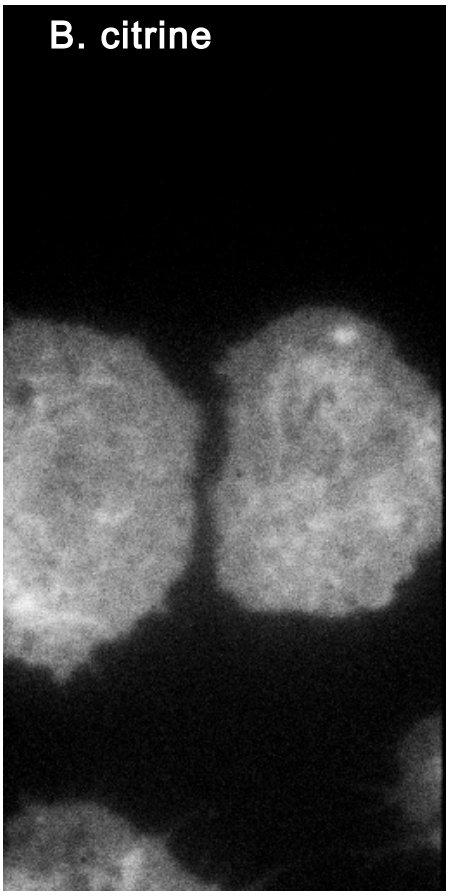
BoNT E	-	-	+	+	-	+
cit-SNAP25 (D179K)	-	+	+	-	+	+
SNAP25(WT)	+	+	+	+	-	-

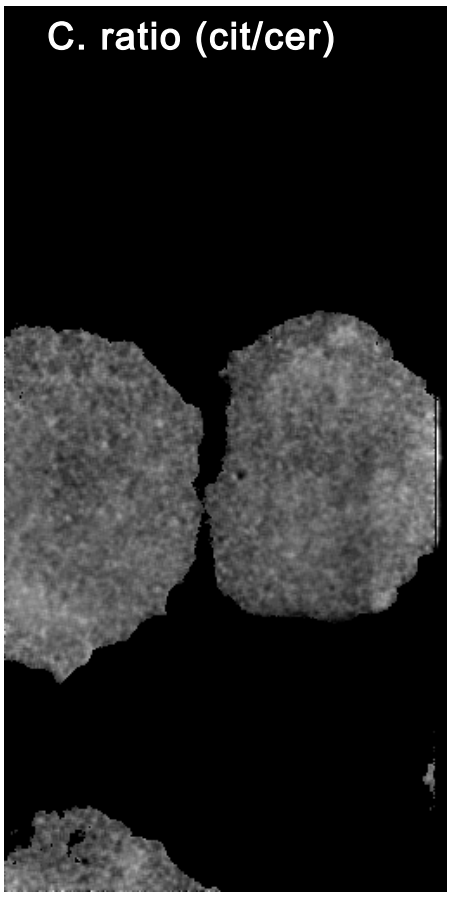
B.

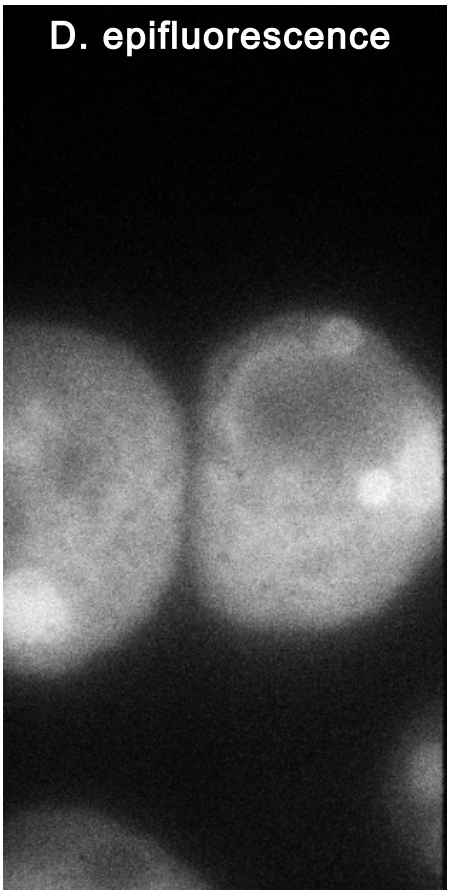
BoNT E	0 nM		10 nM		30 nM		300 nM	
cit-SN2	-	+	-	+	-	+	-	+

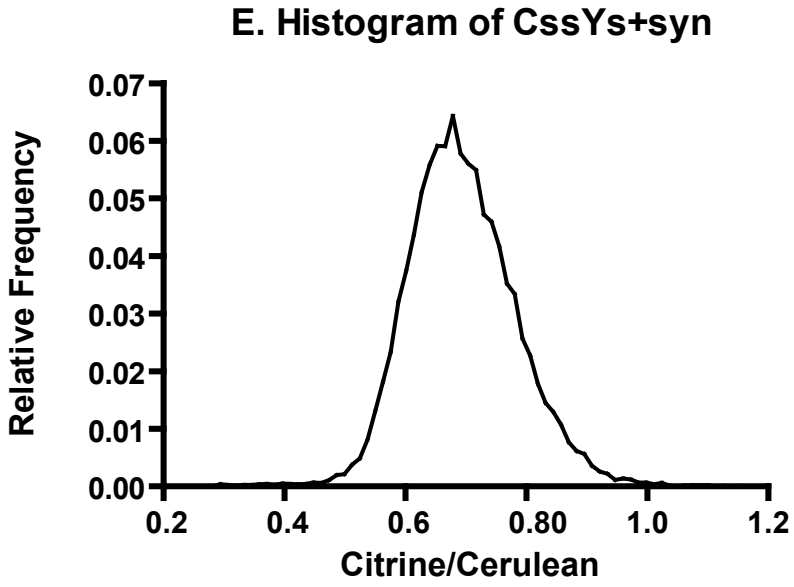
Supplementary Fig. 2





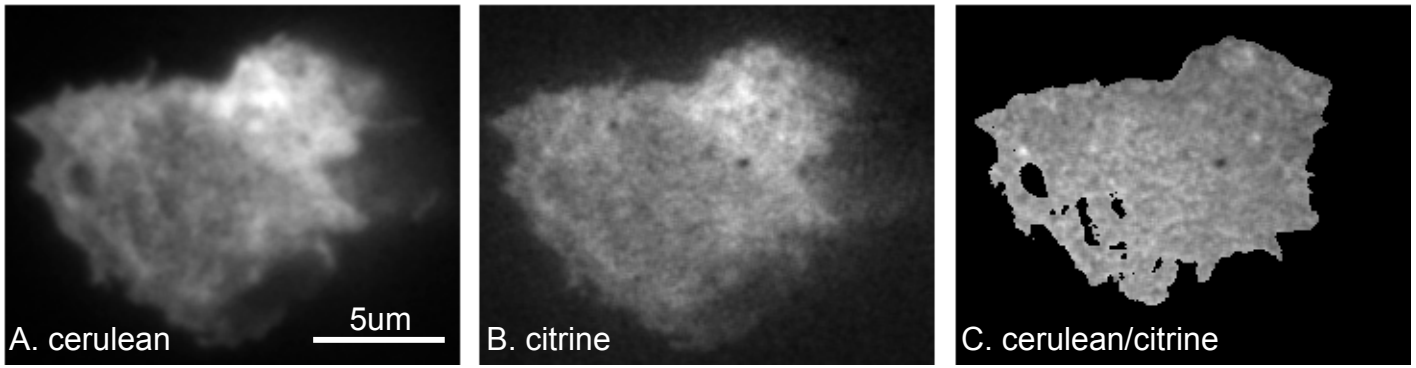




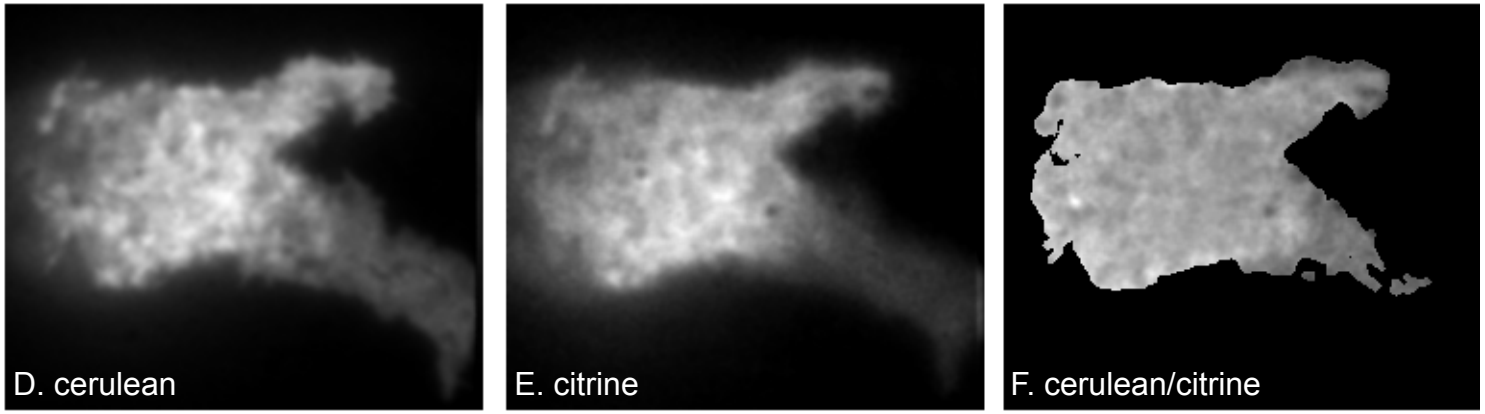


Supplementary Figure 3

CssYs



CssYs+Syn



Supplementary Fig. 4