Figure S1. Cleavage of VAMP, Syx, and SNAP-25 family proteins by En-LC. Related to Figure 1.

(A) GST-tagged VAMP2 (33-86) was incubated with or without En-LC. Samples were analyzed by SDS-PAGE and Coomassie Blue staining.

(B) GST-tagged VAMP2 (33-86) was incubated with En-LC. Samples were then analyzed by LC-MS/MS mass spectrometry.

(C) HA-tagged VAMP1, 3, 5, 7, and 8, Myc-tagged Sec22b and Ykt6, HA-tagged Syx 1A, 1B, 2, 3, 4, SNAP-23, SNAP-25, SNAP-29, and non-tagged VAMP4 were expressed in HEK293 cells via transient transfection. Cell lysates were incubated with En-LC (2 μM, 1 h) and subjected to immunoblot analysis using the HA antibody, Myc antibody, or a VAMP4 antibody. Actin served as a loading control. En-LC cleaved VAMP1, VAMP3, Syx 1B, Syx 4, SNAP-25, and SNAP-23.

(D) Recombinant GST-VAMP2, Syx 1B, and Syx 4 were purified and incubated with En-LC (0.1 μM) for the indicated time. Samples were analyzed by SDS-PAGE and Coomassie Blue staining.

(E) Recombinant Syx 1A, Syx 1B, and Syx 4 were incubated with high concentrations of En-LC (6 μM) for the indicated time. Samples were analyzed by SDS-PAGE and Coomassie Blue staining. The cleavage products were marked with asterisks.

(F, G) The cleavage products described in panel E were analyzed by MS-LS/LS for Syx 1B (panel F, treated with trypsin) and Syx 4 (panel G, treated with chymotrypsin, as the toxin cleavage site contains a lysine). The eluted peptide peaks from the HPLC column are plotted over running time (RT, *X* axis). The upper panel showed the peak exists only in full-length

proteins, while the lower panel showed the peak only found in the cleavage products. The corresponding peptide sequences are shown in each panel.

(H) The sequence alignment of Syx 1A, 1B and Syx 4 around the BoNT/En cleavage site. BoNT/C cleavage site is also labeled.

Figure S2. En-LC cleaves SNAP-25 at a novel site and generating full-length BoNT/En via sortase-mediated ligation. Related to Figure 1.

(A) Recombinant SNAP-25 and SNAP-23 were incubated with En-LC (0.1 μM) for the indicated time. A small portion of SNAP-25 appears to be cleaved and a cleavage product was marked by an asterisk. Cleavage of SNAP-23 was not detectable under this assay condition. These results suggest that En-LC hardly cleaves recombinant SNAP-25 and SNAP-23 *in vitro*. It is possible that the optimal cleavage of SNAP-25 and SNAP-23 by BoNT/En may require a proper cellular membrane environment.

(B) Immunoprecipitation assays were carried out using the SNAP-C antibody, for lysates of HEK293 cells transfected with HA-tagged SNAP-25 followed by incubation with En-LC *in vitro* for 1 h (left side), and for lysates of rat cortical neurons exposed to En-LC-H_N ligated to BoNT/A-H_C (which facilitates the entry of En-LC-H_N into neurons, see panel G) in the medium for 24 h (right side). The antibody pulled down both full-length SNAP-25 and the C-terminal cleavage product (marked by asterisks), which were excised from the SDS-PAGE gel and subjected to mass spectrometry analysis.

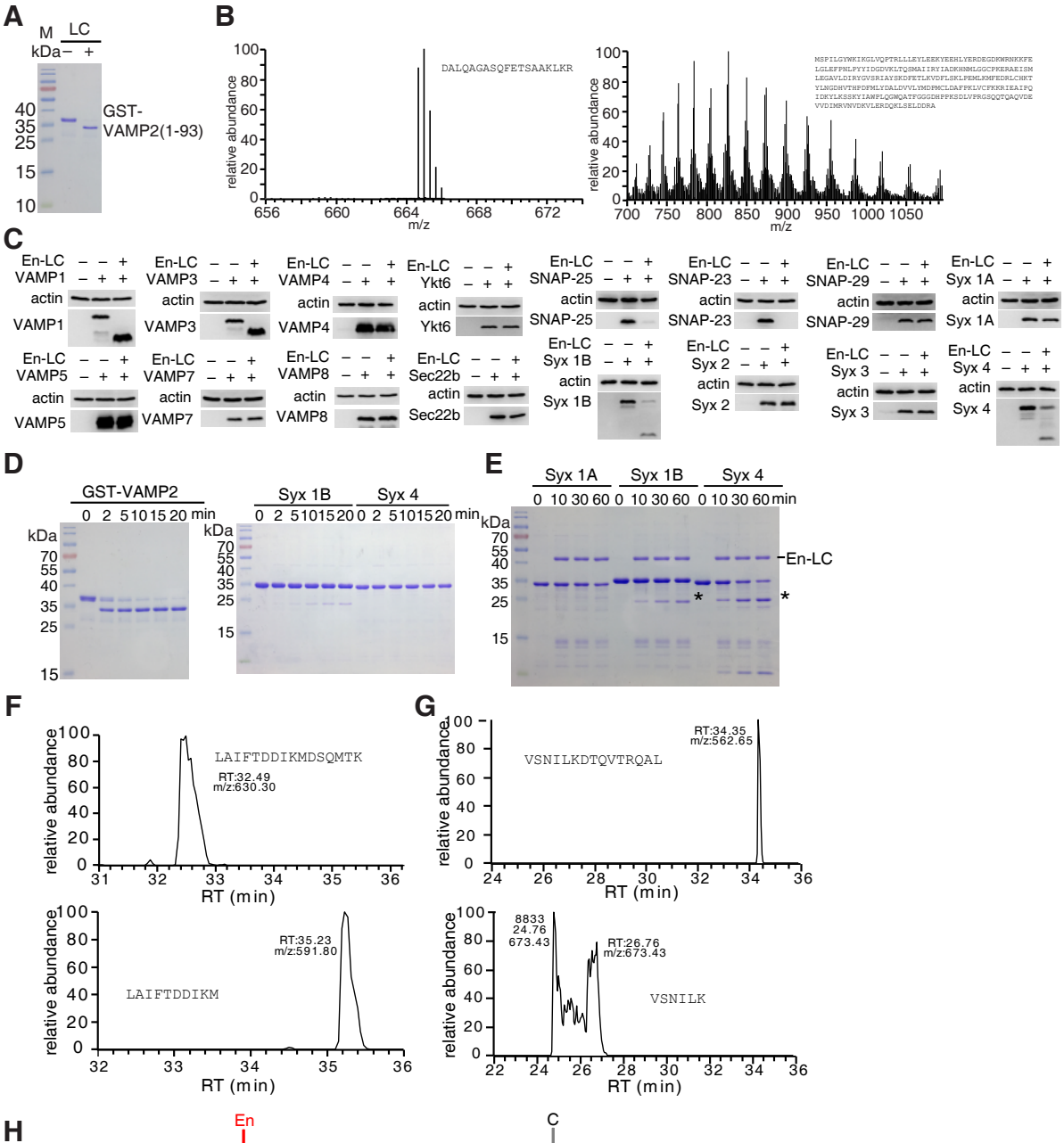
(C) The full-length SNAP-25 and the C-terminal cleavage product isolated by immunoprecipitation as described in panel B were analyzed by MS-LS/LS. The eluted peptide peaks from the HPLC column are plotted over RT. Samples were treated with trypsin (left panels: HA-SNAP-25 isolated from HEK293 cells; right panels: endogenous SNAP-25 isolated from neurons.). The upper panel shows a peak found in full-length protein that is located on the N-terminal site of the cleavage site, thus this peak is missing from the cleavage product. The lower panel shows a peak found only in the cleavage product samples, but not in full-length SNAP-25 samples, suggesting that the starting residue of the cleavage products is D70. The same results were obtained for samples isolated from neurons and for samples isolated from HEK293 cells.

(D) Samples from HEK293 cells were also treated with chymotrypsin and analyzed by MS-LS/LS. The upper panel shows a peptide fragment found in full-length SNAP-25 sample, which covers the cleavage site. The lower panel shows a peptide found only in the cleaved SNAP-25, confirming that the N-terminal residue of the cleavage product is D70.

(E) SNAP-25 with a HA at its N-terminus was expressed in HEK293 cells. Cell lysates were incubated with En-LC and subjected to SDS-PAGE and immunoblot analysis (4-20% gradient gels for samples detected by the SNAP-N and HA antibodies in order to cover lower molecular weight peptides, and 12% gel for the sample analyzed by the SNAP-C antibody). Only the SNAP-C antibody was able to detect a cleavage product, which represents the C-terminal fragment of SNAP-25 generated by En-LC. We note that SNAP-25 in cell lysates showed an additional band with a lower molecular weight than the full-length SNAP-25, which is likely a degradation product.

(F) Sortase ligation reaction mixture and indicated control components, with or without DTT, were analyzed by SDS-PAGE and Coomassie Blue staining. The molecular weight marker is in lane 1 (starting from the left side). Full-length BoNT/En (En-FL) is marked by an asterisk. It separated into two smaller bands in the presence of DTT (lane 7), confirming that it is activated.

(G) An En-A chimeric toxin was generated by ligating En-LC-H_N with A-H_C by sortase, similar to generating En-FL. The sortase ligation mixture and indicated control components were analyzed by SDS-PAGE and Coomassie Blue staining. The ligated En-A chimeric toxin is marked with an asterisk.


A
M
LC
kDa
GST-
VAMP2(1-93)
B
DALQAGASQFETSAAKLKR
relative abundance
m/z
C
En-LC
VAMP1
VAMP3
VAMP4
Ykt6
SNAP-25
SNAP-23
SNAP-29
Syx 1A
Syx 1B
Syx 2
Syx 3
Syx 4
VAMP5
VAMP7
VAMP8
Sec22b
actin
D
GST-VAMP2
Syx 1B
Syx 4
0 2 5 10 15 20 min
kDa
E
Syx 1A
Syx 1B
Syx 4
0 10 30 60 0 10 30 60 0 10 30 60 min
En-LC
F
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RT:32.49
m/z:630.30
LAIFTDDIKM
RT:35.23
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RT (min)
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m/z:562.65
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24.76
673.43
H
En
C
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Syx1B 173.LAIFTDDIKMDSQMTKQALNEI.194...249.DTKKAVKYQSKARRKK.264
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Figure S1

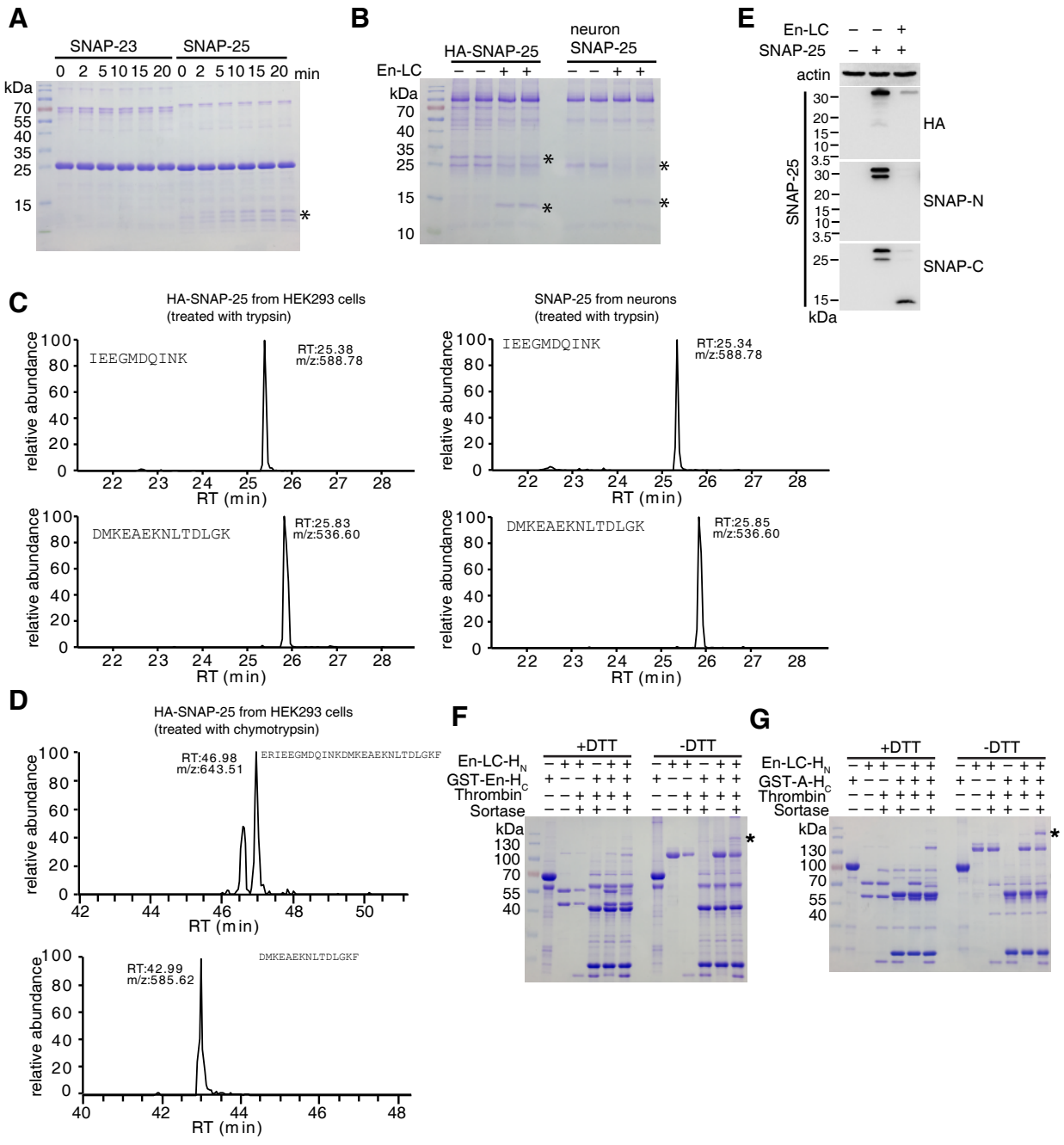
A
SNAP-23
SNAP-25
0 2 5 10 15 20 0 2 5 10 15 20 min
kDa
70
55
40
35
25
15
*
B
HA-SNAP-25
neuron SNAP-25
En-LC
– – + + – – + +
kDa
70
40
35
25
15
10
*
*
*
E
En-LC – – +
SNAP-25 – + +
actin
30
20
15
10
3.5
HA
SNAP-N
SNAP-C
25
15
kDa
C
HA-SNAP-25 from HEK293 cells
(treated with trypsin)
SNAP-25 from neurons
(treated with trypsin)
IEEGMDQINK
RT:25.38
m/z:588.78
RT:25.34
m/z:588.78
DMKEAEKNLTDLGK
RT:25.83
m/z:536.60
RT:25.85
m/z:536.60
relative abundance
RT (min)
D
HA-SNAP-25 from HEK293 cells
(treated with chymotrypsin)
RT:46.98
m/z:643.51
ERIEEGMDQINKDMKEAEKNLTDLGKF
RT:42.99
m/z:585.62
DMKEAEKNLTDLGKF
relative abundance
RT (min)
F
+DTT
-DTT
En-LC-H_N
GST-En-H_C
Thrombin
Sortase
kDa
130
100
70
55
40
*
G
+DTT
-DTT
En-LC-H_N
GST-A-H_C
Thrombin
Sortase
kDa
130
100
70
55
40
*

Figure S2