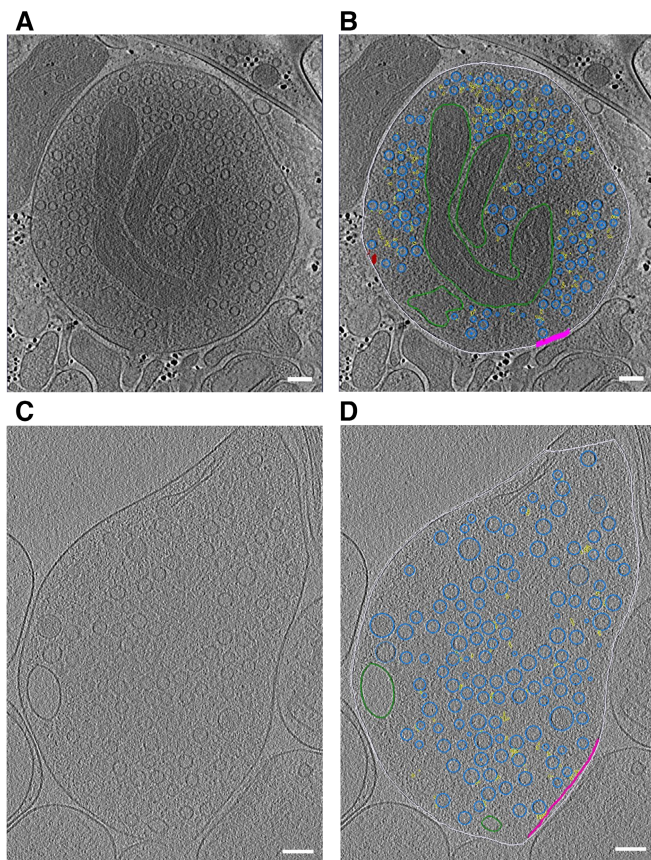


## Expanded View Figures

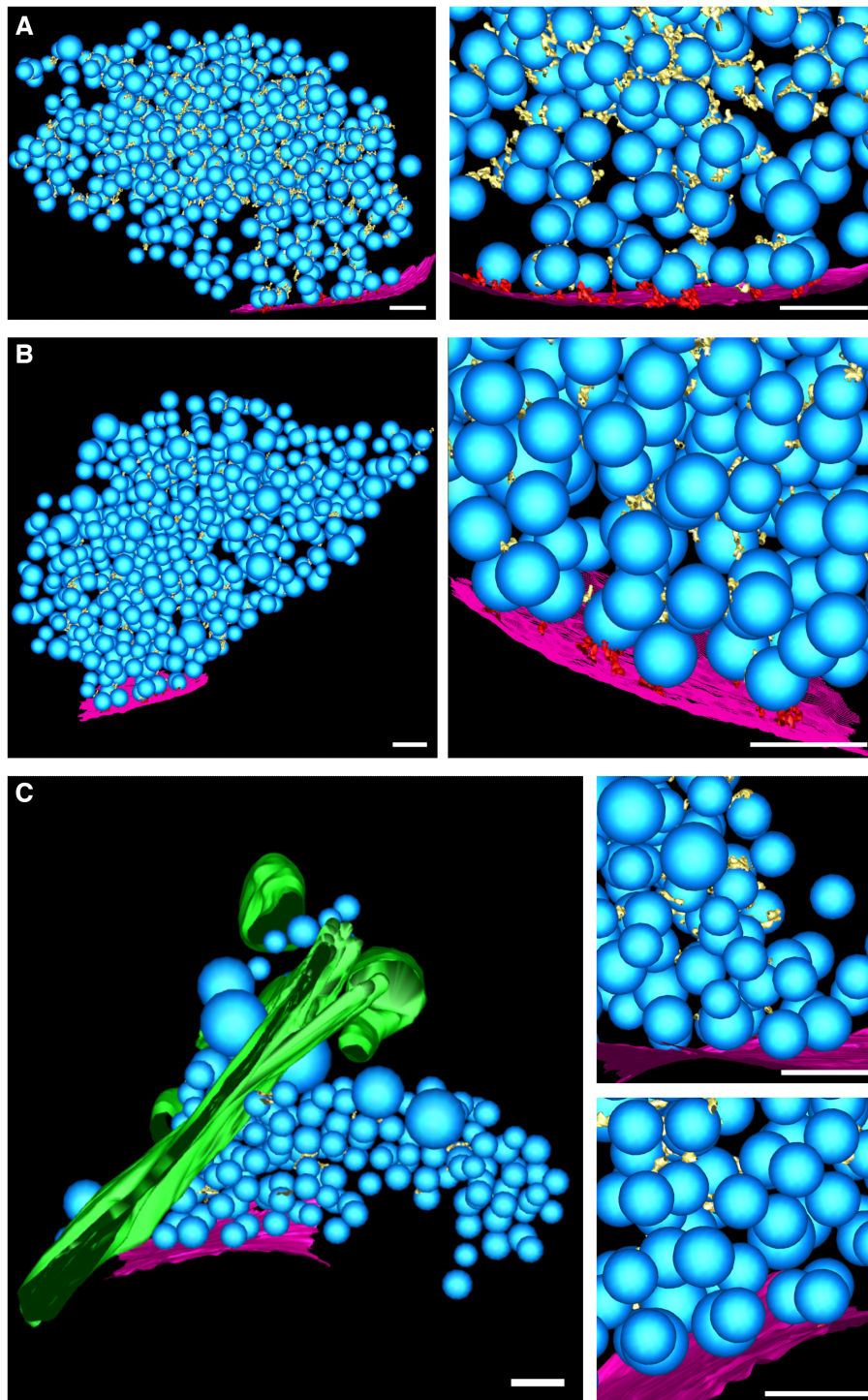


**Figure EV1. Representative slices through tomograms.**

A, B Tomographic slice without (A) and with (B) segmentation of synaptosome with late-fusion events.

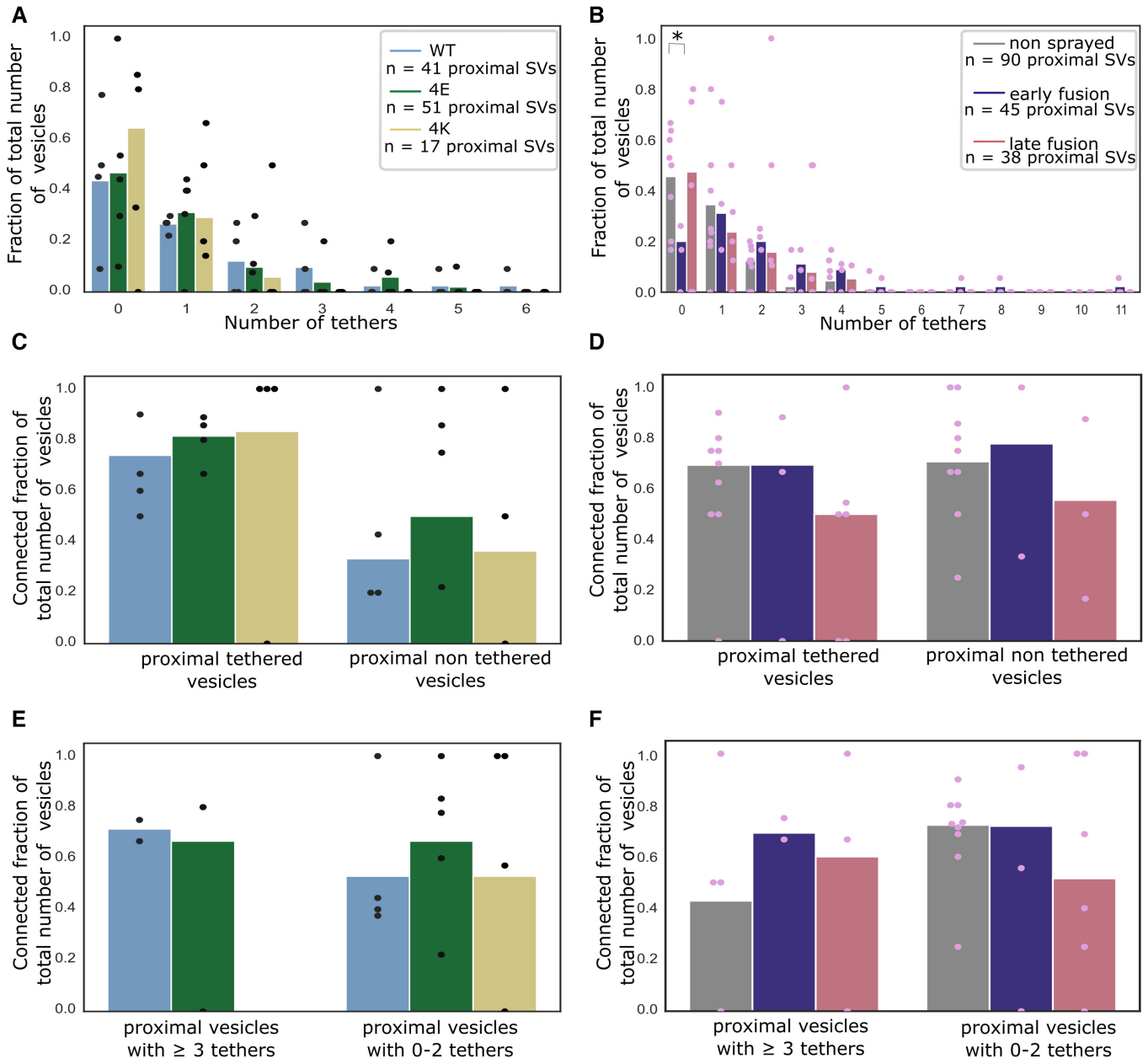
C, D Tomographic slice without (C) and with (D) segmentation of WT SNAP-25 neurons.

Data information: segmentation colors: off-white = cell outline; pink = active zone; blue = synaptic vesicles; green = mitochondria; yellow = connectors and red = tethers. Scale bar, 100 nm.



**Figure EV2. 3-D rendered segmented tomograms of neuron synapses.**

A–C (A) SNAP-25 WT, (B) SNAP-25-4E, and (C) SNAP-25-4K. (left) Overview, (right) detail. Blue: synaptic vesicles; purple: active-zone plasma membrane; green: endoplasmic reticulum-like organelle; yellow: connectors; and red: tethers. Scale bars: 100 nm.



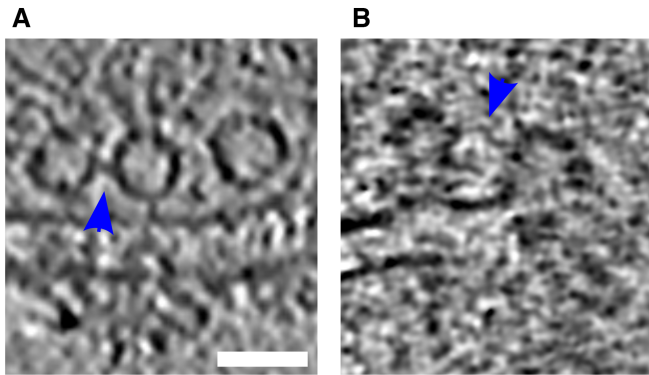
**Figure EV3. Additional SV tethering and connectivity data.**

A, B Histogram of the number of tethers per proximal SV. Statistical test: pairwise  $\chi^2$  test between control and each experimental condition in the zero-tether group with Benjamini–Hochberg correction. \* $P < 0.05$ .

C, D Histogram of connected SV among tethered or non-tethered proximal SVs.

E, F Histogram of connected SV among proximal non-RRP or RRP SVs.

Data information: (A, C, E) Synapses in mouse cultured neurons. (B, D, F) Rat synaptosomes.



**Figure EV4. Tethered connected SVs.**

A, B Tomographic slices showing tethered connected vesicles. Blue arrows highlight the connectors. Scale bar, 50 nm.