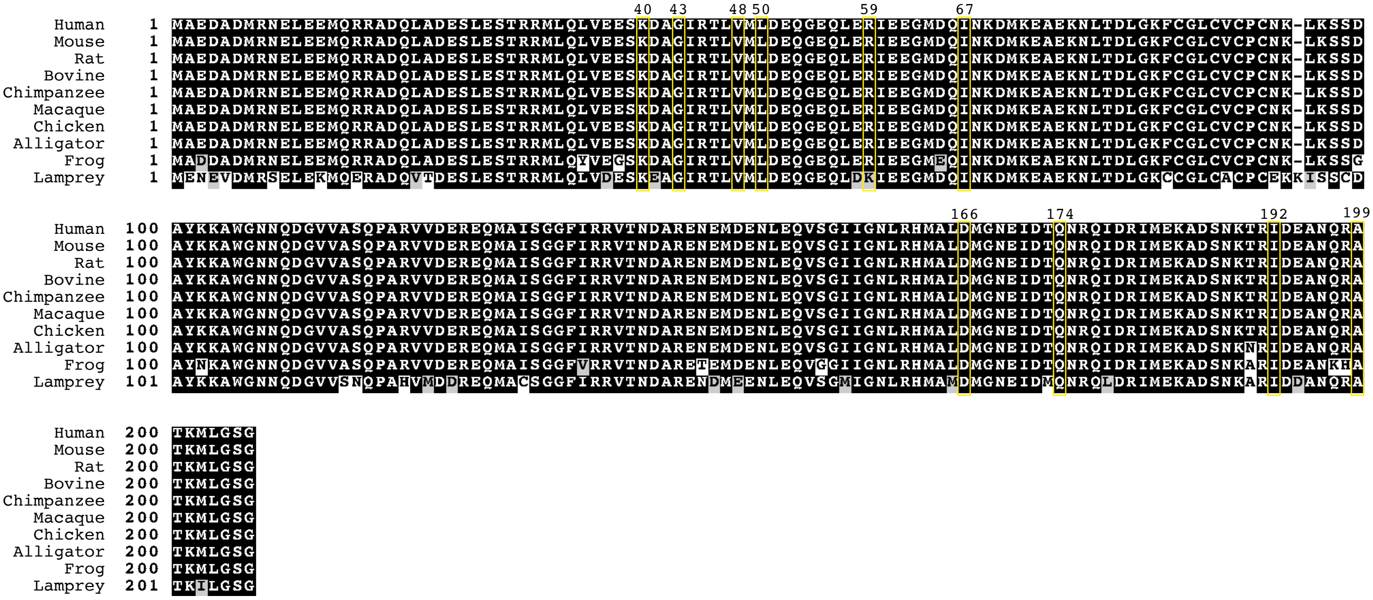
# Supplementary Figure 1



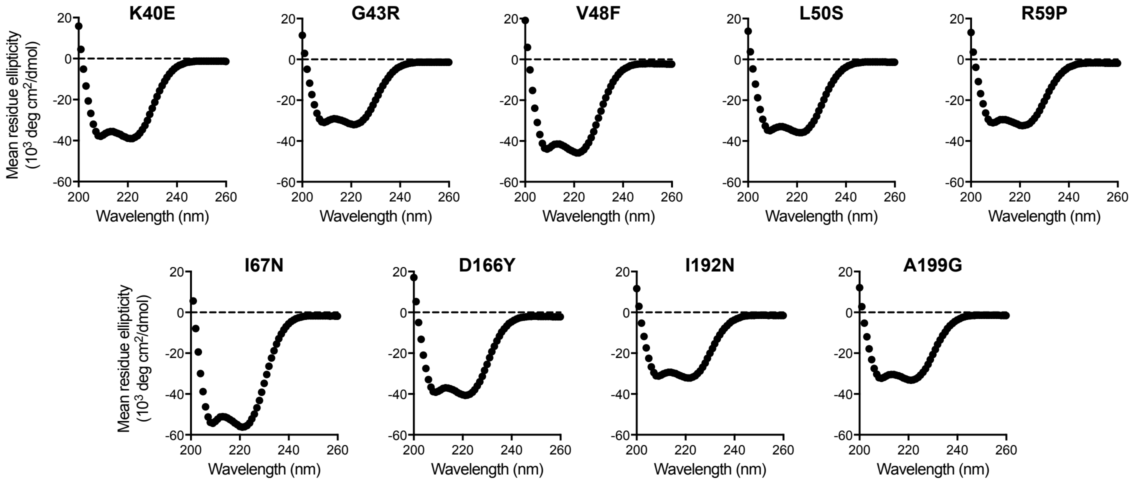
```
                                                                                         40  43    48 50        59        67
     Human    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
     Mouse    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
       Rat    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
    Bovine    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
Chimpanzee    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
   Macaque    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
   Chicken    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
 Alligator    1 MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSD
      Frog    1 MADDADMRNELEEMQRRADQLADESLESTRRMLQYVEGSKDAGIRTLVMLDEQGEQLERIEEGMEQINKDMKEAEKNLTDLGKFCGLCVCPCNK-LKSSG
   Lamprey    1 MENEVDMRSELEKMQERADQVTDESLESTRRMLQLVDESKEAGIRTLVMLDEQGEQLDKIEEGMDQINKDMKEAEKNLTDLGKCCGLCACPCEKKISSCD

                                                                                                166     174               192    199
     Human  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
     Mouse  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
       Rat  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
    Bovine  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
Chimpanzee  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
   Macaque  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
   Chicken  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRA
 Alligator  100 AYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKNRIDEANQRA
      Frog  100 AYNKAWGNNQDGVVASQPARVVDEREQMAISGGFVRRVTNDARETEMDENLEQVGGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKARIDEANKHA
   Lamprey  101 AYKKAWGNNQDGVVSNQPAHVMDDREQMACSGGFIRRVTNDARENDMEENLEQVSGMIGNLRHMAMDMGNEIDMQNRQLDRIMEKADSNKARIDDANQRA

     Human  200 TKMLGSG
     Mouse  200 TKMLGSG
       Rat  200 TKMLGSG
    Bovine  200 TKMLGSG
Chimpanzee  200 TKMLGSG
   Macaque  200 TKMLGSG
   Chicken  200 TKMLGSG
 Alligator  200 TKMLGSG
      Frog  200 TKMLGSG
   Lamprey  201 TKILGSG
```

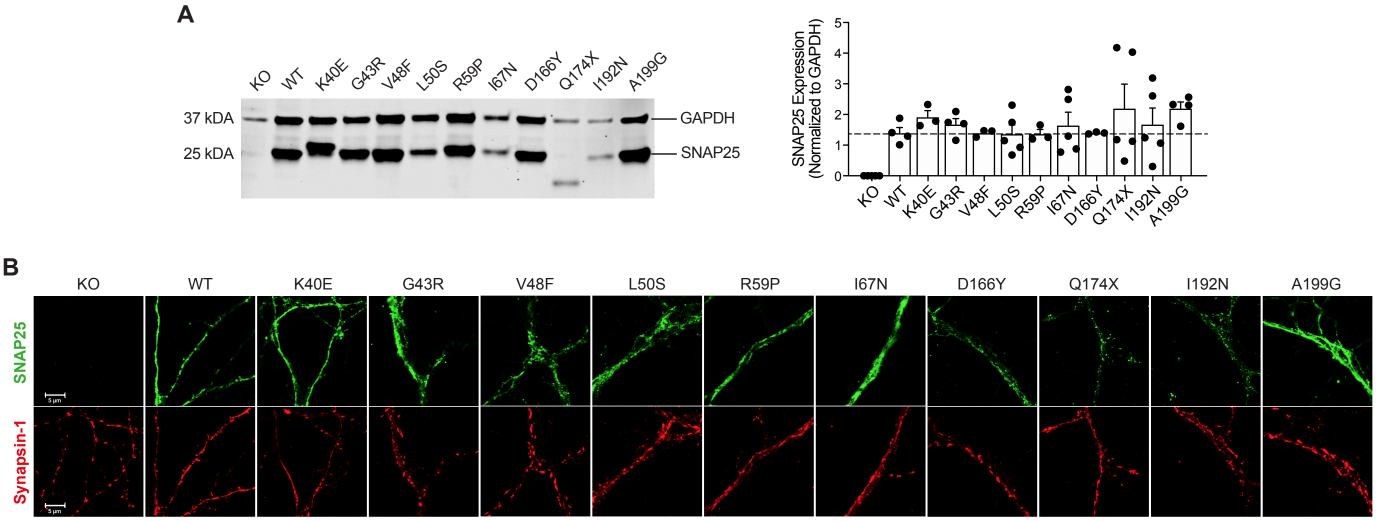
**Supplementary figure 1. Sequence alignments of SNAP25 from multiple species, related to Table 1.** Mutated residues from patients were shown in yellow columns. The alignment was performed using ClustalW2 and the figure was prepared with Boxshade3.21. List of Uniprot accession numbers as follows: P60880 (*Homo sapiens*, human), P60879 (*Mus musculus*, mouse), P60881 (*Rattus norvegicus*, rat), Q17QQ3 (*Bos taurus*, bovine), Q5R1X1 (*Pan troglodytes*, chimpanzee), P60877 (*Macaca mulatta*, rhesus macaque), P60878 (*Gallus gallus*, chicken), A0A151MGV1 (*Alligator mississippiensis*, alligator), Q640W4 (*Xenopus laevis*, frog) and S4RGG0 (*Petromyzon marinus*, sea lamprey).

# Supplementary Figure 2



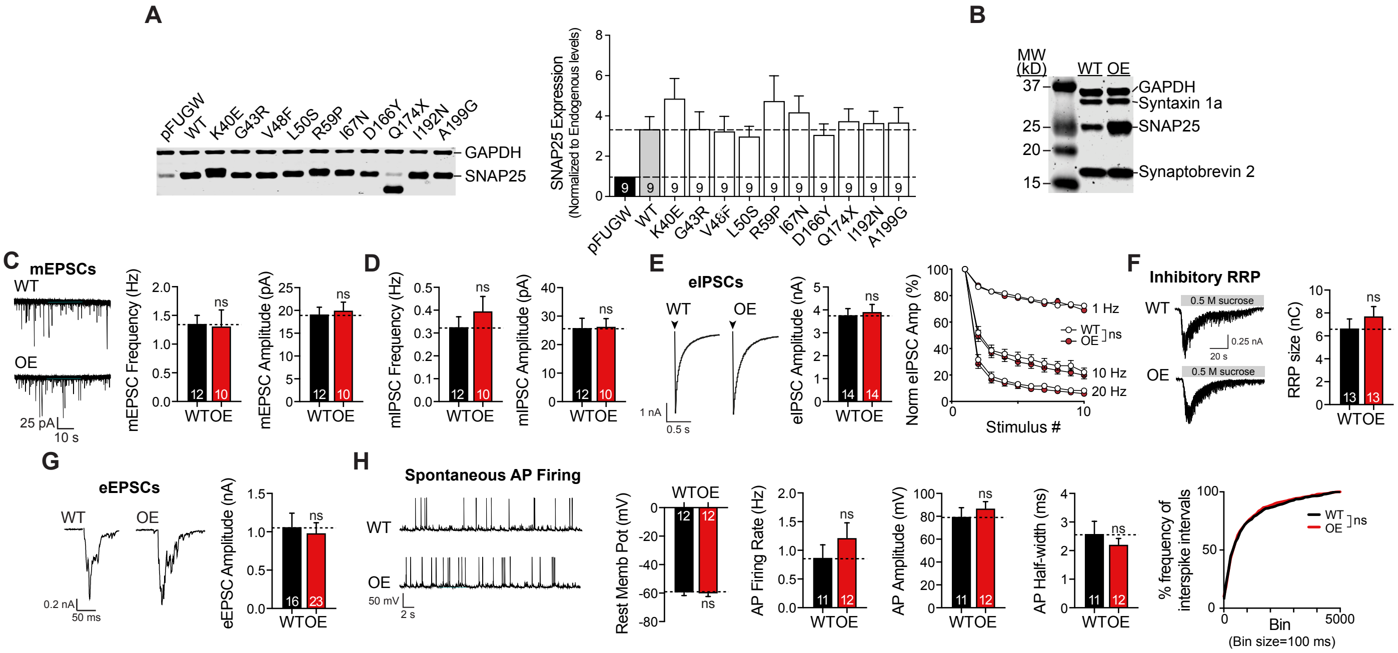
**Supplementary figure 2. CD spectra of SNARE complexes formed with SNAP25 variants, related to Figure 2.** Mean residue ellipticity values at different wavelengths from 200 nm to 260 nm are shown for individual SNARE complexes composed of different SNAP25 variants.

# Supplementary Figure 3



**Supplementary Figure 3. Expression of SNAP25 variants on SNAP25 KO cultures, related to Figure 3 and Figure 4. A.** Representative western blotting immunolabeled against SNAP25 and GAPDH with quantification. Note that Q174X gives rise to a truncated SNAP25 variant of which molecular weight is less than 25 kD. **B.** Coimmunostaining of SNAP25 and synapsin (presynaptic marker) to show that variants were expressed and trafficked normally by lentiviral infected neurons. The scale bar in the KO images are valid for all images and represents 5 µm.

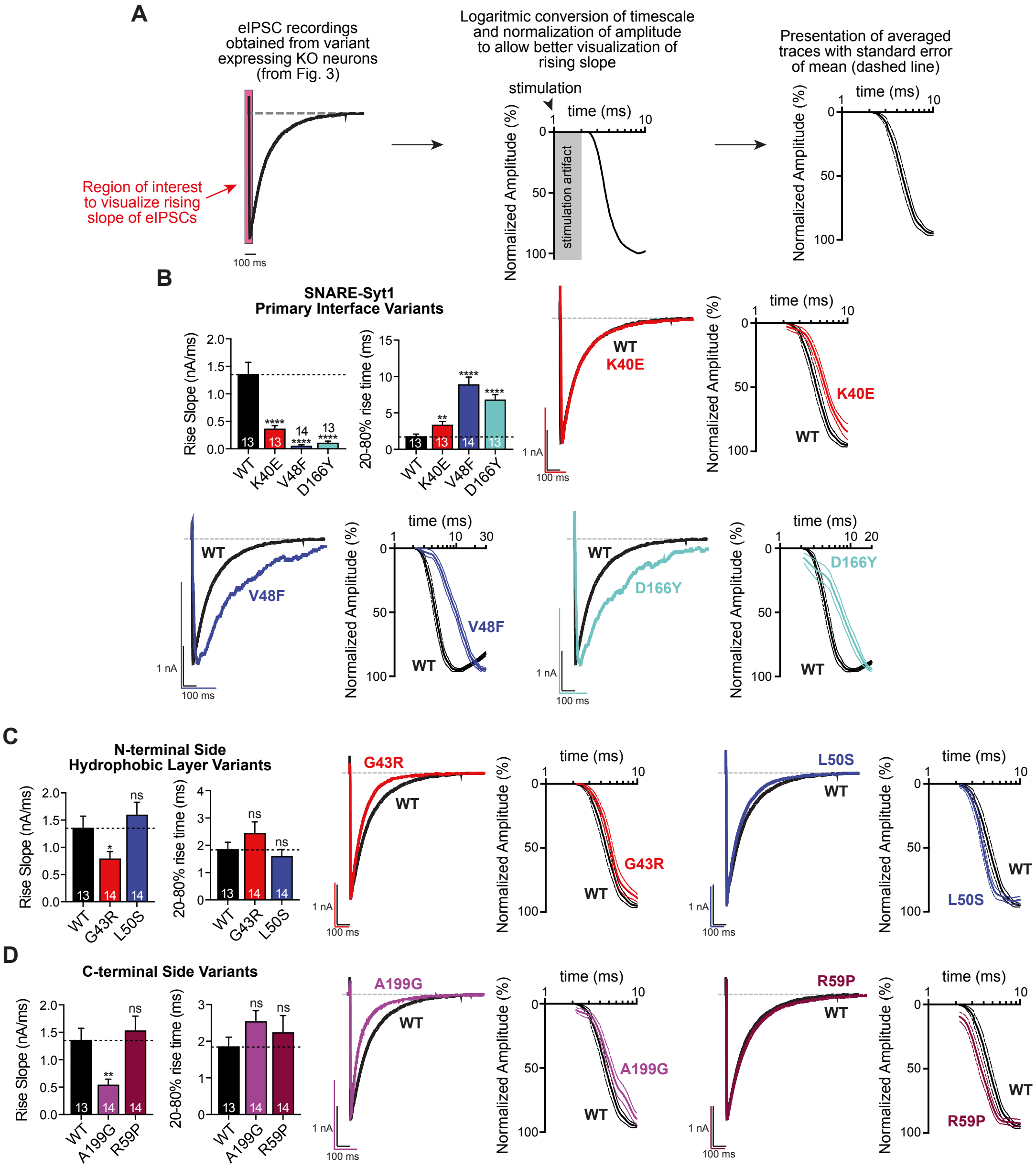
# Supplementary Figure 4



**Supplementary figure 4. Overexpression of WT SNAP25 on WT cultures is electrophysiologically indistinguishable from WT cultures expressing WT SNAP25 only, related to Figure 3, Figure 4 and Figure 7.**

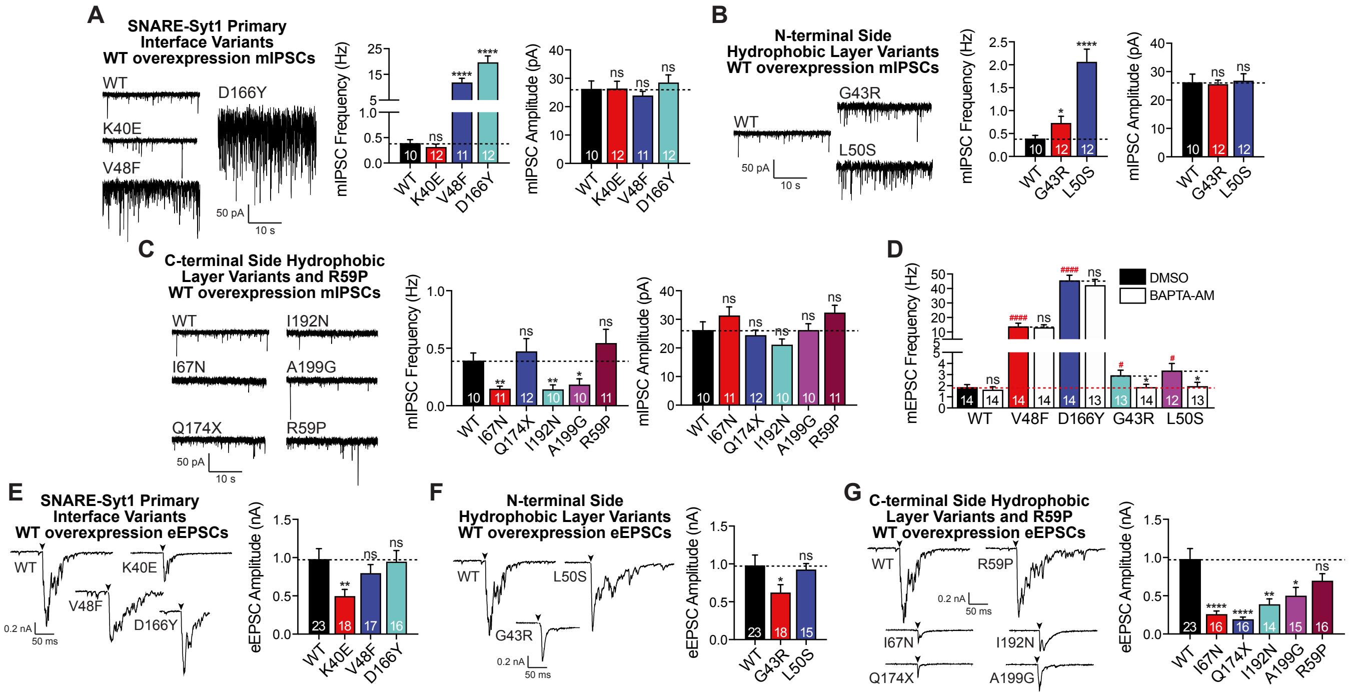
**A.** Representative western blot with quantification of SNAP25 levels obtained from WT overexpression experiments shown in Figures 3, 4 and 7. pFUGW denotes empty lentiviral construct, whereas the others denote the SNAP25 variant that the pFUGW construct carried. **B.** Representative western blot showing WT SNAP25 overexpression without any changes in other canonical SNAREs, syntaxin-1a and synaptobrevin-2. **C-H.** WT denotes cultures without WT SNAP25 overexpression, OE denotes cultures overexpressing WT SNAP25 in addition to endogenous SNAP25. All experiments performed with SNAP25 variant overexpression (Figures 3, 4 and 7) were also performed with WT SNAP25 overexpression and compared to WT cultures without SNAP25 overexpression. WT overexpression does not affect any of the parameter we studied with the variants and we used WT overexpression as the control group. For comparison of the cumulative histogram of interevent intervals, Kolmogorov-Smirnov test was used (Kolmogorov-Smirnov D=0.04194, p>0.05).

# Supplementary Figure 5



**Supplementary figure 5. Representative and averaged traces of eIPSCs to show event kinetics, related to Figure 3. A.** Explanation of the trace conversion. **B-D.** Scaled eIPSCs along with quantification of rise slopes and 20-80% rise times, and their averaged logarithmic traces to emphasize the differences in the rising phase of the eIPSCs.

# Supplementary Figure 6



**Supplementary figure 6. Effects of SNAP25 variants on inhibitory spontaneous neurotransmission and excitatory evoked neurotransmission, and BAPTA-AM responsiveness of the augmented spontaneous release, related to Figure 4 and Figure 6. A-C.** Representative mIPSC recordings along with quantitative analysis of event frequencies and amplitudes. **D.** mEPSC frequencies of variants augmenting spontaneous release after incubated with either BAPTA-AM or vehicle. **E-G.** Effects of SNAP25 variants on evoked excitatory postsynaptic currents (eEPSCs).

## Supplementary Table 1

| | R59P-SNAP25 SNARE complex |
|---|---|
| **Data collection** | |
| Space group | P 1 21 1 |
| Cell dimensions | |
| *a*, *b*, *c* (Å) | 48.76, 45.90, 109.06 |
| α, β, γ (°) | 90, 94.41, 90 |
| Resolution (Å) | 42.29 – 2.11 (2.186 – 2.11) * |
| $R_{\mathrm{merge}}$ | 0.155 (1.602) |
| $R_{\mathrm{PIM}}$ | 0.045 (0.670) |
| $I / \sigma I$ | 11.2/0.8 (0.6/1.3) |
| *CC*1/2 | 0.999 (0.374) |
| Completeness (%) | 89.6% (58.2%) |
| Redundancy | 11.4 (4.8) |
| | |
| **Refinement** | |
| Resolution (Å) | 42.29 – 2.11 (2.186 – 2.11) |
| No. reflections | 19820 (498) |
| $R_{\mathrm{work}}$ / $R_{\mathrm{free}}$ | 0.2003 / 0.2440 (0.2619 / 0.3445) |
| No. atoms | 4654 |
| Protein | 4351 |
| Ligand/ion | 20 |
| Water | 283 |
| B-factors | 34.61 |
| Protein | 34.48 |
| Ligand/ion | 59.48 |
| Water | 34.90 |
| Ramachandran | |
| Favored (%) | 100 |
| Allowed (%) | 0 |
| Outliers (%) | 0 |
| Rotamer outliers (%) | 2.10 |
| Clashscore | 6.58 |
| R.m.s. deviations | |
| Bond lengths (Å) | 0.003 |
| Bond angles (°) | 0.57 |

*Values in parentheses are for highest-resolution shell.

**Supplementary table 1. Data collection and refinement statistics for R59P-SNAP25 containing SNARE complex, related to Figure 2.**