Supplementary information

SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Figure 1. TIR-PALM of tSNAREs on the plasma membrane.**

**a** Sequential frames of a single molecule of PACherry labelled SNAP-25 on the plasma membrane demonstrating the quantal activation and bleaching characteristic of single fluorescent molecules (*upper panel*). Each frame is a 300 ms integration with fluorophore activation by brief 405 nm illumination preceding the first frame. Frames containing detectable fluorescence were averaged and are displayed as an intensity profile plot for the region (*lower panel*). The peak in fluorescence equates to the size of the point spread function of the microscope.  

**b** Intensity plot over time for single molecule activation and bleaching events. The 405 nm activation pulse immediately preceded the first time point. The delay after activation to the generation of a functional fluorophore, the number of photons yielded from the fluorophore and the duration before bleaching exhibited variation due to the stochastic nature of these processes.  

**c** TIRF-PALM of PACherry labeled SNAP-25 showing a non-uniform distribution on the plasma membrane. A summed TIRFM image derived from the entire raw PALM dataset displayed characteristic SNAP-25 clusters on the plasma membrane (*upper left panel*). The region highlighted by the yellow box is shown expanded (*upper right panel*) as a summed, diffraction limited TIRFM image. The same region is shown as a PALM rendered image containing 5,114 molecules in this region (in total 173,475 molecules were localized for the whole cell footprint).  

**d** TIRF-PALM of PACherry labeled syntaxin on the plasma membrane. As in c., the summed TIRFM image was derived from the entire raw PALM dataset (*upper left panel*). The region highlighted by the yellow box is shown expanded (*upper right panel*) as a summed, diffraction limited TIRFM image. The same region is shown as a PALM rendered image containing 3,932 molecules in this region (in total 80,417 molecules...
were localized for the whole cell footprint). The mean localization accuracy in c and d was approximately 12 nm.

Supplementary Figure 2. Quantitative colocalisation analyses of SNAREs in cells.

a Endogenous SNAP-25 (green) and Syntaxin (Syx1, red) were detected by immunostaining (endogenous), or EYFP-SNAP-25_{1-206} (green) and mCer-Syx_{1-288} (red) were expressed in neuroendocrine cells (heterologous), and imaged by CLSM. The two-dimensional histogram represents the intensity for each channel in each voxel with a colour scale representing frequency. The residual map displays weighted residuals from the line fit to the histogram, thus indicating fluorescence channel covariance. The hue is from -1 to 1 with cyan corresponding to a zero residual. b Combining Pearson’s correlation coefficients from multiple cells (as in A) yield no significant difference between endogenous and heterologous t-SNARE covariance (mean ± S.E.M., Mann-Whitney, n=4). c EGFP-SNAP-25 can rescue exocytosis in SNAP-25 functionally deficient cells stably expressing BoNT/E LC. The D179K mutation renders SNAP-25 resistant to cleavage. Basal and ATP stimulated release is expressed as a percentage of total human growth hormone (hGH; mean ± S.D, n=3). d Quantitative colocalisation data describing overlap of endogenous syntaxin1a with synaptobrevin, revealed a 9 ± 1% overlap at the plasma membrane.

Supplementary Figure 3. Analysis and presentation of TCSPC-FLIM data.

In this manuscript we have used both mean-weighted lifetimes (calculated from interacting and non-interacting molecules, and their proportional contributions) and also split coefficients derived from TCSPC data sets. a A representative donor fluorescence decay trace from a cell containing the Cerulean-YFP FRET pair. The detected photons were collected into 256 time bins over a repetitive 12 ns period and the accumulated number of photons in each bin shown (dark grey circles). The
estimated instrument response factor is shown \((\text{light grey})\) and the data were fit by a bi-exponential decay function (black line) using the equation shown in (1). This equation contains the sum of two exponentials each possessing a lifetime \((\tau)\) and an amplitude \((a)\) corresponding to that component’s fractional contribution. One component corresponds to donor molecules participating in FRET \((\tau_{\text{FRET}})\) and the other to donor molecules with a lifetime not statistically different to the non-FRET state in each pixel \((\tau_{\text{NON-FRET}})\). These data lead to the widely accepted (REFS) model where the short component and its amplitude represent interacting donor molecules, and the long component and its fraction represent the non-interacting donor molecules, in each pixel. 

**b** This decay was fit by a bi-exponential decay for every pixel in the image yielding a chi-squared value centred around 1, shown in the frequency distribution \((\text{left panel})\) The intensity (number of photons, grayscale) and the chi-squared values \((0 – 2, \text{false colour})\) can be displayed for the whole image \((\text{right panel})\) ensuring that observed differences in fluorescence lifetime are due to photophysical changes, not sub-optimal fitting.

**c** The four components (lifetime and amplitude for both the FRET and non-FRET states) from the bi-exponential fit can be displayed individually on false colour scales, separating the amplitudes \((0 – 100 \%)\) and the lifetimes \((0 – 2288 \text{ ps})\). This allows analysis of changes in the proportion of molecules undergoing FRET (amplitude, \(a_{\text{FRET}}\)) and conformational changes (lifetime \(\tau_{\text{FRET}}\)). We do not favour the calculation of FRET efficiency using the \(\tau_{\text{FRET}}\) and \(\tau_{\text{NON-FRET}}\) lifetimes, with subsequent calculation of inter-molecular distances, as this is too great an extrapolation of the data. Such calculations are incomparable to intensity based data (and mean weighted lifetime TCSPC-FLIM data) which are a convolution of the FRET and non-FRET states including variation in the proportion of interacting molecules (unknown, in the case of intensity data).

**d** Presentation of the four components derived from the bi-exponential fit can be simplified by the calculation of the weighted mean fluorescence lifetime \((\tau_{\text{MEAN}})\). This involves calculation of the mean of \(\tau_{\text{FRET}}\) and \(\tau_{\text{NON-FRET}}\) compounded by their fractional components \((a_{\text{FRET}}\) and
Supplementary Figure 4. TCSPC-FLIM reports specific protein interactions and is unaffected by molecular crowding
To exclude the possibility that the quenching of the mCerulean donor lifetimes observed in the presence of a proximal acceptor could be due to non-specific molecular crowding, we co-expressed mCer-Syx with EYFP-Syx, in the presence of unfused munc18-1 (to ensure efficient trafficking to the plasma membrane).  

\[ a \]
A representative donor fluorescence decay trace from a cell containing the Cerulean-YFP FRET pair. The detected photons were collected into 256 time bins over a repetitive 12 ns period and the accumulated number of photons in each bin shown (dark grey circles). The data followed a mono-exponential fluorescence decay (fit curve shown in black), with a similar time constant to our control data (compare to Figure 1c). The mono-exponential nature of the decay was confirmed by examination of the fit residuals and reduced chi-squared values compared to more complex fits (grey, displayed below the decay curve). The intensity and FLIM images are shown (right panels), confirming a single, long, donor lifetime.  

\[ b \]
The fluorescence lifetime data from this representative cell are displayed as a frequency distribution histogram. These data have a mean of 2234 ± 66 ps (mean ± SD; compare to 2288 ± 40 ps, mean ± SEM, n = 40, for mCerulean-syntaxin alone(Rickman et al., 2007)). These experiments were repeated 8 times with similar results.

Supplementary Figure 5. Characterization of SNAP-25_{1-206}[G43D] interaction with Syntaxin1a in vitro and in living cells.
\[ a \] Purified, His_{6}-tagged SNAP25_{1-206} or SNAP25[G43D]_{1-206} proteins readily bound the cytoplasmic domain of GST-syntaxin1a. Purified GST-Syxsyx_{1-261} (2 μg) were immobilized on beads and incubated for 1 h with SNAP-25 (2 μg). Bound material
was analyzed, after extensive washing, by SDS-PAGE and Coomassie staining. 

Both wild-type and SNAP-25\textsubscript{1-206}[G43D] readily bind syntaxin and synaptobrevin from rat brain lysates. Rat brain extract was incubated with GST-SNAP-25 immobilized on beads for 1 h at 4\textdegree C. Bound material was analyzed, after extensive washing, by SDS-PAGE, Coomassie staining and western blotting. Native brain GSTs bound directly to the sepharose resin. 

Measurement of the equilibrium dissociation constant for the syntaxin-SNAP-25 complex revealed that SNAP-25\textsubscript{1-206}[G43D] has a decreased affinity for Syx\textsubscript{1-261}. GST-syx\textsubscript{1-261} was immobilized on glutathione beads and incubated with varying concentrations of SNAP-25\textsubscript{1-206} or SNAP-25\textsubscript{1-206}[G43D]. Bound material was analyzed by western blotting. Data were fit with a 4-parameter logistic equation. Error bars represent S.E.M. (n=3). 

d\ EYFP-SNAP25\textsubscript{2-206}[G43D, D179K] fully rescues exocytosis in PC12 cells stably expressing BoNT/E LC. Human growth hormone release assays were performed in triplicate (data are means ± SEM). This mutant, originally isolated as a temperature-sensitive exocytosis defect in Drosophila (Brennwald, 1994 #2223), was clearly shown to disrupt interaction between the isolated C-terminal helix of SNAP-25 with syntaxin in circular dichroism assays (Fasshauer, 1997 #1452). Although this mutant is widely used, these \textit{in vitro} findings were not confirmed using full length mutants in biochemical assays, and our analysis here is the first detailed biochemical and cell based examination of SNAP-25 [G43D]. The small reduction in affinity between SNAP-25\textsubscript{1-206}[G43D] and Syx\textsubscript{1-261}, and the uncharacterized effect of this mutant on tSNARE folding, means that we cannot draw reliable conclusions using this mutant. 

d\ EYFP-SNAP-25\textsubscript{1-206}[G43D] expressed in N2a cells, exhibits an intracellular distribution similar to SNAP-25\textsubscript{1-206}.

\textbf{Supplementary Figure 6. Quantitative reduction in cholesterol has no effect on SNARE cluster morphology or co-variance.}
Filipin staining was used to label endogenous cholesterol at the plasma membrane.

Treatment of the cells with 10 mM MβCD for 15 minutes significantly decreased the intensity of filipin fluorescence, proportional to cholesterol concentration (data are means ± SEM, t-test, n = 15).

Cells were incubated with fluorescent markers of cell viability and the percentage of dead cells in a field of view was calculated, mean values ± SEM n=15 are presented.

The Pearson’s correlation coefficient, describing covariance between labeled t-SNAREs, indicative of stoichiometric binding, was determined. Quantitative reduction in plasma membrane lipid order had no significant effect on tSNARE covariance either throughout the entire 3-D volume of the cell or specifically at the plasma membrane. Bars show mean Pearson’s correlation coefficient ± SEM, n = 4, Mann-Whitney U test.

The density of apparent t-SNARE clusters was also unaffected at this resolution upon MβCD treatment. Graph bars represent mean ± SEM, n >10 cells.

Supplementary References

Rickman, C., C.N. Medine, A. Bergmann, and R.R. Duncan. 2007. Functionally and spatially distinct modes of munc18-syntaxin 1 interaction. J Biol Chem. 282:12097-103.
Supplementary Figure 2

(a) SNAP-25, Syx1, and Residual images for Endogenous and Heterologous conditions.

(b) Pearson's correlation coefficient graph showing hGH release as a function of total.

(c) Mander's coefficient graph for Endogenous, Heterologous, Positive control, and Negative control.

(d) Syx1, Syb, and Merge images for Endogenous conditions.
Supplementary Figure 3

(a) FRET amplitude (aFRET) and Non-FRET amplitude (aNON-FRET) with FRET lifetime (τFRET) and Non-FRET lifetime (τNON-FRET) expressed as:

\[ f(t) = a_{\text{FRET}} e^{-t/\tau_{\text{FRET}}} + a_{\text{NON-FRET}} e^{-t/\tau_{\text{NON-FRET}}} \quad (1) \]

(b) Mean weighted lifetime (τMEAN) calculated as:

\[ \tau_{\text{MEAN}} = a_{\text{FRET}} \times \tau_{\text{FRET}} + a_{\text{NON-FRET}} \times \tau_{\text{NON-FRET}} \quad (2) \]
Supplementary Figure 4

a

![Graph showing residuals and time (ns) with intensity and mean weighted lifetime](image)

b

![Graph showing frequency against lifetime (ns)](image)

- Photons (log10)
- Time (ns)
- Residual
- Lifetime (ns)
- Frequency

Intensity Mean weighted lifetime

1250-2288 ps
Supplementary Figure 5

**a**

| Input | Bound to GST-Syntaxin |
|-------|-----------------------|
|       | SNAP-25\textsubscript{1-206} [G43D] | SNAP-25\textsubscript{1-206} | GST-Sytx1-261 |
| kDa   | 50 | 37 | 25 | 20 |

**b**

Input: 37-50-25-20 kDa

Bound to GST-Syx1-261

IB: Synaptobrevin

Control GST-SNAP-25

GST-SNAP-25 [G43D]

Brain Extract

Native brain GSTS

**c**

Bound SNAP-25 (% of max)

Unbound [SNAP-25\textsubscript{1-206}] (Log\textsubscript{10} M)

G43D > 14.3 μM

S25 = 1 μM

**d**

IB: Syntaxin

IB: Synaptobrevin
