Supplementary Figure Legends

Figure S1. Subsynaptic compartmentalization of APP in functional zones of an excitatory synapse using confocal microscopy: (Related to Fig. 1, 3 and 4) (A) Left panel indicates pseudocolour overlay of presynaptic marker Bassoon (blue), APP (green) and postsynaptic marker Shank2 (Red). In the second panel automated detection of Bassoon clusters is indicated in blue. In the next panel automated detection of APP clusters is indicated in green. In the penultimate panel. automated detection of Shank2 clusters is indicated in Red. The last panel indicates the overlay of segmented regions of Bassoon (blue), APP (green) and Shank2 (red) and the magenta region represents the neuronal processes. Individual synapses are marked as insets in black. The magnified images of overlay and overlap of regions for insets 1, 2, 3, 4 and 5 is represented in the bottom panel. The scale bar in the top right panel is 5 μm and in the bottom right panel indicates 1 μm. (B) Left panel indicates pseudocolour overlay of perisynaptic marker Dynamin (blue), APP (green) and postsynaptic marker Shank2 (Red). In the second panel. automated detection of Dynamin clusters is indicated in blue. In the next panel automated detection of APP clusters is indicated in green. In the penultimate panel. automated detection of Shank2 clusters is indicated in Red. The last panel indicates the overlay of segmented regions of Dynamin (blue), APP (green) and Shank2 (red) and the magenta region represents the neuronal processes. Individual synapses are marked as insets in black. The magnified images of overlay and overlap of regions for insets 1, 2, 3, 4 and 5 is represented in the bottom panel. The scale bar in the top right panel is 5 μm and the bottom right panel indicates 1 μm.

Figure S2. Quantitative estimation of colocalization in functional zones of an excitatory synapse using confocal microscopy: (Related to Fig. 1, 3 and 4) (A) Stereographic representation of APP localization in functional zones of an excitatory synapse. Presynaptic and postsynaptic compartments and APP is indicated by red, blue and green, respectively. (B) Stereographic representation of APP localization in functional zones of an excitatory postsynapse. Perisynaptic and postsynaptic compartments and APP is indicated by red, blue and green, respectively (i) Area of overlap of APP with subsynaptic compartments of an excitatory synapse. (ii) Overlap of APP fluorescence intensity (fractional intensity) with subsynaptic compartments of an excitatory synapse. Significance was determined by one-way ANOVA followed by Tukey's multiple comparison test. n= 20 cells for each category. (iii) Area of Overlap of postsynaptic marker Homer1 with presynaptic marker Bassoon and postsynaptic marker PSD95 with postsynaptic marker Shank2. (iv) Fractional intensity of postsynaptic marker Homer1 on presynaptic marker Bassoon and postsynaptic marker PSD95 on postsynaptic marker Shank2. Significance was determined by unpaired two-tailed Student's t test. n= 12 cells for each category. Data points are mean values with bars showing the standard error of the mean. Scale bar at C indicates 2 μm.

Figure S3. Differential persistence of APP nanodomains on the neuronal processes: (Related to Fig. 2) (i, ii) Distribution of instantaneous diffusion coefficient (median/IQR) for APP-WT and APP-Swe for global, puncta and nanodomain, respectively. Significance was determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. (iii, iv, v) APP-Swe moved significantly slower than the APP-WT in all identified compartments. Significance was determined by unpaired two-tailed Mann–Whitney test. (vi) Local residence time of APP nanodomains indicate that most of them are persistent for less than 5 minutes, indicating a rapid turnover of molecules in these clusters. (A) Time series of evolution of nanodomain depicted in (Fig. 2, nanodomain 1) indicating that this is a stable domain where the molecular density remains constant over the duration of the experiment. Pseudocolour galleries represent super-resolution images for every 20 seconds for a total duration of 400 s. (B) Time series of evolution of an unstable nanodomain which is only present for a short duration during the experiment. Pseudocolour galleries represent images acquired during 400 s every 4 s starting with 28 s and ending with 104 s. Before and after this time period this domain was not detected again (C, D) Time series of evolution of nanodomains which were detected; interestingly the intensity of APP molecules in these domains fluctuates in time and in some cases very few APP molecules were detected. Pseudocolour galleries represent super-resolution images for every 20 seconds for a total duration of 400 s.. n= 3157 trajectories (global), 2146 trajectories (puncta) and 46 trajectories (nanodomain) for APP-WT and 26103 trajectories (global), 3081 trajectories (puncta) and 383 trajectories (nanodomain) for APP-Swe. Scale bar at D indicates 0.265 μm.

Figure S4. Lateral exchange and reversible immobilization of APP in live rat hippocampal neurons and quantification of nanoscale architecture of APP clusters using sptPALM microscopy: (i)

Normalized cumulative distribution of instantaneous diffusion coefficient of Eos::APP-WT (blue), Eos::APP-Swe (magenta) and Eos::APP-Icelandic (green). (ii) Distribution of instantaneous diffusion coefficient (median/IQR) for APP-WT, APP-Swe and APP-Icelandic. Significance was determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. n= 58575 trajectories (APP-WT), 43290 trajectories (APP-Swe) and 52267 trajectories (APP-Icelandic). (iii) (left to right) Diversity in nanodomain length, area and intensity of APP clusters on neuronal processes represented as (median/IQR) using sptPALM microscopy. Significance was determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. n= 172 nanodomains (APP-WT), 112 nanodomains (APP-Swe) and 194 nanodomains (APP-Icelandic).

Figure S5. Lateral exchange and reversible immobilization of APP in live rat hippocampal neurons and quantification of nanoscale size of APP clusters using uPAINT microscopy: (i) Normalized cumulative distribution of instantaneous diffusion coefficient of SEP::APP-WT using uPAINT microscopy (black) and Eos::APP-WT (blue) using sptPALM microscopy. n= 18990 trajectories (SEP::APP-WT) and 58575 trajectories (Eos::APP-WT). (ii) Indicate the distribution of length of APP nanodomains obtained by uPAINT microscopy. n= 437 nanodomains.

Figure S6. Lateral exchange and reversible immobilization of APP in live 3T3 cells: (A) Indicates the super-resolution intensity image of APP obtained by sptPALM. The image is pseudocolour coded from white (0) to black (20) indicated by the scale bar at (A). The cell boundary is indicated by magenta. (B) Indicates the detected individual nanodomains of APP from (A). The cell boundary is indicated by white while nanodomain regions are indicated by magenta. (C) Indicates the trajectories of individual Eos::APP-WT molecules. Each trajectory is pseudocolour coded and represented as a recurring pattern of individual colours. The scale bar at (C) indicates 4.6 µm. (D) Indicates the unicoloured (magenta) trajectories of individual Eos::APP-WT molecules. Scale bar at (D) indicates 4.6 µm. (E) (1, 2, 3. 4. 5. 6) are magnified insets of regions indicated in (D). The single molecule trajectories of Eos::APP-WT are shown in a recurring pattern of magenta. The arrows indicate the transient trapping of APP molecules on the cell membrane. The scale bar at (E) indicates 0.7 µm. (i) Normalized cumulative distribution of instantaneous diffusion coefficient of Eos::APP-WT (blue) and Eos::APP-Swe (magenta). (ii) Distribution of instantaneous diffusion coefficient (median/IQR) for APP-WT and APP-Swe. Significance was determined by unpaired two-tailed Mann–Whitney test. n= 7263 trajectories (APP-WT) and 5313 trajectories (APP-Swe).

Figure S7. Relative nanoscale segregation of excitatory synapse into discrete functional zones observed using STED microscopy: (Related to Fig. 3 and 4) (A) Confocal image of the individual synapses identified by automated detection of postsynaptic marker PSD95 (green) puncta with pseudocolour overlay of Homer1 (magenta). (B) STED image of the individual synapses from (A) with pseudocolour overlay of PSD95 (green) and Homer1 (magenta). The black boundary in (A) and (B) indicate the boundary of the neuronal process. The black insets are individual regions chosen for a magnified observation in gallery (C). The overlap between Homer1 and PSD95 is marked by black. (C) Gallery of individual synapses indicating the extent of overlap between Homer1 and PSD95 in neuronal processes. Upper panel indicates the regions marked by black insets in (A) and lower panel indicates the corresponding regions in STED. (D) Confocal image of the individual synapses identified by automated detection of perisynaptic marker Dynamin (green) puncta with pseudocolour overlay of Homer1 (magenta). (E) STED image of individual synapses from (D) with pseudocolour overlay of Dynamin (green) and Homer1 (magenta). The boundary of the neuronal process is indicated in black in (D and E). The black insets are individual regions chosen for a magnified observation in gallery (F). The overlap between Homer1 and Dynamin is marked in black. (F) Gallery of individual synapses indicating the extent of the overlap between Homer1 and Dynamin in neuronal processes. Upper panel indicates the regions marked by black insets in (D) and lower panel indicates the corresponding regions in STED. Scale bar at E and F indicates 1.5 µm and 0.600 µm, respectively.

Figure S8. Quantification of functional zones marker and nanoscale architecture of APP clusters in different functional zones of a synapse using STORM microscopy: (Related to Fig. 3, 4) (i) (left to right) Comparison of Resolution Scaled Pearson's (RSP) Coefficient and Resolution Scaled Error (RSE) for quantifying colocalization of markers for functional zones of an excitatory postsynapse. (RSP and RSE analysis) n= 10 cells for each category. Significance was determined by one-way ANOVA followed by

Tukey's multiple comparison test. (ii) (left) Indicate the distribution of the observed nearest neighbour distances from Homer1 to PSD95 (blue) and Homer1 to Dynamin (green). The red and brown vertical dotted line corresponds to 40 nm and 100 nm respectively. n= 290 puncta (Homer1-PSD95) and 511 puncta (Homer1-Dynamin). (right) Comparison of nanodomain length for different functional zones marker using dSTORM and STED microscopy indicated as median/IQR. (dSTORM) n= 542 nanodomains (Bassoon), 1438 nanodomains (PSD95), 597 nanodomains (Dynamin). (STED) n= 627 nanodomains (PSD95), 418 nanodomains (Dynamin). (iii, iv) Diversity in nanodomain length, area (iii) and intensity, number of APP molecules per nanodomain (iv) for APP clusters in different functional zones i.e. APP in postsynapse and perisynapse of an excitatory synapse of a pyramidal neuron represented as (median/IQR) using dSTORM. n= 1547 puncta (post), 1671 puncta (peri). Significance was determined by unpaired two-tailed Mann-Whitney test.

Figure S9. Quantification of nanoscale architecture of APP clusters in different functional zones of a synapse using STED microscopy: (Related to Fig. 4) (i) Comparison of RSP and RSE for quantifying colocalization of APP at different functional zones of an excitatory postsynapse i.e. APP in postsynapse and perisynapse by STED microscopy. Significance was determined by unpaired two-tailed Student's t test. (A) Stereographic representation of APP localization within functional zones of an excitatory synapse by STED microscopy. Postsynaptic (left)/perisynaptic (right) compartments and APP is indicated by red and green, respectively. (ii, iii) (left to right) Diversity in nanodomain length, area (ii) and intensity, number of APP molecules per nanodomain (iii) for APP clusters in different functional zones of an excitatory synapse represented as (median/IQR) using STED. n= 10888 puncta (post), 7091 puncta (peri). Significance was determined by unpaired two-tailed Mann-Whitney test. Scale bar at (A) indicates 0.2 µm.

Figure S10. Cross validation of nanoscale distribution of APP in perisynaptic region: (i) (left to right) Diversity in nanodomain length, area and intensity for APP clusters in the perisynapse marked by Dynamin/Clathrin represented as (median/IQR) using STED. n= 7091 puncta (peri/Dynamin) and 8200 puncta (peri/Clathrin). Significance was determined by unpaired two-tailed Mann-Whitney test.

Figure S11. Reconstruction of compositionality of nanoscale organization of APP in an excitatory synapse: (Related to Fig. 5): (A, B, C, D) Realistic reconstruction of APP distribution in functional zones of excitatory synapses. Yellow, white and cyan regions indicate extrasynaptic, perisynaptic (endocytic zone) and postsynaptic density, respectively. Red puncta and clusters represent APP monomers and nanodomains, respectively. Arrows indicate APP nanodomains in post and perisynaptic regions. The representative images of synapses with (A) no APP nanodomain (B) with a postsynaptic nanodomain of APP (C) with a perisynaptic nanodomain of APP and (D) with nanodomains of APP both in post and perisynaptic compartments.

Figure S12. Discrete nanoscale association of APP and β-secretase on neuronal processes using STED microscopy: ((A) Confocal image of APP (magenta) with pseudocolour overlay of β-secretase (green). (B) STED image of APP (magenta) from (A) with pseudocolour overlay of β-secretase (green). (C) Gallery of images from regions indicated in (B). (D) Represents line scans of 25 pixel line (0.360 µm) connecting the centroids, indicating the maximum intensity of the APP and β-secretase distribution from (1, 2, 3, 4, 5, 6, 7, 8, 9) square insets in (C). The square insets in (C) has a lattice of 300 nm. The X and Y-axis in (D) represent the distance (µm) and the normalised distribution of intensity (a.u.) for APP and β-secretase.

Supplementary Movie Legends

Movie S1. Dynamics of individual Eos::APP-WT in two different regions of interest. The arrows indicate the transient trapping of APP molecules on the neuronal membrane, which is indicated by the confined movement of these molecules. The real-time frequency of 50Hz is slowed down to 30Hz for representation.

Movie S2. Dynamics of APP nanodomains in two different regions of interest. The arrows indicate the position of stable nanodomains over 400 seconds. The real-time frequency of 0.25Hz has been augmented to 30Hz for representation.