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Supplemental Information

Packing Density of the Amyloid Precursor Protein in the Cell Membrane

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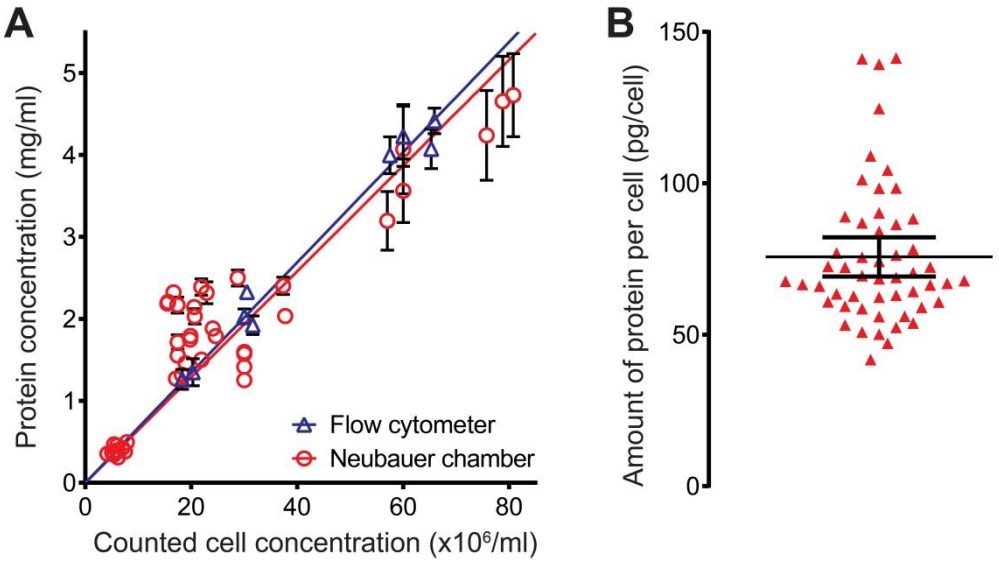
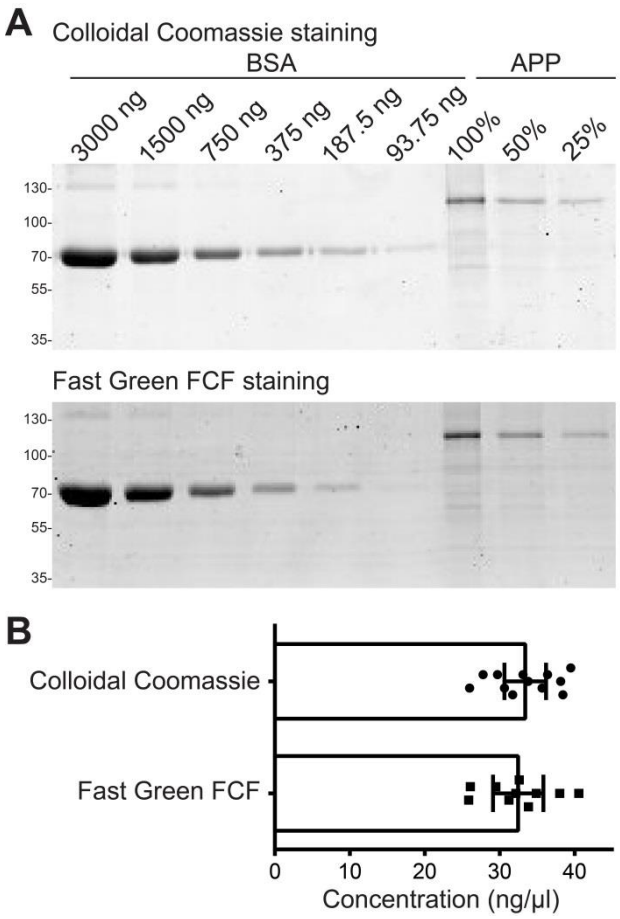


Supporting Figure 1. Amount of protein per SH-SY5Y cell.

(A) SH-SY5Y cells were mechanically detached from the substrate by cell scraping, followed by counting in a Neubauer chamber (red open circles; red regression line) or by a flow cytometer (blue open triangles; blue regression line). The protein concentration of the cell lysates was determined by standardized BCA assays. Both counting methods were in good agreement and indicated linearity between the protein and the cell concentrations. Values are given as means ± SD (n = 3 BCA assay triplicates).

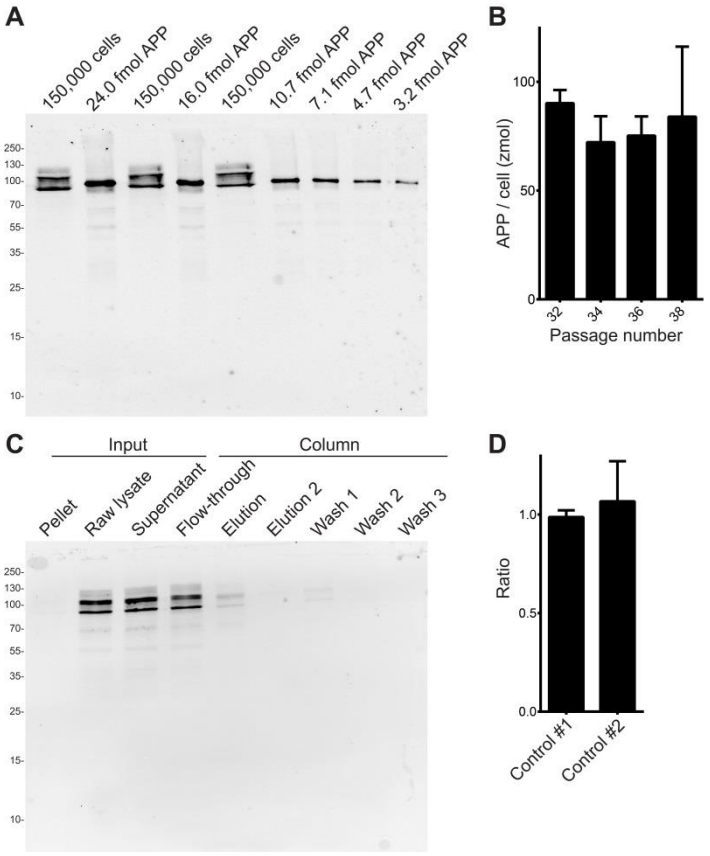
(B) The amount of protein per cell was determined dividing protein concentration by cell concentration. Pooling the values obtained from the two counting methods, the amount of protein per SH-SY5Y cell was 75.7 pg. Error bars indicate the 95% confidence interval (n = 52 independent measurements using the two distinct counting approaches).



Supporting Figure 2. Protein concentration of recombinant His_6-APP_{695}.

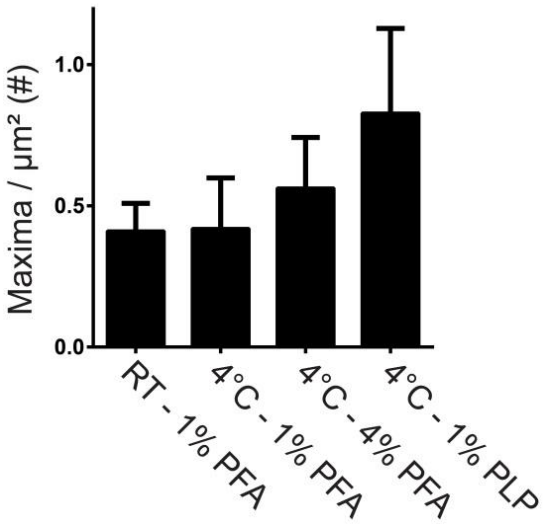
(A) Gels obtained by SDS-PAGE. Shown are serial dilutions of bovine serum albumin (BSA) for obtaining standard curves to which purified His_6-APP_{695} was related. His_6-APP_{695} was loaded at the concentrations as indicated. Top and bottom; gels stained with colloidal Coomassie Brilliant Blue G-250 and Fast Green FCF, respectively. Both dyes showed staining intensities linear with protein concentrations over a 30-fold concentration range.

(B) Concentration of APP with reference to the BSA standard curves. For quantification, only the APP band was related to the BSA standard (not considering the faint additional bands running lower). The results obtained with the two dyes differed by less than 3% and were pooled. The concentration of His_6-APP_{695} was 32.98 ng/µl. Error bars indicate the 95% confidence interval (n = 22 independent measurements, using the two different dyes). Given a theoretical molecular weight of 80,840 Da, which includes the six histidine amino acids and a small linker region, this equates to 407.5 nM.



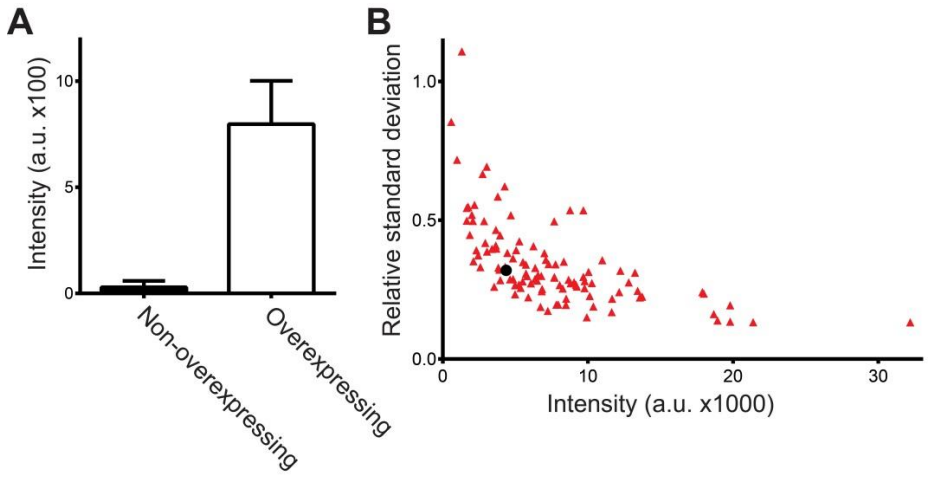
Supporting Figure 3. Additional information to Figure 1.

(A) Entire western blot membranes shown in Figure 1A. (B). APP molecules per cell over passage number, shown for a subset of data including 4 data points in Figure 1C. Cells were obtained at passage 26 and after thawing not used before passage 32. Diluting cells 1:4 upon passaging, the range of passages shown equates to a population doubling level of 16. Values are means ± SD (n = 3 replicates per passage from a continuously passaged culture). (C) Entire western blot membranes from the biotinylation experiment shown in Figure 1C. (D) For the biotinylation experiments included in Figure 1D control ratios were determined that are "1" in case no protein is lost during the procedure. The control ratio #1 (('pellet' + 'supernatant') / 'raw lysate') was 0.9871 ± 0.0342 (n = 6 biological replicates), and the control ratio #2 (('flow-through' + 'elution' + 'wash 1') / 'raw lysate') was 1.067 ± 0.2035 (n = 9 biological replicates). Values are given as means ± SD.



Supporting Figure 4. Evaluation of different fixation protocols.

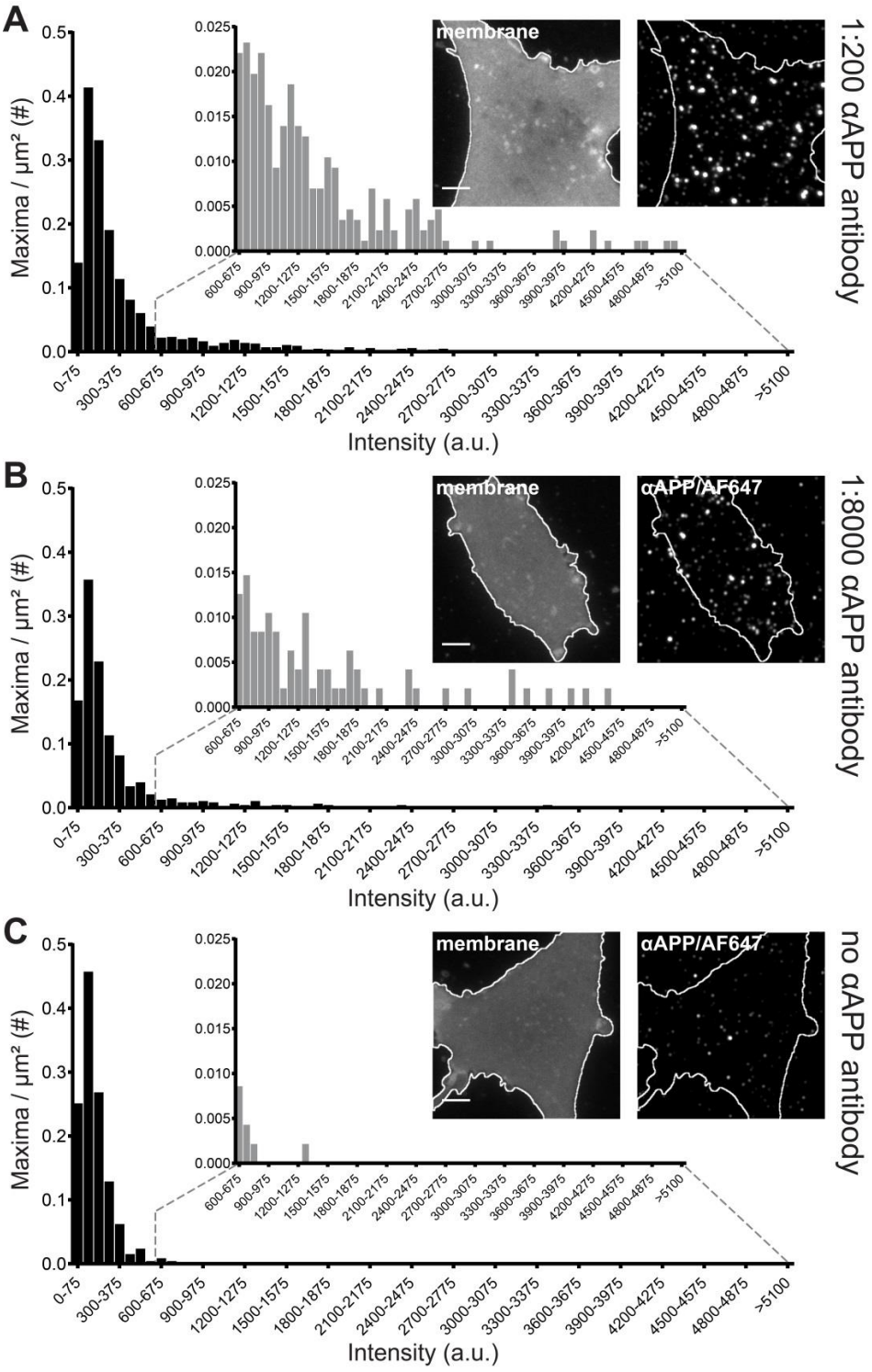
Membrane sheets from non-transfected cells were fixed for 30 min with 1% PFA at room temperature or at 4°C, with 4% PFA at 4°C, or with 1% PLP at 4 °C. Employing super-resolution microscopy, it was evaluated whether the density of detected structures is dependent on the fixative. The experiment was performed exactly as described for Figure 5, though in these experiments no parallel experiments without primary antibody have been performed, which would allow for background correction. Values are given as mean ± SD (n = 3 biological replicates, for each replicate at least 20 membrane sheets were analyzed).



Supporting Figure 5. Additional analyses of the APP antibody staining.

(A) APP immunostaining intensity measured on membrane sheets from APP-GFP overexpressing cells and from in parallel prepared membrane sheets from non-overexpressing cells. Upon APP overexpression the intensity level increases by 27-fold on average. The overexpressing condition includes the same membrane sheets used for the PCC analysis (Fig. 3B; 107 membrane sheets from three experiments). For the non-overexpressing condition, 206 membrane sheets were collected from two independent experiments. Values are given as mean ± SD (n = 2 – 3 biological replicates).

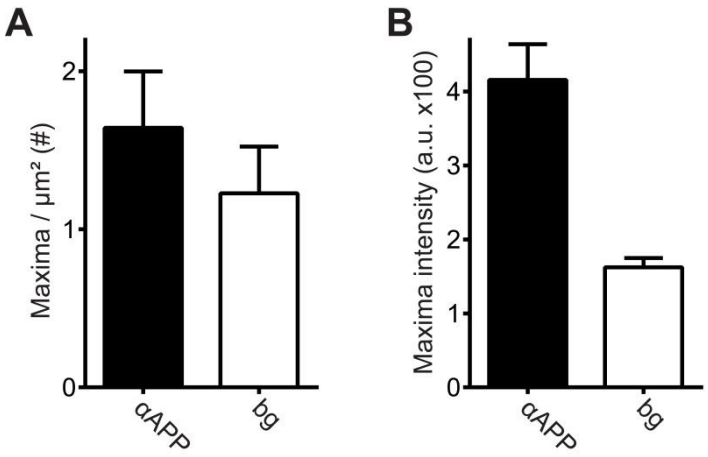
(B) The relative standard deviation is a measure for the degree of clustering and diminishes the more uniform the signal distribution is. From the above mentioned 107 membrane sheets, the relative standard deviation of the APP immunostaining signal is plotted against the average intensity, which increases with higher levels of overexpression. The relative standard deviation decreases with increasing APP levels, suggesting that at higher APP levels the non-crowded signal increases. This saturation effect may be due to the depletion of intracellular components that could be required for aggregation (1,2). The black dot indicates the value obtained for the upper left membrane area shown in Figure 3A.



Supporting Figure 6. Maxima intensity distributions under variable staining conditions.

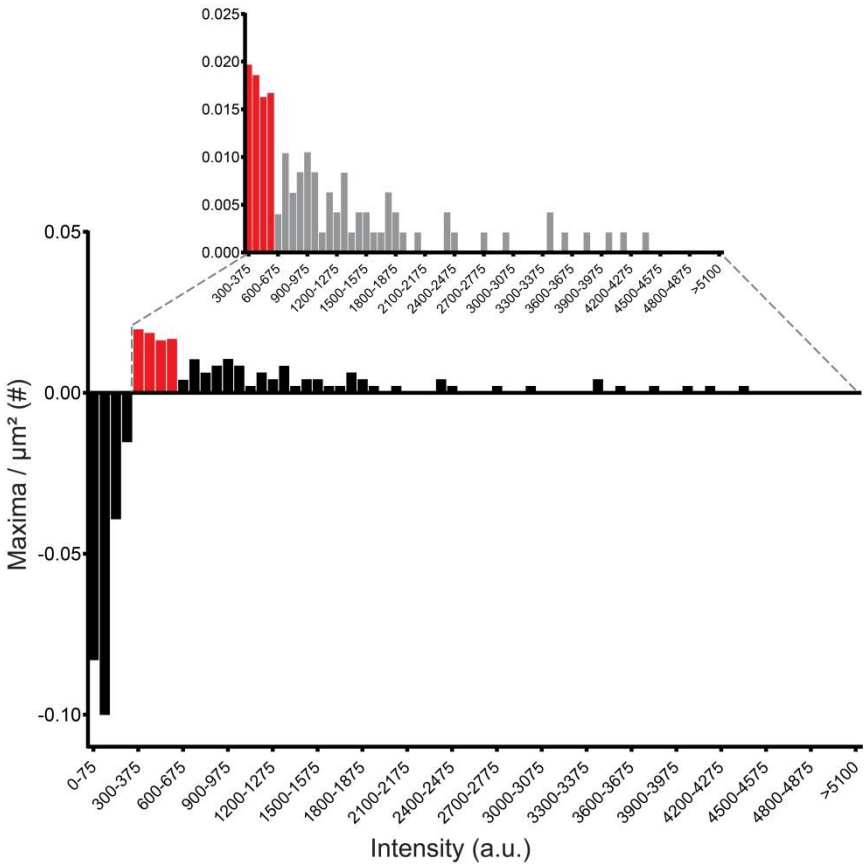
(A) Standard staining in which the primary antibody was diluted 1:200 (in the text referred to as 'αAPP'), (B) stochastic labeling by diluting the primary antibody 1:8,000, or (C) omitting the primary antibody (referred to as 'background' in the text). For each histogram, data from three independent experiments were pooled (1:200, at least 56 membrane sheets per

experiment; 1:8,000 and no antibody, at least 28 membrane sheets per experiment). Histograms with black bars show the intensity distribution over the intensity range spanned by the 1:200 condition. Histograms with grey bars are zoomed-in views of the higher intensity ranges with a different scaling of the y-axis. For each condition one example image is shown. Antibody stains are shown at the same scaling to allow direct comparison of the intensities. For the histogram in Figure 4B, the histogram 'no αAPP antibody' ('background') was subtracted from the histogram '1:200 αAPP antibody' ('αAPP'). Figure 4A shows the same images as in (A), although at a different scaling of the antibody stain to better illustrate the dynamics in intensity from the brighter spots. Scale bars, 2 µm.



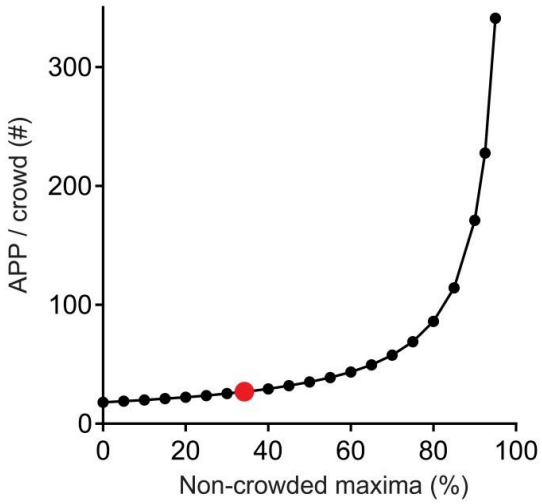
Supporting Figure 7. 'Maxima per imaged µm²' and 'maxima intensity' in epifluorescence micrographs.

(A) Maxima per imaged μm^2 in the presence ('αAPP') and absence (background, 'bg') of primary antibody. Background correction of 'αAPP' yields 0.414 APP maxima per imaged μm^2. (B) Background maxima were on average 2.6-fold dimmer than 'αAPP' maxima, which include the 'bg' maxima. Values are shown as means ± SD (n = 3 independent experiments).



Supporting Figure 8. Intensity of stochastically labeled structures after background subtraction.

Histogram obtained after subtracting the histogram ‘no αAPP antibody’ (Fig. S6C; in the text referred to as ‘background’) from the histogram ‘1:8000 αAPP antibody’ (Fig. S6B). A first population of primary antibody dependent maxima arises at intensities between 300 and 600 counts (red bins in the histogram). The histogram with the grey bars is a zoomed-in view with a different scaling of the y-axis.



Supporting Figure 9. Dependence of the average copy number per crowd from the fraction of non-crowded molecules.

We determined a surface concentration of 9 molecules per µm² (see text) and 0.5 maxima per µm² (see legend of Fig. 5B). Based on these values, the number of molecules per crowd is a function of the fraction of non-crowded molecules, as stated in the following equation:

$$\#\,\text{APP / crowd} = \frac{\text{total molecules / µm²} \;-\; \text{non-crowded molecules / µm²}}{\text{total maxima / µm²} \;-\; \text{non-crowded maxima / µm²}}$$

The calculation is explained for the value of 34% of 'non-crowded' APP maxima (red dot in graph; this value was determined in Fig. 4B). This means that the 0.5 maxima per μm^2 divide into 0.17 'non-crowded' APP (34%) and 0.33 'crowded' APP maxima per µm². The 'non-crowded' APP maxima equates the 'non-crowded' APP molecule density. Subtracting this density from the 9 molecules per μm^2, we are left with 8.83 'crowded' APP molecules per μm^2, which are distributed over the 0.33 'crowded' APP maxima per µm². This yields on average 27 APP molecules per crowd. The average number of molecules per crowd does not change much with the percentage of monomers over a range from 0 - 50% (please note the graph's small slope in the range from 0 – 50%).

SUPPORTING REFERENCES

1. Schneider, A., L. Rajendran, M. Honsho, M. Gralle, G. Donnert, F. Wouters, S. W. Hell, and M. Simons. 2008. Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. J. Neurosci. 28:2874–2882.

2. Schreiber, A., S. Fischer, and T. Lang. 2012. The amyloid precursor protein forms plasmalemmal clusters via its pathogenic amyloid-β domain. Biophys. J. 102:1411–1417.