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Supplemental Information

Deleterious Effects of Amyloid β Oligomers Acting as an Extracellular Scaffold for mGluR5

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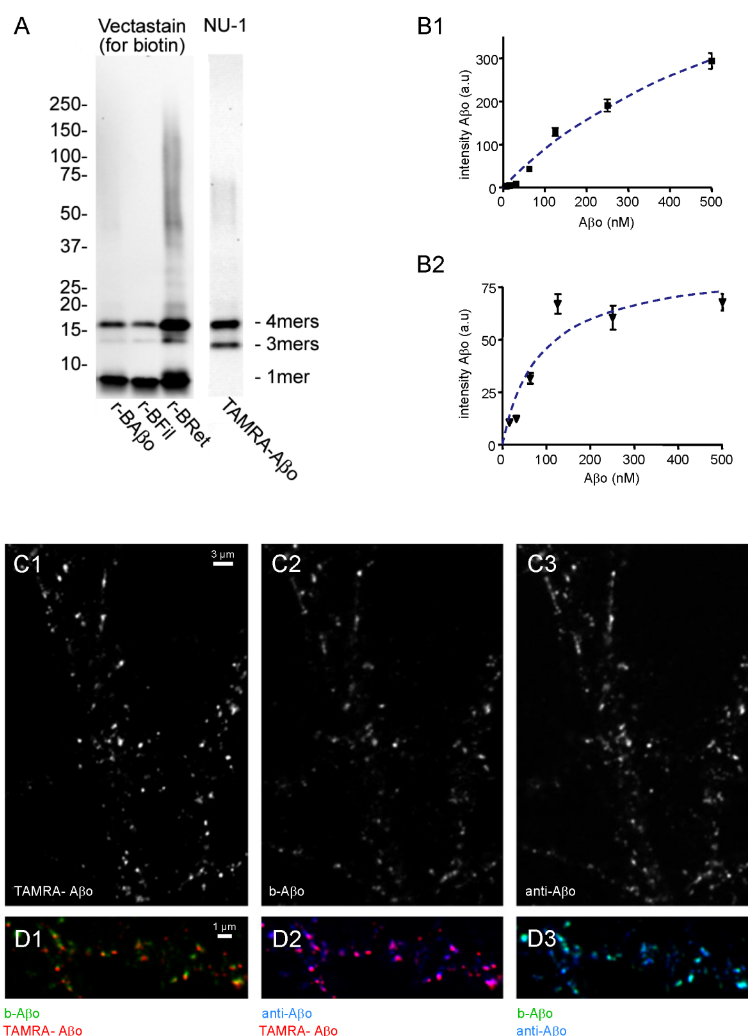
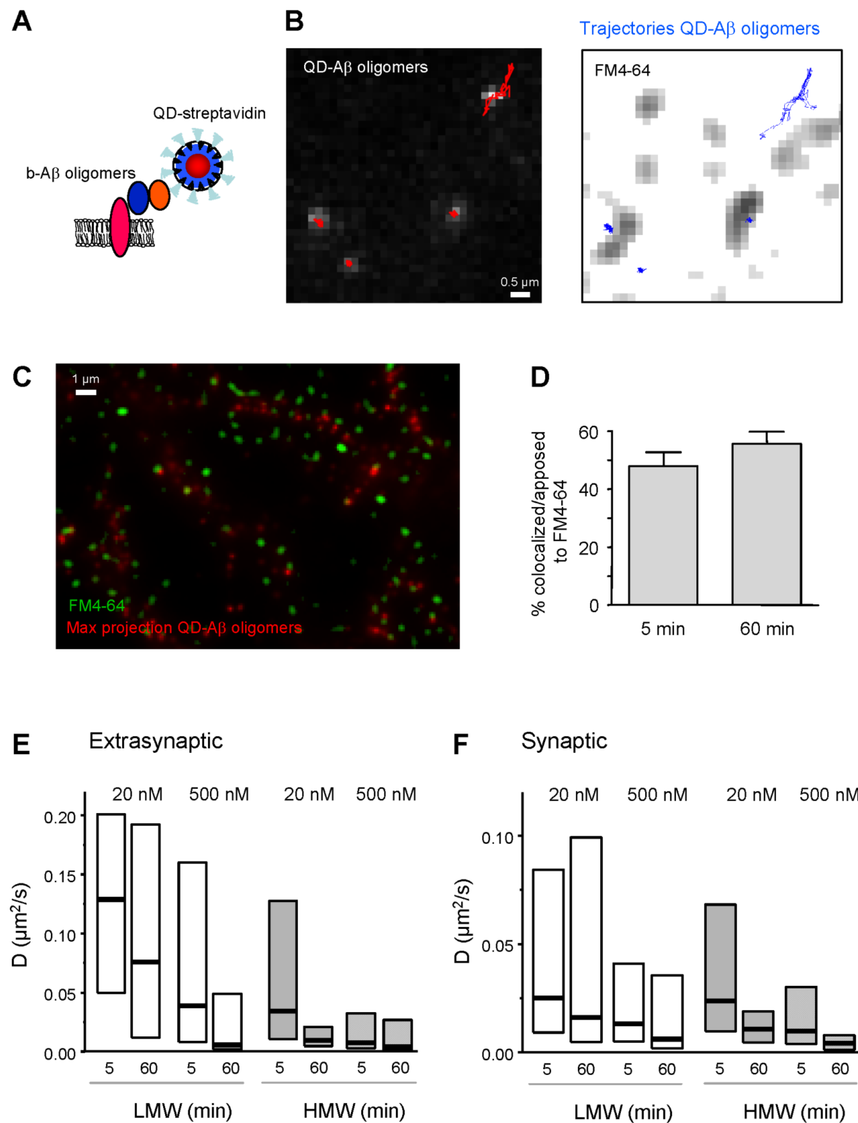
Fig. S1: Aβo species and detection (related to Figure 1)



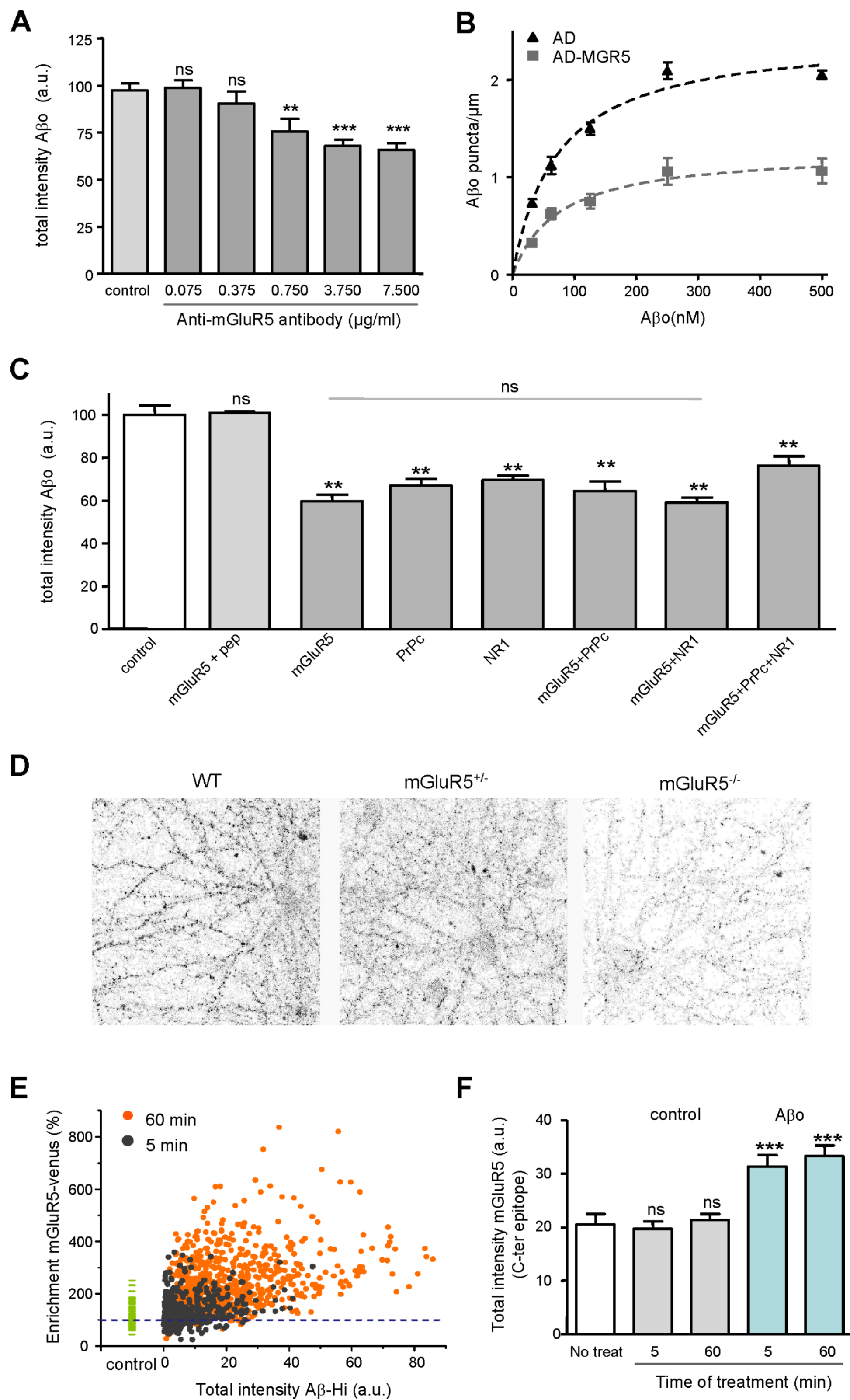
A: Dried and reconstituted whole solutions of b-Aβo (r-BAβo), fractionated solutions of b-Aβo (species <50kDa: r-BFil; species >50kDa: r-BRet) or TAMRA-Aβo were separated using SDS-PAGE and analyzed using avidin detection (Vesctastain) or immunoblotting using NU-1 (a monoclonal antibody raised against Aβo). Fractionation of biotinylated Aβo generated tetramers, trimers and monomers in r-BFil fractions while residual higher molecular weight species were present in r-BRet fractions in accordance with our previous description in Chromy et al., 2003. TAMRA-Aβo preparations show patterns similar to unmodified Aβo with the presence of trimers, tetramers and trace residual high molecular weight oligomers. **B1-2**: Dose-response curves of FAM-Aβo binding to mature hippocampal neurons for 15 min at 4°C (B1) and 37°C (B2) (mean ± s.e.m. from 25 individual dendritic branches at each concentration). Data are expressed as integrated fluorescence intensity and were fit by nonlinear regression (dashed lines). **C1-3**: Fluorescence of TAMRA-Aβo (C1), b-Aβo detected by streptavidin (C2) and immunoreactivity of both (NU1 anti-Aβo antibody, C3) in cells that were treated with a mixture of TAMRA-Aβo and b-Aβo (250 nM each) for 5 min. **D1-3**: Co-localization of TAMRA-Aβo and streptavidin labeling of b-Aβo (D1), TAMRA-Aβo and immunoreactivity of NU-1 (D2) and b-Aβo and NU-1 (D3).

Fig. S2: QD labeling and single particle tracking (SPT) of b-Aβo (related to Figure 3)



A: Cartoon of the labeling of b-Aβo with streptavidin-QDs. **B**: Left: Image of QDs (bright spots) and the trajectories (red lines) obtained by tracking them over 3 s. Right: corresponding image of synapses, labeled with FM4-64 (grey), and the final trajectories after 7.5 s of recording (blue). **C**: Maximum projections of QD images over a stack of 250 frames (red), overlaid to synaptic staining by FM4-64 (green). Neurons were labeled with high concentration of QDs (50 times more than for SPT) to check the distribution of QD-bound Aβo. **D**: Quantification of the colocalization of QD-Aβo and the synaptic staining as in C, after 5 min or 60 min of b-Aβo application (mean ± s.e.m., n=5 neurons). **E-F**: Diffusion coefficient D (median, 25% and 75% IQR) for low-molecular-weight Aβo (LMW-Aβo, white boxes) or high-molecular-weight Aβo (HMW-Aβo, gray boxes) on extrasynaptic (E) or synaptic (F) membranes (extrasynaptic: n=601-1017, synaptic: n=267-779). Aβo were applied at the indicated concentrations and times. Note the concentration and time-dependent reduction of diffusion.

Fig. S3: Inhibition of Aβo binding and effect of Aβo on the distribution of mGluR5 (related to Figure 4)

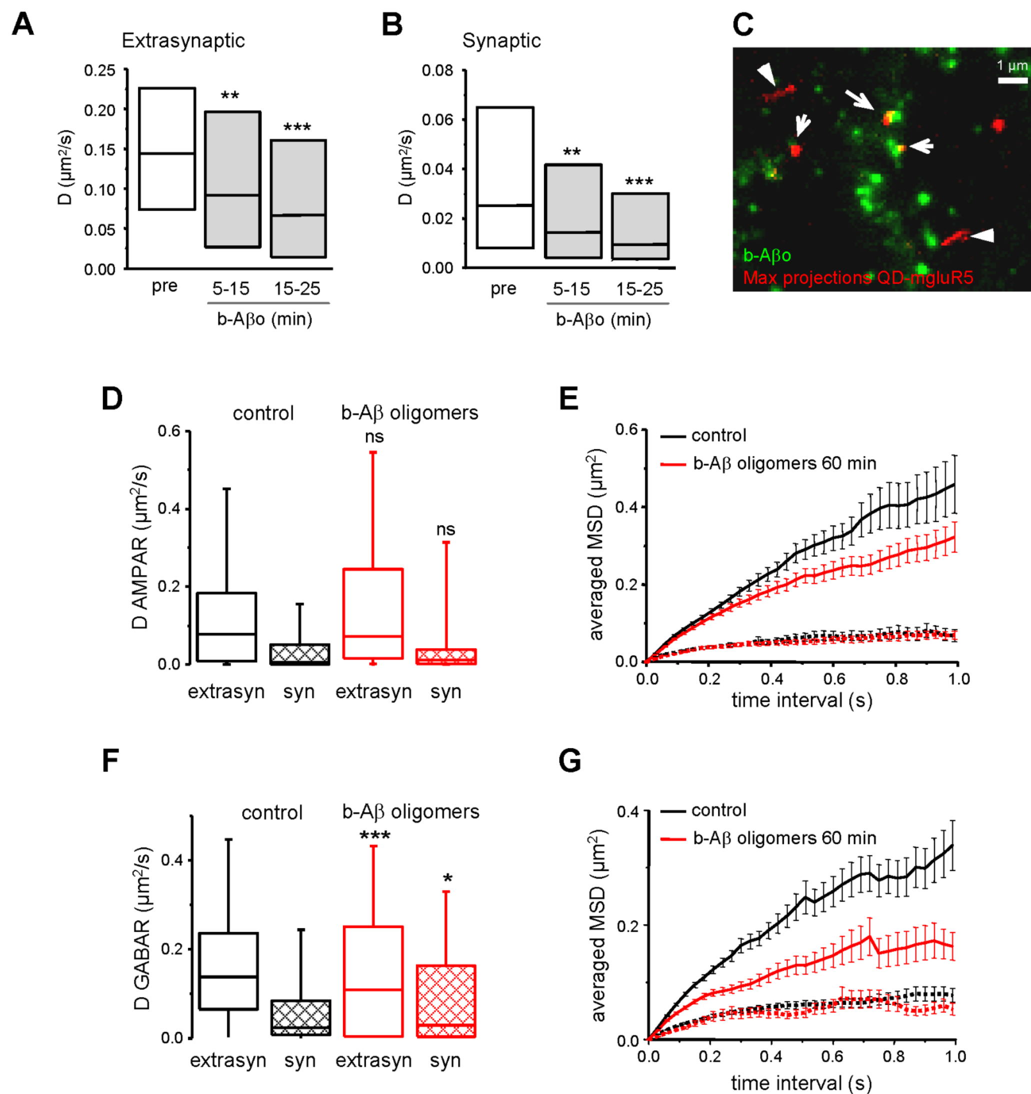


A: Quantification of streptavidin-labeled b-Aβo in cells that were previously incubated with the indicated amounts of N-ter anti-mGluR5 antibody (mean ± s.e.m., n=16-21 dendritic regions, t-test, ns: not significant, **: $p<0.01$, ***: $p<0.001$). **B:** Quantification of FAM-labeled Aβo binding in the presence (AD-MGR5) or absence (AD) of mGluR5 antibody. FAM-Aβo were applied for 15 min at the indicated concentrations (mean ± s.e.m., n= 25). **C:** Quantification of 500nM FAM-labeled Aβo binding in the presence of 5ug/ml of antibodies raised against the extracellular portion of mGluR5 (mGluR5), Prion (antibody 6D11; PrPc), NMDAR subunit 1 (NR1) or a combination of these antibodies compared to binding in the absence of antibody (control) or the binding in presence of mGluR5 antibody pre-absorbed with its blocking peptide (mGluR5 + pep) (mean ± s.e.m., n=25 independent dendritic branches in each group, Newmann-Keuls test, ns: not significant, **: $p<0.01$). **D:** Fluorescence images in inverted hue of FAM-Aβo applied for 15 min to hippocampal neurons from wild type (WT), heterozygous (+/-) or homozygous (-/-) mGluR5 KO mice. **E**: Quantification of the enrichment of mGluR5-Ve in spines vs the fluorescence intensity of HiLyte555 tagged Aβo (Hi-Aβo) after the application of the oligomers for 5 min (grey) or 60 min (orange) (n= 531 and 776 spines of 25-43 neurons, respectively). In green, control cells (n=194 spines of 10 neurons). Enrichment was quantified as the total intensity on top of spine divided by the total intensity outside spines on the same cell (see Experimental Procedures). **F**: Quantification of total intensity of mGluR5 clusters using an antibody directed to an intracellular epitope of the receptor, on non-treated neurons (No treat) or after 5 or 60 min of application of the control solution (control, grey bars) or 500nM b-Aβo (Aβo, light blue bars) (mean ± s.e.m., n= 15-24 dendritic regions, t test, ns: not significant, ***: $p<0.001$).

Supplemental video (related to Figure 5)

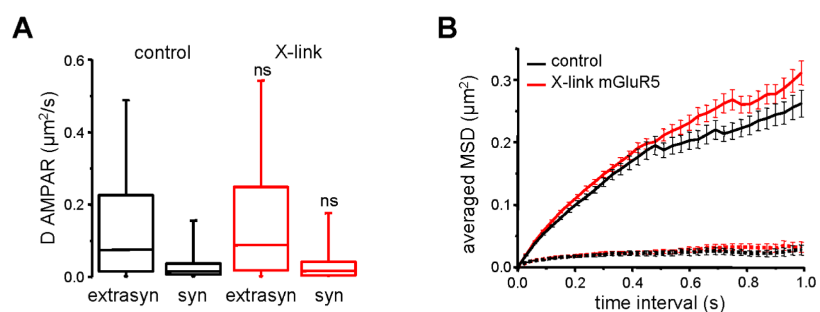
SupVideo.mov: Neurons were transfected with Homer-EGFP (as in Renner et al., 2009) to identify synapses. b-Aβo were applied at 20 nM for 10 min and still display fast diffusion outside synapses. Dual-color SPT was performed on a SPT experimental set-up equipped with an image splitter (DualView, Photometrics, Germany), which allows simultaneous dual-color fluorescence imaging. To visualize two QD emitting at different wavelengths, an appropriate set of filters and dichroic lens were used (D455/70x, HQ605/20m, Chroma Technologies, VT, USA; FF01-655/15-25, Semrock, NY, USA; 630DRLP, Omega Optical, VT, USA). mGluR5 and b-Aβo were tracked as described in the Experimental Procedures section, using QDs emitting at 655 nm for mGluR5 and QDs emitting at 605 nm for the oligomers. Individual QDs were recognized by their blinking behavior (on and off states). In the video, one QD-mGluR5 (red) diffuses together with a QD-Aβo (green) outside synapses (blue) before being both stabilized at a synaptic site.

Fig. S4: Effect of Aβo on the distribution and mobility of mGluR5, AMPAR and GABAR (related to Figure 5)



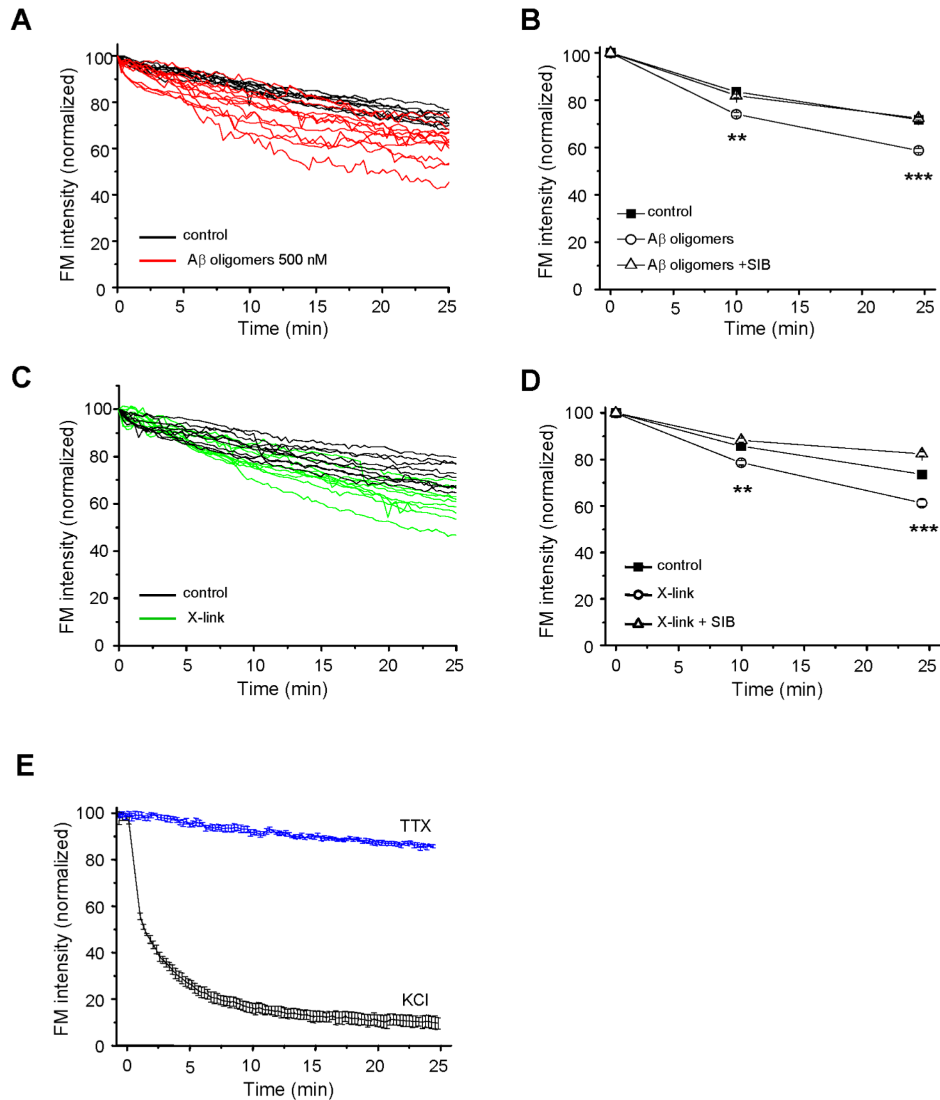
A-B: Diffusion coefficient D (median, 25% and 75% IQR) for QD-mGluR5 trajectories on extrasynaptic (C) and synaptic (D) membranes before (pre) or after the indicated times of b-Aβo application (500 nM) (extrasynaptic: n=126-355, synaptic: n=55-198, KS test; **: $p<0.01$, ***: $p<0.0001$). **C:** Fluorescence of streptavidin-labeled b-Aβo (green) and the maximum projections of QD-mGluR5 (red) after the SPT experiment. QD-mGluR5 were applied before b-Aβo. Some of the QDs colocalize with b-Aβo (arrows). Note that the diffusion of these QDs is low in comparison to others that do not colocalize with b-Aβo (triangles). **D**: Diffusion coefficient D (median, 25% and 75% IQR, 5-95% confidence intervals) for QD-AMPAR trajectories on extrasynaptic (extrasyn) and synaptic (syn) membranes in control cells (black) or after 60 min of Aβo application (500 nM; red) (KS test; ns: not significant). **E**: Averaged MSD (mean ± s.e.m.) plot of the same trajectories analyzed in D. Solid lines: extrasynaptic trajectories. Broken lines: synaptic trajectories. Same color coding as in D. **F**: Diffusion coefficient D (median, 25% and 75% IQR, 5-95% confidence intervals) for QD-GABAR trajectories on extrasynaptic (extrasyn) and synaptic (syn) membranes in control cells (black) or after 60 min of Aβo application (500 nM; red) (KS test; *: $p<0.05$, ***: $p<0.0001$). **G**: Average MSD (mean ± s.e.m.) plot of the same trajectories analyzed in F. Solid lines: extrasynaptic trajectories. Broken lines: synaptic trajectories. Same color coding as in F.

Fig. S5: Effect of mGluR5 cross-link on the lateral diffusion of AMPAR (related to Figure 6)



A: Diffusion coefficient D (median, 25% and 75% IQR, 5-95% confidence intervals) for QD-AMPAR trajectories on extrasynaptic (extrasyn) and synaptic (syn) membranes in control cells (black) or after 60 min of cross-linking of mGluR5 (red) (KS test; ns: not significant). **B**: Averaged MSD (mean ± s.e.m.) plot of the same trajectories analyzed in A. Solid lines: extrasynaptic trajectories. Broken lines: synaptic trajectories. Same color coding as in A.

Fig. S6: Impact of Aβo and cross-linking of mGluR5 on FM4-64 unloading (related to Figure 8)



A-D: FM unloading during continuous application of Aβo (500 nM) (A-B) or mGluR5 cross-linking (C-D). **A-C:** characteristic examples of the FM4-64 intensity measurements performed in control (black) or treated (red or green) cells. **B**: Comparison of FM4-64 cluster intensity at three time points in control conditions (squares), and after Aβo (circles) or Aβo together with the mGluR5 antagonist SIB 1757 (Aβo+ SIB, triangles) treated neurons (mean ± s.e.m., t test; **: $p<0.01$, ***: $p<0.0001$; n=13-47 fields of observation). **D**: Same as in B in control conditions (squares), and after mGluR5 cross-linking (circles) or mGluR5 cross-linking together with SIB (X-link+SIB, triangles) treatments (mean ± s.e.m., t test; **: $p<0.01$, ***: $p<0.0001$; n=15 fields of observation). **E**: FM unloading depends on the overall activity of cultured neurons. The addition of KCl (40 mM) to the cellular medium at time=0 rapidly reduced FM fluorescence (black) in comparison to the basal line obtained in presence of TTX (blue) (mean ± s.e.m, n=15 fields of observation).

Supplemental experimental procedures

Neuronal cultures

Hippocampal neurons from 18-day-old Sprague-Dawley rat embryos were cultured at a density of $6x10^4$ cells/cm^2 on coverslips pre-coated with 80 µg/ml poly-D,L-ornithine (Sigma Aldrich, France). Alternatively, cells were plated at a density of $2x10^4$ cells/ cm^2 on coverslips coated with 0.002% poly-L-lysine (Sigma Aldrich, USA). In case of mGluR5 KO mice, cultures were done from P1 pups. Each pup was genotyped as described in Xu and collaborators (2009). Freshly dissociated cells were first plated in culture media with 5% fetal calf serum (Invitrogen, Carlsbad, CA) for 45 min, and then maintained in serum-free neurobasal medium supplemented with B27 (1X) and glutamine (2 mM). Pure neuron cultures were obtained plating cells at $10x10^4$ cells/cm^2 on coverslips pre-coated with 80 µg/ml poly-D,L-ornithine (Sigma Aldrich, France) and maintaining them in neurobasal medium previously conditioned by astrocyte cultures and supplemented with AraC (5 µM).

Cultures of astrocytes were obtained from dissociated cortex from 18-day-old Sprague-Dawley rat embryos, plated at a density of $20x10^4$ cells/cm^2 on 10 cm dishes pre-coated with 15 µg/ml poly-D,L-ornithine (Sigma Aldrich, France). Cells were maintained in MEM supplemented with fetal bovine serum (10%), Na-pyruvate (1 mM) and glutamine (2 mM). After they became confluent, cultures were treated with AraC (5 µM) for 48 h to eliminate microglia and they were then used to pre-condition neurobasal medium for pure neuron cultures.

Mature neuronal cultures with complete synaptic maturation (21-27 days in vitro, DIV) were used in all experiments.

Unmodified and modified Aβ oligomer (Aβo) preparations and SDS-analysis

The following Aβ1-42 peptides were used to prepare synthetic Aβ oligomers (Aβo): unmodified or amino-terminus biotin-tagged peptide (American Peptide, Sunnyvale, CA); tetramethylrhodamine (TAMRA)-, fluorescein amidite (FAM)-, or HiLyte555-labeled peptides (AnaSpec®, San Jose, CA). The protocol was described previously (Klein 2002; Lacor et al., 2004, 2007). Briefly, Aβ1-42 was dissolved in hexafluoro-2-propanol (HFIP) which was then removed by lyophilization and stored at –80°C. Aβ1-42 was dissolved in anhydrous DMSO to make a 5 mM solution, then added to cold F12 medium (Invitrogen) to make a 100 µM solution. This solution was incubated at 4°C for at least 24 h and then centrifuged at 14,000 x g for 10 min. The supernatant contained Aβ oligomers. For modified (biotinylated and fluorescent) Aβo, DMSO solutions of modified and unmodified Aβ were combined in a 1:3-1:5 ratio before dilution in F12 medium. Molecular weight fractionation of Aβo was obtained using Microcon YM-50 (Amicon, Bedford, MA) as reported (Lacor et al., 2007). Preparations were dried for shipping purpose by speed vacuuming. Aβo used here are mostly species above 50kDa (HMW) otherwise indicated in the text. After reconstitution of previously dried Aβo preparations, we conducted SDS-polyacrylamide gel electrophoresis on biotinylated or fluorescently-tagged Abo preparations (r-BAβo and TAMRA-Aβo respectively) as well as biotinylated Aβo fractionated by 50kDa cut-off filter (species <50kDa: r-BFil; species >50kDa: r-BRet) (Fig. S1A). Aβo species were analyzed using avidin detection (Vesctastain) or immunoblotting using NU-1 (a monoclonal antibody raised against Aβo; Lambert et al, 2007).

Aβo modified with TAMRA fluorophore formed clusters together with biotinylated Aβo (b-Aβ oligomers) (Fig. S1 B,C). When applied together at 250 nM each, they formed clusters co-localizing in 89.78 ± 1.05% (Fig. S1C1). NU1 antibody recognized 95.46 ± 0.56% of TAMRA-Aβ oligomer clusters (Fig. S1 C2) and 97.72 ± 0.14% of b-Aβo clusters (Fig. S1 C3). The difference from 100% represents the percentage of clusters that did not incorporate Aβo with the fluorescent or biotin moiety.

Immunocytochemistry and quantification of synaptic proteins and Aβ oligomers.

Cells were fixed for 15 min at room temperature in 4% (w/v) paraformaldehyde (Serva Feinbiochemica, Germany) and permeabilized or not for 2 min with 0.3% (v/v) Triton X-100. After 15-min incubation at room temperature in 3% (w/v) bovine serum albumin (BSA, Sigma Aldrich, France) in PBS, they were incubated for 30 min at room temperature with primary antibodies in 3% BSA. After washing they were incubated for 30 min at room temperature with secondary antibodies, washed, and mounted on slides with Prolong (Invitrogen, USA). Images were filtered by segmentation using a multi-dimensional image analysis interface (Racine et al., 2007) implemented in Metamorph (Molecular Devices Sunnyvale, CA).

Co-isolation of synaptic proteins and Aβ oligomers

Synaptosomes (2 mg, 0.5 mg/ml) from rat cerebral cortex were treated on a rotator with 300 nM Aβo in F12 for 1 h at 37°C, centrifuged at 6000 x g for 15 min at 4°C and washed 3 times by resuspending in F12 medium for 10 min at 4°C and centrifuged ad previously described (Lacor et al., 2007). The washed synaptosomes were resuspended to 1 mg/ml in F12 with 2.5 µg/ml NU-2 anti-Aβ oligomer IgG, incubated on a rotator for 1 h at 4°C, centrifuged and washed as above. Synaptosomes were resuspended to 1 mg/ml in 1% NP40, 0.5% DOC, 0.1% SDS in 50 mM Tris-HCl, pH 8, 150 mM NaCl and incubated on ice for 30 min. The suspension was incubated on a rotator with goat anti-mouse IgG M450 Dynabeads (175 µl/mg protein) overnight at 4°C. The Dynabeads were washed 6 times with the detergent mixture for 10 min each at RT. The bound protein complex was released with 2 x 0.1% SDS at RT for 30 min each and pooled (total elution 120 µl). Samples (10 µl elution plus 5 µl 2X Laemmli buffer) were treated with 50 mM dithiothreitol at 100°C for 10 min and analyzed by Western blot with antibodies to PSD-95 (1:3000, ABR), NR1 (1:500, Santa Cruz), NR2A/B (1:500, Chemicon), GluR1 (1:50, Calbiochem), AChRα7 (1:250, Santa Cruz), glycine receptor (1:2000, Synaptic Systems), EphB2 (1:500, Santa Cruz), mGluR5 (1:1000, Chemicon), Homer (1:2000, Synaptic Systems), and neuroligin (1:2000, Synaptic Systems), using HRP-linked anti-mouse or anti-rabbit IgG (Amersham) at 1:20,000 as the secondary antibody.

Similarly, the kinetic of mGluR5 and Aβo co-isolation in triton-resistant DOC-extractable fraction was studied on neurons in culture. Cells were treated with vehicle or unfractionated Aβo preparations at different concentration (52, 125 and 300nM) for 5 or 60 min at 37°C in conditioned media, cells are washed and subsequently incubated with anti-Aβo IgG (NU-2) at 1µg/ml and kept on ice for 1hr. After washed, cells are treated with 0.15% Triton X-100 in PHEM Buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgCl2, pH 6.9) for 10 min at RT. Cells are treated with DOC pH 9 buffer (50mM Tris pH9, 1% sodium deoxycholate, 50mM NaF, 1mM Na_3VO_4, 20µM ZnCl, 0.5mM PMSF, 2 µg/ml Aprotinin, 2 µg/ml Leupeptin), a buffer described for NMDAR-complex isolation from synapses (Husi and Grant, 2001), for 30 min and collected (Lysates). Lysates were boiled in SDS sample buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins in the gel were transferred to a nitrocellulose membrane which was subsequently probed with antibodies and visualized by enhanced chemiluminescence detection (SuperSignal, ThermoScientific). mGluR5 and Aβo were detected respectively by anti-mGluR5 (Alomone) or NU-1 antibody at 1:1,000 dilution.

FM unloading

These experiments were performed using pure neuronal cultures. In each experiment, 5-8 fields containing typically 3-4 cells were imaged. The decrease of FM4-64 fluorescence, that depends on the overall activity of neurons (Fig. S4E), was monitored following the protocol described in Gaffield and Betz (2006) modified as follows. Neurons were loaded with FM4-64 by incubating them at 37°C with the FM solution (2 µM FM4-64, 40 mM KCl) for 1 min, rinsed and incubated at 37 °C in MEMr for 5 min to allow complete loading of synaptic vesicles with the dye. After recordings prior to the treatments to establish the initial baseline, neurons were treated for the corresponding experiment (Aβo or cross-linking) for 5 min and then the intensity of FM was registered by time-lapse recordings (one image each 15 sec) for an additional 25 min in the presence of the drugs. When SIB1757 was used, cells were previously incubated with this drug (3 µM) for 30 min. For each recording, the total fluorescence intensity of 10 FM4-64 spots chosen randomly was averaged. The figures S4 A,C show examples of the averaged recordings in one experiment. The application of Aβo significantly increased the rate of FM unloading (two-tailed t-test; after 10 min, $p=0.0086$; after 25 min, $p<0.0001$) (Fig. S 4B). This effect was prevented when the neurons were previously treated with SIB1757 (Fig. S 4B). Similar results were obtained after mGluR5 cross-linking (t-test; after 10 min, $p=0.0066$; after 25 min, $p<0.0001$) (Fig. S 4D).

Supplemental References

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Racine, V., Sachse, M., Salamero, J., Fraisier, V., Trubuil, A., and Sibarita, J. B. (2007). Visualization and quantification of vesicle trafficking on a three-dimensional cytoskeleton network in living cells. J Microsc 225, 214-228.