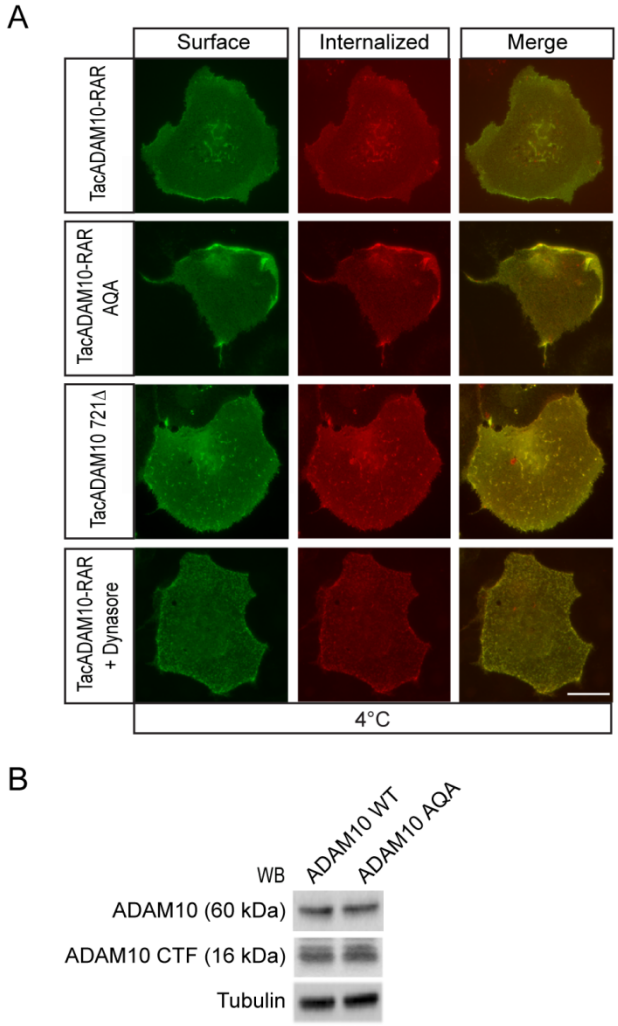
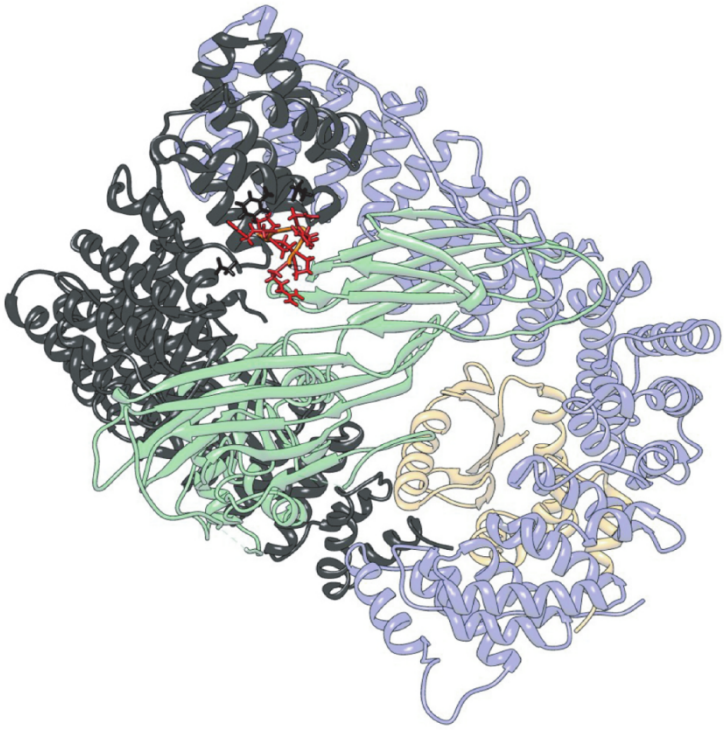
Suppl. Fig. 1



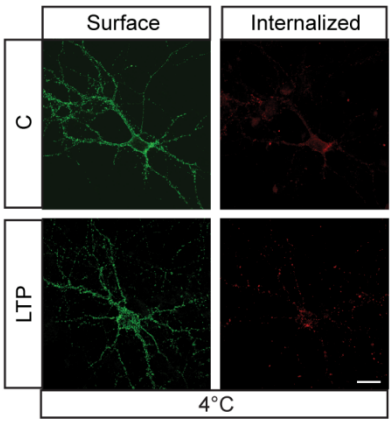
Suppl. Figure 1. Evaluation of the internalization in COS7 cells kept at 4°C and ADAM10 shedding. (A) Antibody uptake assays were performed on COS7 cells transfected with either TacADAM10-RAR or TacADAM10-RAR AQA or the deletion mutant Tac721Δ. Representative images of surface (green) and internalized (red) Tac chimera expressed in COS-cells kept at 4°C. As expected, at 4°C there was no internalization. Scale bar, 20 µm. **(B)** WB analyses of lysates from HEK293 cells transfected with ADAM10 WT and ADAM10 AQA. The levels of ADAM10 CTF are not modified by the lack of AP2 binding of ADAM10 AQA mutant.

Suppl. Fig. 2



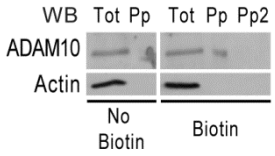
Suppl. Figure 2. Ribbon representation of the AP2 complex with the peptide bound to the β2 subunit represented in dark grey. The colour code for the peptide and the interacting amino acids is as in Figure 4B and 4E, respectively.

Suppl. Fig. 3

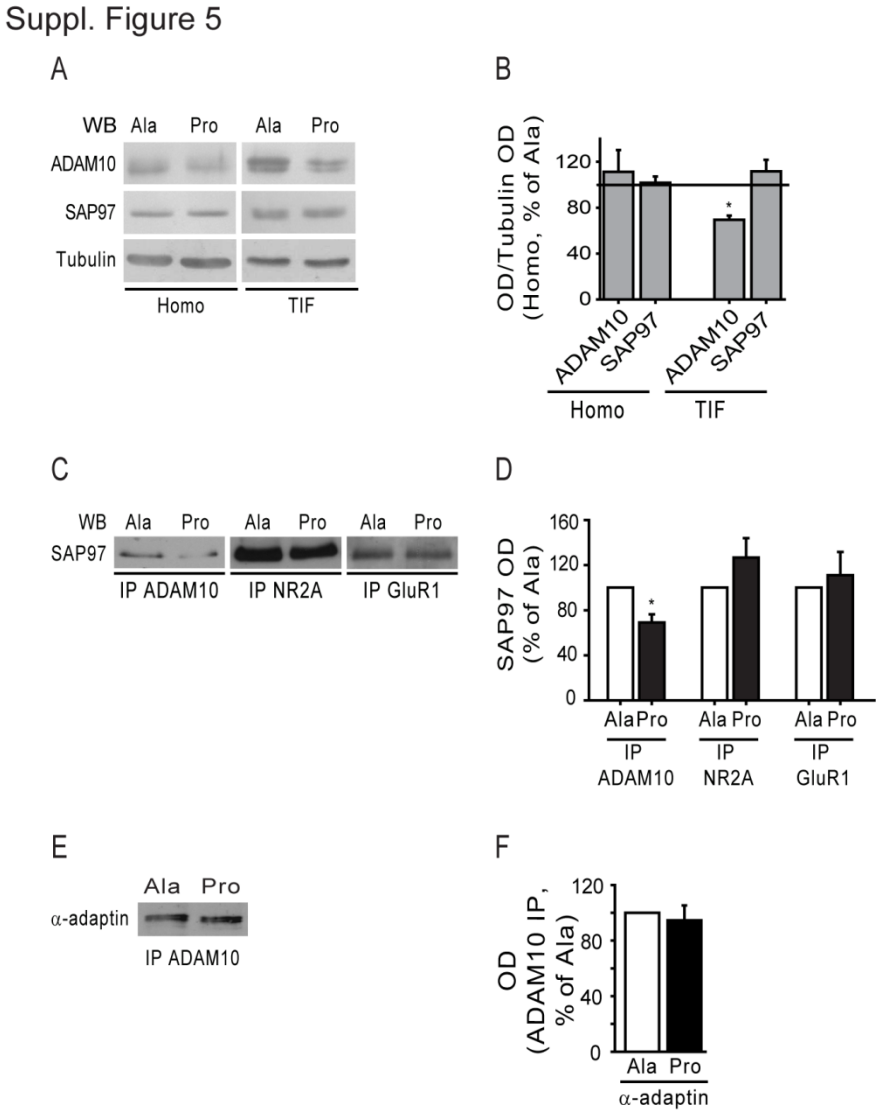


Suppl. Figure 3. Analysis of the internalization in neurons kept at 4°C. Antibody uptake assays were performed on neurons expressing TacADAM10-RAR either exposed or not exposed to cLTP treatment. Neurons kept at 4°C show membrane expression of TacADAM10-RAR (green), and low levels of internalization (red). No significant differences between control and cLTP were observed when trafficking was arrested.

Suppl. Figure 4



Suppl. Figure 4. Evaluation of the efficiency of the biotinylation assay. In total membrane fraction (tot), a band corresponding to ADAM10 is detectable, while no signal is visible in neutravidin-precipitated samples (Pp) of cells not exposed to biotin. When cells are incubated with biotin, in the neutravidin-precipitated samples (Pp) the mature form of ADAM10 is present, demonstrating that it is at the surface. The precipitation is efficient because no ADAM10 is detected when the supernatant recovered from the precipitation was reprecipitated with neutravidin beads, and the additional neutravidin-precipitated material was loaded as well (Pp2). Moreover, virtually no actin is detected in the neutravidin-precipitated material, indicating that only cell surface proteins were recovered.



Suppl. Figure 5. Evaluation of the efficiency and specificity of Pro peptide. (A) WB analysis of homogenate and TIF of hippocampal slices incubated with either Pro or Ala cell-permeable peptides (1μM, 30 min). ADAM10 but not SAP97 localization in TIF is reduced by Pro peptide treatment. Total protein levels are not affected by Pro peptide. **(B)** Quantification of OD of experiments in **A** (TIF; ADAM10=- 31±3.7%, P=0.0004 Pro versus Ala, SAP97=+11±10%, P>0.05 Pro versus Ala, n=6; Homo; ADAM10=+11±19.6%, SAP97=+2.1±6%, P>0.05 Pro versus Ala, n=6). **(C)** Coimmunoprecipitation assays were carried out from total homogenate of slices treated with either Pro or Ala cell-permeable peptides (1μM, 30 min). Samples were incubated with antibody raised against ADAM10 or NR2A or GluR1 and the presence of SAP97 in the immunocomplex was evaluated by WB. Pro peptide specifically interferes with ADAM10/SAP97 association, but does not affect the binding of SAP97 to NR2A or GluR1. **(D)** Quantification of OD of experiments in **C** (IP ADAM10: -31±8%, P=0.009 Pro versus Ala; IP NR2A: +26.5±19.3%, IP GluR1: +11±21.3%, P>0.05 Pro versus Ala, n=6). **(E)** Co-immunoprecipitation experiments carried out from hippocampal slices treated with either Ala or Pro peptide (1μM, 30 min). α-adaptin binding to ADAM10 is not affected. **(F)** Quantification of ADAM10 OD of experiments in **E** (a-adaptin: -5.4±10.7%; P>0.05, Pro versus Ala, n=6).

SUPPLEMENTAL METHODS

Antibodies and cell-permeable peptide. The following antibodies were used: mAb 4G8 (against 17-24 aa of Aβ) was purchased from Covance; pAb recognizing ADAM10 N-terminal and pAb anti APP domain 653-662 (Ab2072) from Abcam; rat mAb against ADAM10 ectodomain from R&D; pAb recognizing ADAM10 C-terminal domain from Proscience; pAb anti-GluR1 from Calbiochem; mAb 22C11 recognizing the N-terminal domain of APP (MAB348) and anti-GluR1 phosphoSer845 (04-1073) from Millipore; mAb anti-SAP97 from Stressgen; pAb anti-Actin, mAb anti-Tubulin were purchased from Sigma; mAb anti-ADAM22, mAb anti-GST, mAb anti-GFP from Neuromab; mAb anti-NR2A from Invitrogen Life Technologies; mAb against α-adaptin, ε-adaptin, γ-adaptin, μ2 subunit were purchased from BD; pAb against β2-adaptin, from Santa Cruz Biotechnology; mAb anti-Tac antibody (clone 7G7) was kindly provided by Dr. Bonifacino (National Institutes of Health, Bethesda, MD 20892, USA). AlexaFluor 488 and 555 secondary antibodies were purchased from Invitrogen; peroxidase conjugated secondary antibodies from Pierce. The cell-permeable peptides Pro and Ala (Pro: NH2-YGRKKRRQRRRPKLPPPKPLPGTLKRRRPPQP-COOH, Ala: NH2- YGRKKRRQRRRAKLAAAKALAGTLKRRRAAQA-COOH) were synthesized by Bachem.

DNA constructs. ADAM10 cDNA was provided by Dr. Saftig (Biochemical Institute, Christian-Albrechts-University Kiel, Kiel, Germany). Generation of chimeras of the extracellular domain of the human interleukin receptor Tac with the intracellular C-terminal domain of mouse ADAM10 (TacADAM10), the mutant TacADAM10-RAR and the deletion mutant Tac721Δ have been previously described (1). Generation of GST ADAM10 Ct fusion protein has been previously described. Deletion mutants GST747Δ, GST741Δ, GST737Δ, GST734Δ and GST721Δ were generated by PCR amplification using the same forward primer and different reverse primers. After digestion with BaMHI and SalI, PCR fragments were ligated into the linearized pGex-KG vector. TacADAM10-RAR AQA and GST ADAM10 Ct point mutants were generated using the quick-change site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. All constructs were verified by sequencing.

Acute hippocampal slices, cell cultures and transfection. Acute hippocampal slices were prepared as previously described (2, 3). Hippocampal neuronal cultures were prepared from E18-E19 rat hippocampi as described (4). Neurons were transfected using the calcium phosphate precipitation method at 10 days in vitro. COS7 cells were transiently transfected by Superfect® Transfection Reagent (Qiagen) with cDNA expression constructs for 2-3 h at 5% CO_2, 37°C, and afterwards cells were washed twice with PBS. COS7 cells were grown for 24 h before fixation for immunocytochemistry.

SDS-PAGE. Samples were separated by electrophoresis on SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with the corresponding primary Ab followed by incubation with appropriate horseradish peroxidase-conjugated secondary Ab (Pierce). In order to analyze APP C-terminal fragments (CTFs), TIF proteins were separated by 15% Tris-Tricine SDS-PAGE and WB analysis was performed with 4G8 antibody.

Determination of sAPPα release. For the evaluation of sAPPα released from control and LTD-treated cells into the cell media after 20 min of recovering, an ELISA kit was used (IBL). Before the assay, to concentrate the sample, the media were lyophilized and dissolved in water. The test was performed as indicated in the instructions of the manufacturer: a standard curve ranging from 1 to 640 pg/ml was obtained with each point corresponding to the average of two measurements. The release of sAPPα was evaluated by reference to the standard curve, and normalized to the total content of proteins of the medium sample. To detect soluble sAPPα released in one hour of recovering from control and LTP/LTD-induced hippocampal neurons, cell media were desalted by using DG10 columns (Bio-Rad), lyophilized, resuspended in sample buffer. The evaluation of sAPPα was achieved by loading the total lyophilisate and analyzed by WB with pAb anti APP, recognizing the domain 653-662.

Immunoprecipitation. Aliquots of either human hippocampi or mouse brain homogenate, either cLTP or cLTD-treated neurons homogenate, either Pro peptide or Ala peptide (1 μM, 30 min) treated neurons homogenate/acute hippocampal slices homogenate were incubated in RIA buffer

(NaCl 200 mM, EDTA 10 mM, Na2HPO4 10 mM, NP-40 0.5%, SDS 0.1%) in a final volume of 150 µl with antibody against ADAM10 (AbCaM) overnight at 4°C. Protein A/G-agarose beads were added and incubation was continued for 2 h, at room temperature, shaking. The beads were washed three times with RIA buffer. Sample buffer for SDS-PAGE was added and the mixture was heated for 3 min. Beads were collected by centrifugation and a volume of supernatants was applied onto SDS-PAGE; immunocomplex precipitated was revealed by mAb anti-SAP97, pAb anti-GluR1, mAb anti-NR2A, mAb anti α-adaptin, pAb anti β2-adaptin, mAb anti µ2, mAb anti γ-adaptin, rat Ab anti ADAM10.

GST-fusion protein production and pull down assay. GST-fusion proteins were expressed in Escherichia Coli, purified on Glutathione Agarose beads (Sigma) as previously described (2). Aliquots of mouse brain homogenate were diluted in Hepes 10 mM, NaCl 150mM, Triton 0.5% to a final volume of 1 ml and incubated for 2 h with glutathione-agarose beads saturated with fusion proteins or GST alone. After incubation period, the beads were extensively washed with Hepes 10 mM, NaCl 150 mM, Triton 0.1%. Bound proteins were resolved by SDS-PAGE and subjected to immunoblot analysis with mAb anti α-adaptin, ε-adaptin, γ-adaptin and µ2 subunits antibodies. To pull-down SAP97 from mouse brain homogenate, experiments were carried out as previously described (1).

Immunocytochemistry and colocalization analysis. The analysis of ADAM10 WT/ADAM10 AQA surface and total staining was carried out as previously described (5). To evaluate colocalization degree of surface ADAM10 WT/ADAM10 AQA and α-adaptin, transfected COS7 cells were fixed with 4% PFA, 4% sucrose in PBS pH 7.4, and surface-expressed ADAM10 WT/ADAM10 AQA were labeled with anti ADAM10 antibody. To visualize surface expression, cells were then blocked with 4% normal serum and followed by a 555-conjugated secondary antibody. Afterwards, cells were permeabilized with 0.2% saponin and α-adaptin was labeled with anti- α-adaptin antibody followed by a 488-conjugated secondary antibody. After either cLTP or cLTD induction, hippocampal neurons were fixed in 100% methanol at -20°C for 15 min. Antibody

against ADAM10 and α-adaptin and secondary antibodies were applied in GDB buffer (30 mM phosphate buffer [pH7.4] containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl) (6). cLTD was induced in GFP-transfected neurons after preincubation with either Ala or Pro peptide. Neurons were then fixed in 4% PFA, 4% sucrose in PBS pH7.4 for 15 min and immunostained for GFP. Primary and secondary antibodies were applied in GDB buffer. Fluorescence images were acquired by using the confocal LSM510 Meta system (Zeiss) with a 63x objective and a sequential acquisition setting at 1024 x 1024 pixel resolution.

Structural characterization. The sequence of the region of ADAM10 involved in the interaction was searched against all the protein sequences available in the Protein Data Bank (PDB) (www.pdb.org) (7) using BLAST (8). The BLAST best hit corresponds to the NMR structure of the c-Src-SH3 domain in complex with a peptide (PDB:1QWE) (9). As for the AP2 complex, we used its X-ray structure at 2.6 Å resolution (PDB:1GW5) (10). Structural superposition was performed via the superpose3D program (11), while the interactions were calculated using the P.I.C. (Protein Interaction Calculator) web server (12). Figures were made using the UCSF Chimera program (13).

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