Supplementary Material and Methods

Expression Constructs

pCDNA3.1 and pMT (Invitrogen) constructs of N-terminally myc/HA-tagged human APP and APLP1, or C-terminally myc/HA-tagged APLP2 together with corresponding deletion constructs were generated by PCR or have been described previously (Paliga et al., 1997; Scheuermann et al., 2001). In detail, following APP/APLP deletions constructs have been generated by PCR with appropriate primers: APPΔE1 (lacking aa 31–192 of APP-695), APPΔE2 (lacking aa 292-502), APPΔEC (lacking aa 19-599), APPΔCT (described in Fossgreen et al., 1998); APLP1ΔE1 (lacking aa 38-212), APLP1ΔE2 (lacking aa 284-494), APLP1ΔEC (lacking aa 38-543), APLP1ΔCT (lacking aa 46-243), APLP2ΔE1 (lacking aa 46-243 of APLP2-763), APLP2ΔE2 (lacking aa 364-581), APLP2ΔEC (lacking aa 24-664), APLP2ΔCT (lacking aa 720-763). N-terminally myc-tagged APP , C-terminally myc-tagged APLP1 (Paliga et al., 1997), APLP2 (Eggert et al., 2004), and Notch (Loewer et al., 2004) were subcloned into the pUAST vector. Gal4 was PCR-amplified and cloned into the pMT/V5-His-TOPO vector (Invitrogen). pCEP4 APP695, APLP1, and APLP2-763 constructs have been described previously (Eggert et al., 2004; Paliga et al., 1997). The identity of all PCR-amplified constructs was confirmed by sequencing.

Coimmunoprecipitation of myc/HA-tagged APP family proteins

COS7 cells were cultivated in DMEM (Sigma) supplemented with 10 % FCS (Biochrom) according to standard cell culture techniques. Cells were transiently co-transfected with myc/HA-tagged pCDNA3.1-APP, -APLP1, -APLP2, or empty vector with Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. Transfected COS7 cells were lysed 24 h post-transfection (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, Complete Protease Inhibitor mix (Roche Molecular Diagnostics)) for 20 min on ice. Lysates

were centrifuged at 16.000x g for 10 min and 1/25 of the supernatant was denatured for the direct load. The remaining supernatant was incubated with either anti-myc-Agarose (Sigma) or anti-HA-Sepharose beads (Roche) overnight at 4 °C. The beads were washed three times with lysis buffer and finally denatured with SDS sample buffer. Total lysates and immunoprecipitates were analyzed on 4-12 % Bis-Tris gels (Invitrogen) and probed by immunoblotting for HA- and myc-tagged APP family proteins.

Crosslinking of APP family proteins

Cells were transfected with pCEP-APP, -APLP1, or -APLP2 with Lipofectamine Plus according to the manufacturers protocol (Invitrogen) and selected for stable expression with Hygromycine (PAA Laboratories). Confluent cells were labeled with 200 µCi ^{35}S-Methionine for 4h, washed twice with ice-cold PBS-CM (PBS containing 1 mM $CaCl_2$, 2 mM $MgCl_2$), and incubated with 1 mM DTSSP (3,3´-Dithiobis[sulfosuccinimidyl propionate], Pierce) in PBS-CM or PBS-CM only for 1 h at 4 °C to crosslink cellular proteins present at the cell surface. The crosslinker was quenched with 50 mM Tris pH 7.4 for 30 min on ice. The cells were washed with PBS-CM and lysed with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, Complete Protease Inhibitor mix (Roche Molecular Diagnostics)) for 20 min on ice. Lysates were centrifuged at 16.000x g for 10 min and preincubated with Protein A Sepharose for 30 min at 4 °C. The resulting lysates were then incubated at 4 °C overnight with Protein A Sepharose and anti-APP (22734), anti-APLP1 (57) or anti-APLP2 (D2-II) antibodies, respectively. The beads were washed twice with buffer A (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2 % NP-40, 0.05 % SDS), buffer B (buffer A with 500 mM NaCl), buffer A´ (buffer A with 0,2 % SDS) and buffer C (10 mM Tris pH 7.4). The samples were denatured under nonreducing conditions and analyzed on 3-8 % Tris-Acetate gels (Invitrogen) and by autoradiography. The crosslinked dimers were excised from the gel and extracted with 0.2 M $(NH_4)_2CO_3$/20 % Acetonitril at 37 °C over night. Protein extracts

were denatured under reducing conditions and analyzed on 3-8 % Tris-Acetate gels (Invitrogen) and by autoradiography.

S2-cell aggregation assay

S2-cells were transiently cotransfected with pMT-Gal4 and either pUAST- APP, APLP1, or APLP2 as described (Klueg et al., 2002) or transfected with pMT-APP/APLP full length and deletion constructs using Effectene according to the manufacturers protocol (Qiagen). 48h after transfection, expression was induced with 500 µM $CuSO_4$ for 16h. For analysis of homotypic interactions, $4x10^5$ cells in single cell suspension were aggregated in a 24-well for 2 h at 80 rpm on a horizontal shaker. For heterotypic aggregation, two separately transfected pools of S2- cells were mixed ($2x10^5$ cells each) as single cell suspensions and aggregated as described above. Afterwards, cells were transferred to glass cover slips and fixed in 4% PFA in PBS for 20 min. Cells were then permeabilized with 0.1 % NP40/PBS for 15 min, blocked with 5 % goat serum for 1 h, and incubated with appropriate primary antibodies over night at 4 °C. After washing with PBS, cells were incubated with appropriate fluorescent dye coupled goat secondary antibodies (Alexa 488/Alexa 594, Molecular Probes) for 1 h, washed with PBS and mounted in Mowiol.

For quantification, at least three low magnification fields with equal cell density were taken from each experiment in a blinded fashion and the immunostained cells were assayed for aggregation. For homotypic aggregation experiments, clusters of three or more transfected cells were scored as positive. For heterotypic aggregation experiments, transfected cells with direct heterotypic cell contact were scored as positive. In total, 300-800 transfected cells were counted per experiment, and the data from at least three independent experiments were evaluated. Statistical significance was tested with the unpaired t-test.

MEF cell aggregation assay

Cell aggregation of MEF cells was performed in Calcium- and Magnesium free Hank´s balanced salt solution (HBSS) essentially as described (Miura et al., 1992). Briefly, MEFs were treated with 1 mM EDTA in HBSS/1 % BSA for 15 min at room temperature. Cells were gently suspended and washed three times with HBSS. Subsequently, single cell suspensions (10^6/ml) in HBSS/1 % BSA were aggregated in BSA coated 6 well dishes (Nunc) for 60 min at 80 rpm.

For co-aggregations, MEF cells were labeled with 2.5 µM Calcein or 2.5 µM Calcein red-orange (Invitrogen) in HBSS/1 mM EDTA for 15 min. Cell suspensions (10^6/ml) of two differentially labeled cell lines were mixed and aggregated as described above.

Stable clonal Dko cells expressing APLP1 (DkoAPLP1re) or APLP2 (DkoAPLP2re) and APP-/- cell lines expressing APP (APP-/-APPre) were generated by transfection of according pCEP constructs with Superfect (Qiagen) according to the manufacturer's instructions. Stable clones were selected with Hygromycine and analyzed for expression of APP/APLPs.

For quantification of cellular aggregation, the number of cell particles was counted in duplicate per indicated timepoint and the ratio of particles after 15, 30, and 60 min (N_t) compared to starting conditions at t=0 (N_0) was calculated (N_t/N_0) and plotted against time. Experiments were performed in triplicates and statistical significance was tested with an unpaired t-test. Cell viability was >90% for all cell lines as tested by Trypan blue exclusion or Calcein labeling.

Brain extraction, coimmunoprecipitation and synaptic plasma membrane preparation

Whole mouse brain extracts were prepared from 6-9 months old (for Co-IPs and SPM preparation) or 4 and 8 months old (for age related APP/APLP levels) age matched wild type (C57/BL6 X 129 (F_x)), APP -/-, APLP1 -/-, and APLP2 -/- mice by homogenization in TBS

buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, Complete protease inhibitor mix (Roche)). NP-40 was added to a final concentration of 1 % and the homogenate was incubated for 1 h at 4 °C, followed by centrifugation at 5000x g for 10 min. The total protein content of lysates was assayed with the BCA method (Sigma), and the extracts were further used for immunoprecipitation or denatured in SDS sample buffer. For coimmunoprecipitations, lysates were first centrifuged at 16.000x g for 10 min and preincubated with Protein A Sepharose for 1 h at 4 °C. The extracts were then incubated either with the C-terminal anti-APLP1 antibody 57, or the N-terminal anti-APP antibody 22734 and 30 µl Protein A-Sepharose overnight at 4 °C. Sepharose-beads were washed three times with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40), denatured in reducing sample buffer, and analyzed on 7 % or 8% Tris-Glycine gels.

Preparation of synaptic plasma membranes (SPMs) was performed as previously described (Beher et al., 1999). 20 µg of total protein were analyzed by SDS-PAGE and immunoblotted.

For coimmunoprecipitation, synaptic plasma membranes and brain homogenates were incubated with Protein A Sepharose and either preimmune serum or the anti-APLP1 antibody 57 overnight at 4 °C, and further processed as described above.

Supplementary references

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