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Soluble Oligomers of Amyloid-β Peptide Disrupt Membrane Trafficking of α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor Contributing to Early Synapse Dysfunction*[S]

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β-Amyloid (Aβ), a peptide generated from the amyloid precursor protein, is widely believed to underlie the pathophysiology of Alzheimer disease (AD). Emerging evidences suggest that soluble Aβ oligomers adversely affect synaptic function, leading to cognitive failure associated with AD. The Aβ-induced synaptic dysfunction has been attributed to the synaptic removal of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (AMPARs). However, the molecular mechanisms underlying the loss of AMPAR induced by Aβ at synapses are largely unknown. In this study we have examined the effect of Aβ oligomers on phosphorylated GluA1 at serine 845, a residue that plays an essential role in the trafficking of AMPARs toward extrasynaptic sites and the subsequent delivery to synapses during synaptic plasticity events. We found that Aβ oligomers reduce basal levels of Ser-845 phosphorylation and surface expression of AMPARs affecting AMPAR subunit composition. Aβ-induced GluA1 dephosphorylation and reduced receptor surface levels are mediated by an increase in calcium influx into neurons through ionotropic glutamate receptors and activation of the calcium-dependent phosphatase calcineurin. Moreover, Aβ oligomers block the extrasynaptic delivery of AMPARs induced by chemical synaptic potentiation. In addition, reduced levels of total and phosphorylated GluA1 are associated with initial spatial memory deficits in a transgenic mouse model of AD. These findings indicate that Aβ oligomers could act as a synaptic depressor affecting the mechanisms involved in the targeting of AMPARs to the synapses during early stages of the disease.

Alzheimer disease (AD)[2] is an age-dependent neurodegenerative disorder and the first cause of dementia in the elderly. AD is thought to involve changes in excitatory synaptic transmission in brain regions that are critical for cognitive function and memory encoding (1). Synaptic dysfunction in AD occurs apparently long before synapse and neuron loss is observed. Several findings suggest that it is caused by accumulation of soluble oligomers of amyloid-β (oAβ) peptides, also referred as amyloid-β-derived diffusible ligands (1–3) that have been described as potently toxic species for synapses (4–6).

Excitatory synaptic transmission is tightly regulated by total number and activation of AMPA receptors (AMPARs) present at the synapse. The NMDA receptor (NMDAR) has a central role in synaptic plasticity events, such as long term potentiation (LTP) or long term depression (LTD), depending on the extent of the $[Ca^{2+}]_i$ rise and activation of downstream intracellular cascades (7). On the other hand, cell surface AMPARs localization is closely related with NMDAR activity. AMPARs are inserted into the postsynaptic membrane during LTP and removed from the membrane during LTD (8). On the other hand, phosphorylation of AMPAR modulates its trafficking and activity (9, 10), which finally affects synaptic transmission (11). Phosphorylation of GluA1 at Ser-845 contributes specifically to the recruitment of new AMPAR into extrasynaptic sites from where, due to coincident synaptic activity, it will be delivered to the synapsis. Increasing the extrasynaptic pool of AMPAR has been proposed to be a critical event for the establishment of LTP (12). Recent reports have shown that oAβ could alter AMPAR endocytosis by modulating downstream kinases and phosphatases (13–15).

Soluble oAβ has been shown to disrupt synaptic structure (3, 16, 17). Accordingly, several reports have shown that oAβ inhibited LTP and facilitated LTD (5, 6, 17–19), probably by

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[2] The abbreviations used are: AD, Alzheimer disease; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AMPAR, AMPA receptor; DAPT, *N*-(*N*-(3,5-difluorophenacetyl)-l-alanyl)-*S*-phenylglycine *t*-butyl ester; NMDA, *N*-methyl-D-aspartic acid; NMDAR, NMDA receptor; oAβ, amyloid-β oligomer; ADDL; LTP, long term potentiation; LTD, long term depression; APP, amyloid precursor protein; ACSF, artificial cerebrospinal fluid; CaMKII, calcium/calmodulin-dependent protein kinase II; F/R, forskolin plus rolipram; BAPTA-AM, 1, 2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis; cLTP, chemical LTD.

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inducing internalization of NMDAR and AMPAR (13, 16, 17). Although changes in $[Ca^{2+}]_i$ are important to trigger LTP or LTD (7), the effect of oAβ on the intracellular calcium levels and its contribution to the deregulation of LTP and LTD are still a controversial issue (20).

In this study, we show that oAβ induces the removal of AMPAR from the surface of culture neurons and reduces the levels of phosphorylated GluA1 at Ser-845. The oAβ-mediated dephosphorylation of AMPAR modifies the interaction between GluA1 and GluA2 subunits, affecting the composition of heteromeric AMPARs at the cell surface. The effect of oAβ is mediated by calcium influx through ionotropic glutamate receptors resulting in the activation of calcineurin, which in turn dephosphorylates Ser-845 of GluA1. Our results further show that oAβ blocks the increase in synaptic AMPAR mediated by chemical LTP. Moreover, neurons from transgenic $APP_{Sw,Ind}$ mice show reduced levels of AMPARs, whereas decreased phosphorylation of GluA1 at Ser-845 correlates with the development of early memory deficits. These results provide functional evidence between initial accumulations of oAβ and AMPAR deregulation at early synaptic deficits of AD.

EXPERIMENTAL PROCEDURES

Primary Neuronal Culture—Neurons were cultured from E14.5–15.5 C57BL/6 wild-type mouse embryo forebrains or embryos of heterozygous $APP_{Sw,Ind}$ x non-transgenic crossings. Cells were enzymatically and mechanically disrupted in the presence of trypsin and DNase I before plating in poly-D-lysine (100 μg/ml)-coated 24-well plates, 35- to 60-mm dishes, or on coverslips. Cells were seeded at a density of 5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, heat inactivated), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 30 mM glucose. Three hours after seeding, medium was replaced with serum-free Neurobasal medium supplemented with 2% B27 (Invitrogen), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, 30 mM glucose, which yielded nearly pure neuronal cultures (21). Culture medium was partially replaced every 3–4 days with fresh Neurobasal supplemented with B27. Cell cultures were kept at 37 °C in a humidified incubator with 5%CO_2/95%air, and neurons were used for experiments after 12–14 days *in vitro*.

Aβ Oligomer Preparation—Synthetic $A\beta_{1-42}$ (Bachem, United Kingdom) was dissolved in hexafluor-2-propanol and kept at −80 °C after evaporation of hexafluor-2-propanol. Aβ oligomers (oAβs) were prepared freshly by dissolving the peptide film with DMSO and cold F-12 medium without phenol red to yield a 100 μM stock as previously described (22). Samples were incubated at 4 °C for 24 h and centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant containing a mixture of oAβ was biochemically analyzed by SDS-PAGE and electron microscopy. For negative staining analysis, 5 μl of the sample was placed on copper grids covered with carbon and counterstained with 2% uranyl acetate, using the droplet technique, and examined in a JEOL JEM-2011 transmission electron microscope.

Cell Stimulation and Lysate Preparation—Cultures were first incubated in ACSF for 30 min at room temperature (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 33 D-glucose, and 25 HEPES (pH 7.3), followed by stimulation with 5 μM oAβ in ACSF (no $MgCl_2$). After 10 min of stimulation, neurons were replaced in regular ACSF and then subjected to different procedures at indicated time points. For total lysate preparation, Cultures were then washed once with ice-cold PBS and scraped in cold 1% Nonidet P-40 homogenization buffer (in mM: 20 Tris, pH 7.5, 150 NaCl, 5 EDTA, 1 PMSF, 1 Na_2VO_4, 1 × Sigma protease inhibitor and phosphatase inhibitor cocktails) to obtain cell lysates. Mouse hippocampi were lysed in 0.2 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1% Nonidet P-40, 0.1% SDS, 1 mM Na_3VO_4, 50 mM NaF, and 1 mM PMSF) supplemented with protease and phosphatase inhibitors. Tissue was homogenized during 20 s using a pellet pestle (Sigma) and kept 1 h at 4 °C. Lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the protein in the supernatant was quantified by a DC protein assay kit based on the Bradford method (Bio-Rad Laboratories, Inc.).

Immunoblotting—Primary antibodies were: anti-phospho-Ser-831-GluA1, anti-phospho-Ser-845-GluA1, anti-GluA1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Ser-845-GluA1, anti-GluA1, and anti-GluA2 (1:1,000, Millipore); anti-phospho-Ser-880-GluA2 (1:1,000, Abcam); anti-Calcineurin A and B (1:1,000, BD Bioscience); anti-GAPDH (1:40,000, Ambion Inc.); and anti-β-amyloid (1:1,000, 6E10, Signet). Samples were separated on 7.5 or 10% SDS-PAGE and transferred onto Hybond-C Extra, nitrocellulose membranes (Amersham Biosciences). Blots were blocked at room temperature for 1 h with 10% dry milk, 0.1% BSA (fraction V), pH 7.4, in PBS and incubated at 4 °C overnight with primary antibody in PBS 0.1% BSA, pH 7.4. After washing, blots were then incubated with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer and developed using the ECL™ Western blotting Detection Reagents (Amersham Biosciences). Semi-quantitative analysis of immunoblots was performed by densitometry using ImageJ (National Institutes of Health, Bethesda, MD), and protein levels were corrected for corresponding loading control.

Surface Biotinylation—After oAβ stimulations, cultured neurons were transferred to ice-cold PBS-Ca^{2+}-Mg^{2+} buffer (pH 7.4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$), followed by biotinylation in 1 mg/ml biotin (EZ-Link Sulfo-NHS-SS-Biotin, Pierce) for 30 min with slow agitation. Free biotin was quenched by 3× wash in cold PBS-Ca^{2+}-Mg^{2+} + glycine (0.1 M). Cell cultures were immediately scraped in cold 1% Triton X-100 homogenization buffer (in mM: 50 NaCl, 10 EDTA, 10 EGTA, 1 Na_3VO_4, 50 NaF, 25 $NaPP_i$, 1β-glycerophosphate, 1 PMSF, 1 × protease inhibitor mixture, 1 × phosphatase inhibitor mixture, and 50 HEPES, pH 7.5). Solubilization was performed in 1% Triton X-100 to avoid solubilization postsynaptic densities (23) and is, therefore, selective for extrasynaptic AMPARs. Homogenates from cultures were centrifuged at 10,000 × g for 20 min to pellet insoluble fraction. 75 μl of the supernatant was mixed and heated with 25 μl of 4× SDS sample buffer to determine total fraction of GluA1 (surface plus internal). Biotinylated surface proteins in the remaining supernatant (~225 μl) were pulled down with 40 μl of 50% avidin-agarose beads (ImmunoPure

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Immobilized Avidin, Pierce) overnight at 4 °C. The beads were pelleted, and 75 μl of the supernatant (internal fraction) was mixed and heated with 25 μl of 4× SDS sample buffer. The beads were then rinsed three times with 1% Triton X-100 homogenization buffer and heated in 100 μl of 2× SDS sample buffer (surface fraction). Equal volumes of the total, internal, and biotinylated fractions were subjected to 10% SDS-PAGE, probed for total GluA1, and normalized to GAPDH.

Immunoprecipitation—Neurons were washed in ice-cold PBS and immediately scraped in cold 1% Nonidet P-40 homogenization buffer (400 μl/2 × 60-mm plate). Homogenates from cultures were centrifuged at 10,000 × g for 10 min to pellet insoluble fraction. 75 μl of the supernatant was mixed and heated with 25 μl of 4× SDS sample buffer (total homogenate). The remaining supernatants (~300 μl) were immunoprecipitated with 1 μg of anti-GluA1 (Millipore) overnight at 4 °C. Incubations continued for 1 h at 4 °C in the presence of 40 μl of 50% slurry protein-G-Sepharose beads (Amersham Biosciences). The beads were pelleted, and 75 μl of the supernatant (unbound fraction) was mixed and heated with 25 μl of 4× SDS sample buffer. The beads were then rinsed three times with 1% Nonidet P-40 homogenization buffer and heated in 100 μl of 2× SDS sample buffer (immunoprecipitation fraction). Equal volumes of each fraction were detected by immunoblotting.

Calcium Imaging—Primary neurons grown onto poly-lysine-coated coverslips for 12 days were loaded with Fura-2/AM (4 μM, Molecular Probes, Invitrogen) for 1 h at room temperature. Coverslips were washed with ACSF buffer and mounted in a static chamber at room temperature on an inverted Nikon TE2000U microscope. Cells were excited alternatively at 340 and 380 nm using a monochromator (Cairn Research Ltd.), and emission light was collected at 510 nm every 4 or 20 s. Images were acquired by using a 12-bit CCD ERG ORCA Hamamatsu camera and processed with Metafluor (Universal Imaging). When appropriated, cells were treated with oAβ (5 μM) in ACSF (no $MgCl_2$). $N > 50$ cells were analyzed in each experiment (three independent experiments were performed). Data were analyzed with Excel (Microsoft, Seattle, WA) and Prism (GraphPad, San Diego, CA) software.

Calcineurin Activity—Calcineurin activity was determined with the calcineurin cellular activity assay kit (Calbiochem). Briefly, neurons were homogenized in lysis buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 50 μM EDTA, 50 μM EGTA, and 0.2% Nonidet P-40). Free phosphate was eliminated using a desalting column, and an equal amount of protein was incubated with the calcineurin substrate RII phosphopeptide (1.64 mg/ml) for 30 min at 30 °C. The reaction was stopped by adding 100 μl of GREEN TM reagent, and fluorescence was measured at 620 nm using a microtiter plate reader.

APP Transgenic Mice and Morris Water-maze Test—$APP_{Sw,Ind}$ transgenic mice (line J9, C57BL/6 background) expressing mutant human APP695 isoform harboring the FAD-linked Swedish (K670N/M671L) and Indiana (V717F) mutations under the neuronal PDGFβ promoter have been previously described (24). Mice were age-matched littermate males obtained by crossing heterozygous $APP_{Sw,Ind}$ x non-transgenic (WT) crossings. The Morris water-maze test was performed as previously described (25, 26). Experimenters of the behavioral tests were blind to the genotypes of the mice. Animal procedures were performed in accordance with institutional and national guidelines following approval by the Animal Care and Ethical Committee (CEEAH) of the Universitat Autònoma de Barcelona.

Statistical Analysis—Statistical analysis of the biochemical experiments was performed using one-way analysis of variance and the Newman-Keuls multiple comparison post hoc test. The behavioral data were analyzed using two-way analysis of variance with repeated measures and the Scheffé test for post hoc comparisons. Data were shown as the mean ± S.E. Differences with $p < 0.05$ were considered significant.

RESULTS

oAβ Induce Ser-845-GluA1 Dephosphorylation and a Decrease in Surface Expression of GluA1—We first evaluated the effects of oAβ on critical phosphorylation sites of AMPAR subunits that are important for cell surface receptor expression and regulation of synaptic plasticity (8). Primary neuronal cultures were treated with freshly prepared oligomers of Aβ (oAβ 5 μM, supplemental Fig. S1) prepared as described under "Experimental Procedures," and phosphorylation levels of Ser-831 and Ser-845 in GluA1 and Ser-880 in GluA2 were analyzed by immunoblotting. We found that oAβ significantly reduced phosphorylation levels (~35%) of GluA1 Ser-845 in a time-dependent manner but had no effect on GluA1 Ser-831 or GluA2 Ser-880. There were no significant changes in the total amount of GluA1 and GluA2 in the cells treated with oAβ (Fig. 1, *A–C*).

It is well established that PKA-dependent phosphorylation of GluA1 at Ser-845 increases cell surface expression of AMPARs, whereas NMDA-induced AMPAR dephosphorylation triggers its internalization (12, 27). Because dynamic changes in this phosphorylated site seem to be crucial in the modulation of AMPAR trafficking and synaptic plasticity, we next examined the effect of oAβ on surface GluA1 subunit of AMPAR in primary hippocampal neurons. We found that treatment of cultured neurons with oAβ significantly decreased cell surface GluA1 expression (~43%) and induced a slight but not significant reduction of GluA2 (Fig. 1, *D* and *E*). As a result, the GluA1/GluA2 ratio at the cell surface was significantly decreased (~40%) after oAβ treatment (Fig. 1*F* and supplemental Fig. S2).

oAβ Negatively Affects the Interaction between the AMPAR Subunits GluA1 and GluA2—It has been recently reported that ~80% of synaptic and >95% of somatic extrasynaptic receptors are GluA1/GluA2 heteromers (28). GluA2/GluA3 receptors continuously cycle in and out of synapse, preserving the number of synaptic AMPAR (constitutive pathway), whereas GluA1/GluA2 are added into synapses in an activity-dependent manner during synaptic plasticity (regulated pathway) (29). Because oAβ alters the GluA1/GluA2 ratio of AMPAR across the cell surface, we next tested the possibility that oAβ could be affecting the global subunit composition of AMPARs. For this purpose, we performed immunoprecipitation assays with anti-GluA1 antibodies in neurons treated with oAβ and examined the bound and unbound GluA2. Surprisingly, the levels of GluA2 were decreased in the bounded fraction and increased in the unbounded fraction (~30%) 60 min after treatment (Fig. 2,

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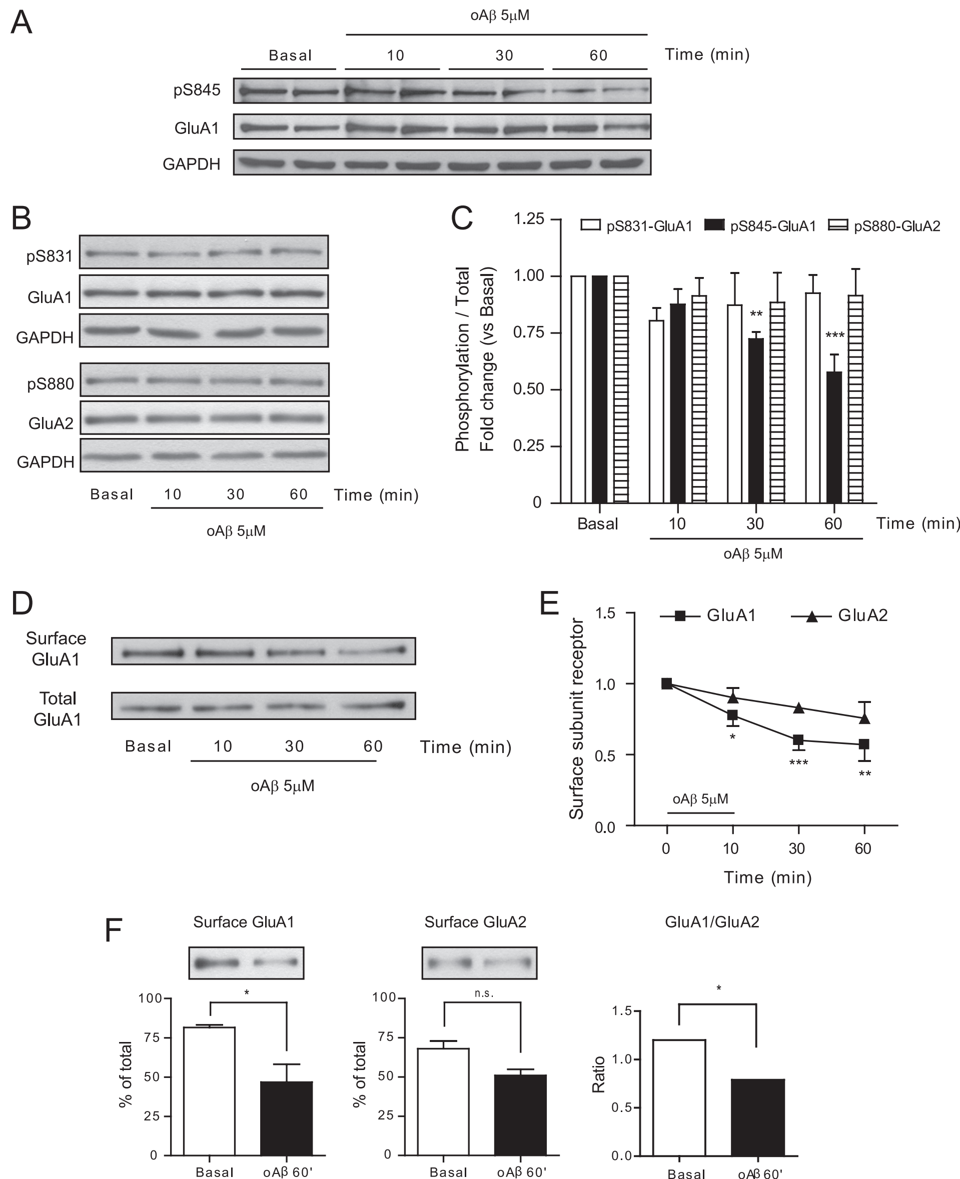

FIGURE 1. **oAβ induces Ser-845 dephosphorylation in GluA1 and a decrease in cell surface AMPARs.** *A*, Western blots showing phosphorylated levels of Ser-845 (*upper blot*), total GluA1 (*middle blot*), and GAPDH protein levels (*lower blot*) as loading control. *B*, Western blots showing phosphorylated levels of Ser-831 and Ser-880 (*upper blot*), total GluA1 and GluA2 (*middle blot*), and GAPDH protein levels (*lower blot*) as loading control. *C*, *graph* represents quantification of phosphorylated AMPA subunits in response to oAβ at different times after stimulation compared with baseline. Represented values are the ratio of the levels of phosphorylated AMPA subunits *versus* normalized total levels (related to GAPDH) of the corresponding AMPA subunit. **, $p < 0.01$; ***, $p < 0.001$ ($n = 8$); *error bars* indicate ± S.E. *D*, surface proteins were analyzed by biotinylation. *Blots* show surface GluA1 (*upper blot*) and total GluA1 (*lower blot*). *E*, quantified changes in surface fraction of GluA1 and GluA2 in response to oAβ at different times after stimulation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *F*, Western blot and percentage of GluA1 (*left*) and GluA2 (*middle*) and GluA1/GluA2 ratio (*right*) in basal and oAβ at 60 min. Surface values are the ratio between surface and total amounts of each subunit. oAβ alters the GluA1/GluA2 ratio ($n = 3–6$). *Error bars* indicate ± S.E. *, $p < 0.05$.

A–C). These data revealed that soluble oAβ induced a dissociation of GluA1/GluA2 complexes, which led to a significantly decrease of the GluA2/GluA1 ratio (~30%) in oAβ-treated neurons (Fig. 2*D*).

oAβ Induces Calcium Influx into Neurons and Reduces Surface Expression of GluA1 through Ionotropic Glutamate Receptors and Calcineurin Activity—Synapse loss, induced by oAβ in cultured neurons (3), is thought to result from an initial excitotoxicity mediated by oxidative stress and increased $[Ca^{2+}]_i$ (30, 31). The increase in $[Ca^{2+}]_i$ has been linked to increased NMDAR responsiveness induced by oAβ treatment (32, 33). In support of this idea, our data show that oAβ (5 μM) caused a glutamate receptor-dependent increase in $[Ca^{2+}]_i$ (Fig. 3, *A* and *B*). Experiments with the membrane-permeable calcium chelator BAPTA-AM (20 μM) and the NMDA and AMPAR antagonists, MK-801 (10 μM) and CNQX (50 μM), revealed that the increase of $[Ca^{2+}]_i$ and activation of ionotropic receptors were required for oAβ-mediated AMPAR internalization (Fig. 3, *C* and *D*).

When cytosolic calcium reaches critical concentrations, certain LTD-related signaling pathways are activated (34). For instance, NMDAR-dependent LTD in the CA1 region recruits calcineurin (protein phosphatase 2B) (35). Because PKA and calcineurin have been implicated in the regulation of AMPAR

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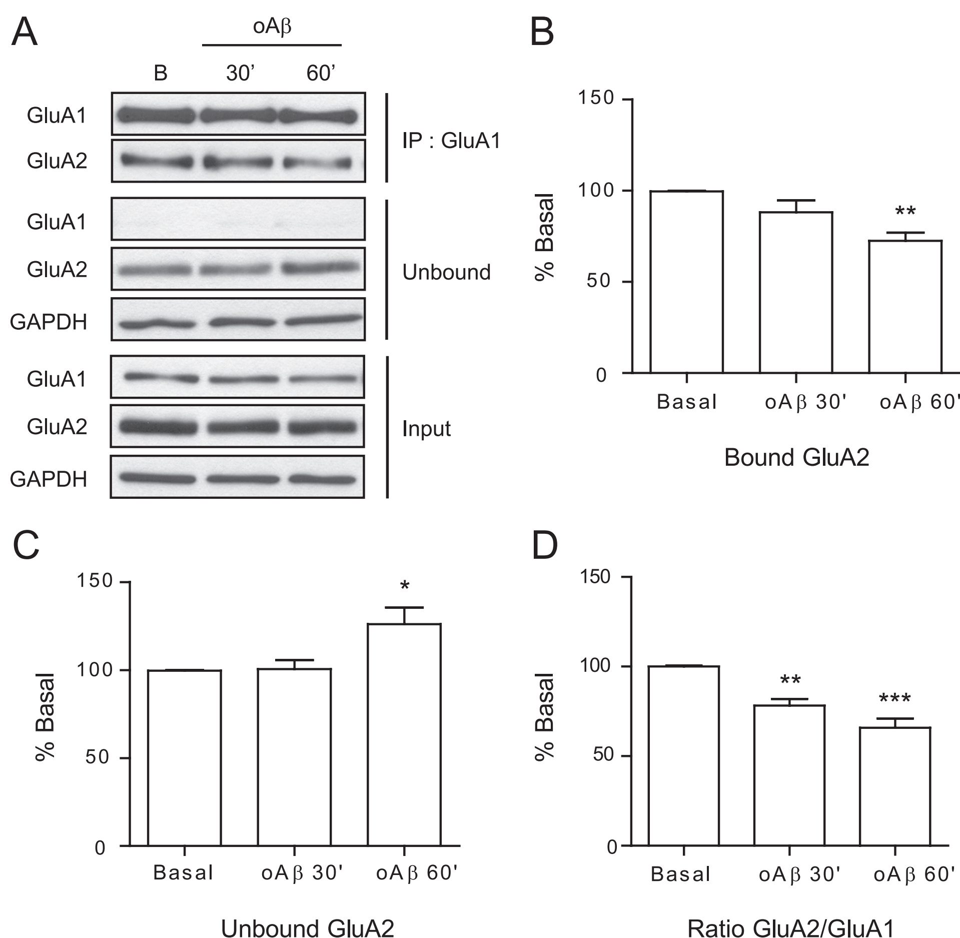

FIGURE 2. **oAβ affects the interaction between GluA1 and GluA2.** *A*, representative immunoblots for GluA1 immunoprecipitation (*IP*) experiments. Almost all GluA1 subunits were pulled down by the GluA1 C-terminal antibody, as observed with no GluA1 signal in the unbound lane. GluA2 subunits in the unbound lane are likely GluA2/3 heteromers. *B–D*, *graphs* represent quantified changes in GluA1/2 heteromers. oAβ affects interaction between GluA1 and GluA2, inducing a decrease of GluA1/2 heteromers. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. ($n = 6$). *Error bars* indicate ± S.E.

trafficking during synaptic plasticity (35–37) and PKA-dependent phosphorylation of GluA1 Ser-845 is reduced by oAβ, we decided to explore the possibility that calcium influx would results in an increase in calcineurin activity. As shown in Fig. 4*A*, calcineurin activity was significantly increased in the presence of oAβ (~25% at 30 min). Western blotting analysis revealed unchanged levels of the calcineurin calmodulin-binding catalytic and Ca^{2+}-binding subunits in oAβ-treated neurons (Fig. 4*B*).

Together these results provided evidences indicating that oAβ increased calcium influx and calcineurin activity in an ionotropic glutamate receptor-dependent manner. To establish the relationship between these data and the decrease in phosphorylation levels of GluA1 Ser-845 and cell surface GluA1, we performed experiments in the presence of FK-506, a calcineurin inhibitor (10 μM). We observed that FK-506 was able to prevent the decrease in the levels of phosphorylated Ser-845-GluA1 and cell surface expression of GluA1 induced by oAβ (Fig. 4, *C–E*).

oAβ Blocks the Extrasynaptic Delivery of AMPAR Mediated by Chemical Potentiation—Although the effect of Aβ on LTD has been less examined than its effects on LTP, there is compelling evidence that high amounts of Aβ actually induce a "chemical" LTD (cLTP) (17, 38). The most notable physiological disruption of synaptic function by synthetic and natural Aβ is the inhibition of LTP, and this effect seems to be caused specifically by oligomeric forms of Aβ (6, 39). Because the mechanisms by which Aβ oligomers inhibit LTP are unclear, we focused our attention in the possible effect of oAβ on inhibition of LTP. To induce cLTP, we used a chemical stimulation protocol of forskolin plus rolipram (F/R) that results in prolonged NMDAR-dependent LTP (40) and increases phosphorylation of GluA1 at Ser-845 and cell surface GluA1 levels (12) (supplemental Fig. S2). Treatment of neuronal cultures with F/R (50 μM/0.1 μM) for 10 min increased basal GluA1 Ser-845 phosphorylation at 30 min (~70%, Fig. 5*A*), which correlated with a significant increase in the levels of cell surface expression of GluA1 but not GluA2 (Fig. 5*B* and supplemental Fig. S3). Bath application of oAβ to cultured neurons prior to F/R treatment partially blocked F/R-mediated Ser-845 phosphorylation and completely blocked surface delivery of GluA1. These results support the hypothesis that oAβ is affecting the bidirectional process of LTP and LTD through the regulation of GluA1 phosphorylation at Ser-845.

Naturally Secreted Aβ Reduces Surface AMPA Receptors—To compare the effects on AMPAR of synthetic oAβ and naturally secreted Aβ, we established primary neurons from a β-amyloid precursor protein (APP) transgenic mouse ($APP_{Sw,Ind}$) that develops age-dependent amyloid pathology and memory deficits (24, 25). Neurons from $APP_{Sw,Ind}$ embryos

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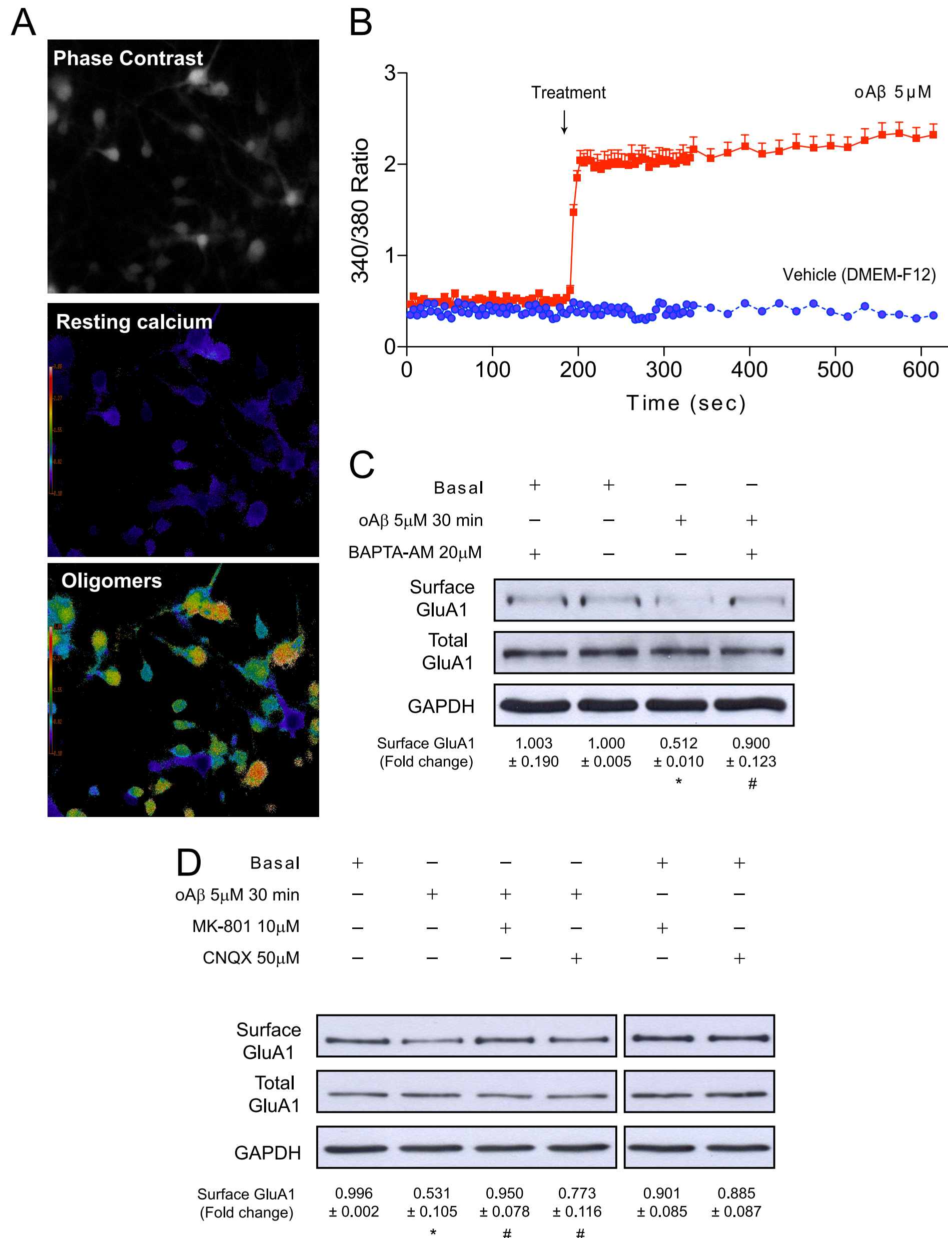

FIGURE 3. **oAβ increases intracellular calcium into primary neurons and reduces surface expression of AMPAR through ionotropic glutamate receptors.** *A*, oAβ induces a rapid and sustained increase in $[Ca^{2+}]_i$ in primary neurons. Cells were loaded with fura2/AM and subjected to calcium imaging. *Images* show a phase-contrast image and $[Ca^{2+}]_i$ levels before (resting calcium) and after treatment with oAβ (oligomers). *B*, *traces* correspond to 52 representative neurons ($n \geq 150$ cells in three experiments) for oAβ treatment. In vehicle control, cells were treated with DMEM/F-12. *C*, effect of calcium chelator BAPTA-AM on GluA1 internalization. Neurons were pre-treated with 20 μM BAPTA-AM followed by treatment with 5 μM oAβ for 30 min. The amounts of surface GluA1 were assessed by surface biotinylation. *Values* indicate mean ± S.E. normalized to basal.*, $p < 0.05$ basal *versus* oAβ; #, $p < 0.05$ oAβ *versus* oAβ plus BAPTA-AM ($n = 4$). *D*, ionotropic glutamate receptor antagonists (MK-801 for NMDAR and CNQX for AMPAR) prevent oAβ-induced surface GluA1-containing AMPAR loss. Primary cultures were treated with oAβ for 30 min in the presence or absence of MK-801 (10 μM) and CNQX (50 μM). Changes in surface GluA1 subunit were examined by surface biotinylation. *Values* indicate mean ± S.E. normalized to basal. *, $p < 0.05$ basal *versus* oAβ; #, $p < 0.05$ oAβ *versus* oAβ plus MK-801 and oAβ plus CNQX ($n = 5$).

expressed human APP (~2-fold) and released soluble Aβ peptides without causing gross morphological synaptic changes (26). Using the biotinylation assay, we found that surface expression of GluA1 was reduced in 12 days *in vitro* cultured neurons from $APP_{Sw,Ind}$ mice compared with neurons from wild-type littermates, although total GluA1 levels were not affected (Fig. 6). Surface expression of GluA1 in neurons from $APP_{Sw,Ind}$ was ~60% of that observed in wild-type neurons. Treatment of $APP_{Sw,Ind}$ neurons with the γ-secretase inhibitor DAPT for 72 h restored surface GluA1 to that of control neurons.

To further investigate potential mechanisms underlying synaptic dysfunction between $APP_{Sw,Ind}$ and wild-type mice, we analyzed the levels of phosphorylated levels of GluA1 Ser-845 in GluA1 in hippocampus from 6-month-old wild-type and $APP_{Sw,Ind}$ mice, when initial hippocampal Aβ accumulation and spatial memory deficits are detected (24, 25). We examined spatial learning and memory in the Morris water maze, a hippocampal-dependent spatial memory task. After the second trial day, $APP_{Sw,Ind}$ mice required significantly longer latencies to locate the platform during training (Fig. 7*A*; two-way analysis

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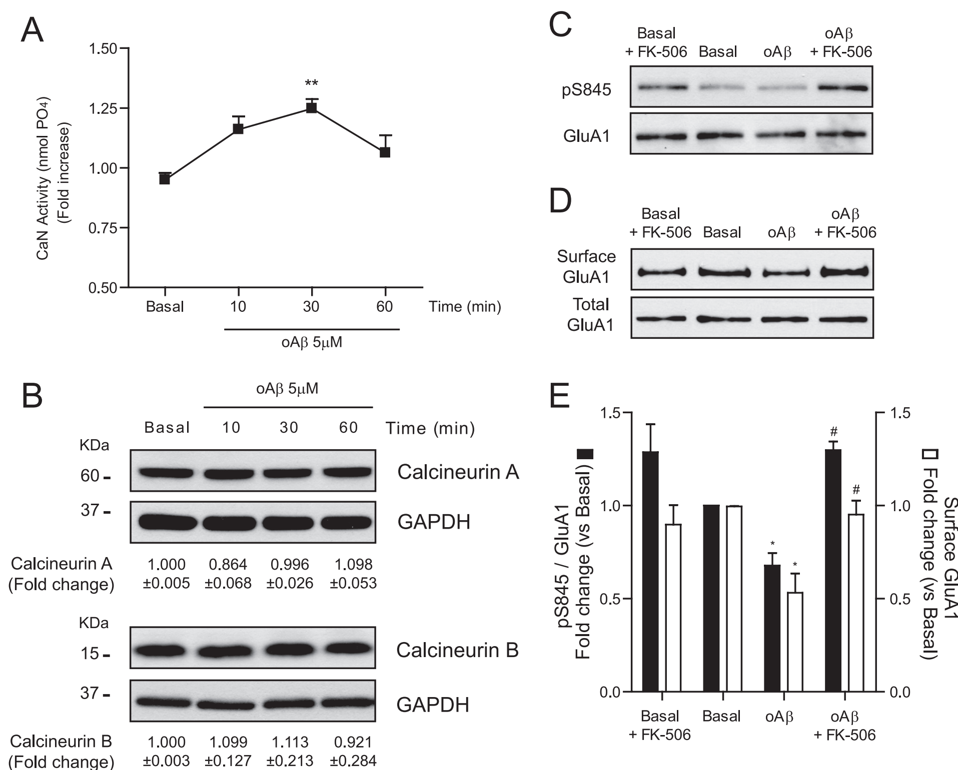

FIGURE 4. **oAβ reduces surface expression and dephosphorylation at Ser-845 of GluA1 in a calcineurin-dependent manner.** *A*, increased calcineurin activity in cultured neurons in presence of oAβ (5 μM). Neurons were treated with oAβ at indicated times and calcineurin activity was determined (see "Experimental Procedures" for details). *B*, immunoblots showing expression of calcineurin in total lysates from cultured neurons treated with oAβ (5 μM). Neither catalytic subunit (calcineurin A; *upper panel*) nor regulatory subunit (calcineurin B; *lower panel*) amounts were affected by oAβ treatment. *Values* indicate mean ± S.E. normalized to basal ($n = 4$). *C* and *D*, effect of a calcineurin-selective inhibitor (FK-506) on GluA1 internalization. Neurons were pre-treated with 10 μM FK-506 followed by treatment with 5 μM oAβ for 30 min. *C*, representative blot showing phosphorylation levels of Ser-845 (*upper blot*) and total GluA1 (*lower blot*). *D*, representative blots showing surface GluA1 (*upper blot*) and total GluA1 (*lower blot*). Note that FK-506 alone is affecting basal levels of Ser-845 phosphorylation but has no effect on surface GluA1 compared with basal levels. *E*, *graph* represents quantified changes in phosphorylated Ser-845 (*left axis*) and surface GluA1 (*right axis*) in response to oAβ. *, $p < 0.05$ basal *versus* oAβ; #, $p < 0.05$ oAβ *versus* oAβ plus FK-506. ($n = 5$); *bars* represent mean ± S.E. normalized to basal.

of variance; latencies: genotype effect, $F_{(1)} = 10.71$; day effect, $F_{(4)} = 24.38$; $p < 0.0001$). Interestingly, levels of phosphorylated GluA1 Ser-845 were increased after the second day of training in the hippocampus of control mice, whereas that increased was not observed in $APP_{Sw,Ind}$ mice. In addition, total hippocampal GluA1 levels were decreased (45%) in $APP_{Sw,Ind}$ mice after 2 days of training (Fig. 7, *B* and *C*). These results revealed a relationship between initial accumulation of Aβ, memory deficits, and deregulation of AMPARs.

DISCUSSION

It is well established that accumulation of oAβ impairs synapse function (41), but the molecular mechanisms involved are largely unknown. AMPARs are essential for excitatory synaptic transmission and play key roles in LTP and LTD, cellular mechanisms of plasticity that are believe to underlie learning and memory (36). Previous studies have shown that oAβ can facilitate the removal of AMPAR from the cell surface depending on the subunit composition of the receptors. Thus alteration in the functionality of these receptors could be involved in the early cognitive dysfunction observed in experimental models of AD. Although, the mechanisms involved in oAβ-mediated effect on AMPAR have not been fully elucidated yet, it is know that different kinases and phosphatases are involved (13, 14, 17). Our results strongly indicate that AMPAR is one of the receptors involved in excitatory synaptic transmission affected by Aβ, which agrees with recent evidence suggesting that oAβ alters the functionality of ionotropic glutamate receptors (16, 17). In the present study, we have further examined the mechanisms underlying oAβ-induced AMPAR internalization.

Among the different subunits present in AMPARs, GluA1 is the one whose trafficking depends on neuronal activity, whereas GluA2 is more prone to undergo constitutive, activity-independent recycling (42). Membrane insertion of GluA1 is regulated by two phosphorylation sites in the intracellular C-terminal tail: Ser-845 and Ser-831, which are phosphorylated by PKA and CaMKII, respectively (43). Phosphorylation at Ser-845 contributes specifically to the recruitment of new AMPARs to extrasynaptic sites, a critical event for the establishment of LTP (11, 12), whereas its dephosphorylation is essential for NMDA receptor-dependent LTD (44). Our results show that oAβ reduces phosphorylation of GluA1 Ser-845 levels leading to a decrease of surface AMPAR. By contrast, oAβ does not affect the phosphorylation of Ser-831 in GluA1 and Ser-880 in GluA2. However, we cannot rule out the possibility that longer oAβ treatments could also cause changes in the phosphorylation of these residues.

Several reports have shown that oAβ alters homeostasis of $[Ca^{2+}]_i$ (45, 46), whereas activation of the calcium-dependent

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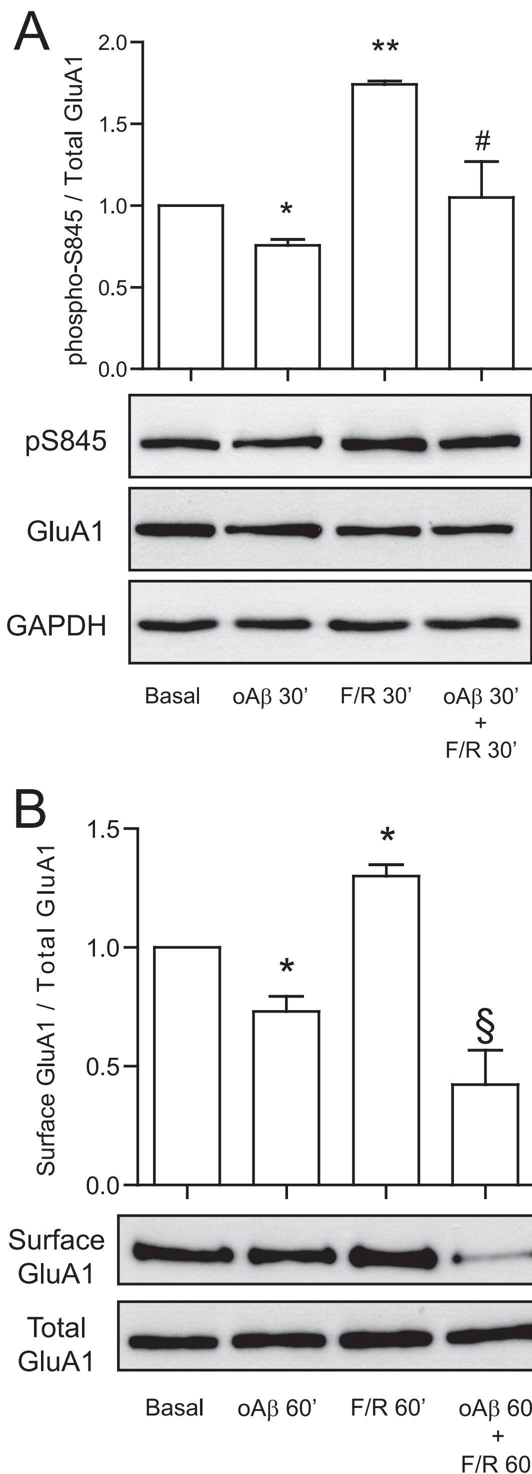

FIGURE 5. **oAβ-mediated block of phosphorylation at Ser-845 impairs AMPAR priming for synaptic incorporation.** Neurons (pre-treated or not with 5 μM oAβ during 30 min) were stimulated with forskolin/rolipram (*F/R*; 50 μM/0.1 μM) for 30 or 60 min. *A*, sample blots showing phosphorylation levels of Ser-845 (*upper blot*), total GluA1 (*middle blot*), and GAPDH protein level (*lower blot*) as loading control. The *graph* represents quantified changes in phosphorylated subunit in response to F/R stimulation compared with baseline. *B*, sample blots showing surface GluA1 (*upper blot*) and total GluA1 (*lower blot*). The *graph* represents quantified changes in surface GluA1 in response to F/R stimulation compared with baseline. oAβ treatment during 30 min impair Ser-845 phosphorylation induced by F/R stimulation affecting GluA1 surface delivery. *, basal *versus* oAβ or F/R; # and §, F/R *versus* oAβ plus F/R. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.05$; §, $p < 0.001$ ($n = 6$); *bars* represent mean ± S.E. normalized to basal.

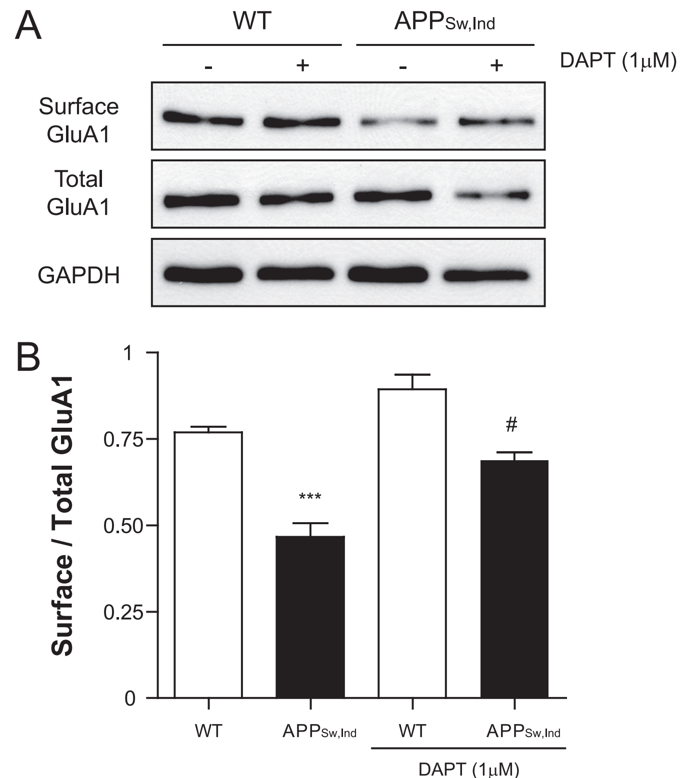

FIGURE 6. **Reduced cell surface AMPARs in $APP_{Sw,Ind}$ neurons.** Primary neurons were prepared from $APP_{Sw,Ind}$ mice or wild-type littermates in the presence or absence of DAPT (1 μM during 72 h). Surface expression of GluA1 subunit was analyzed by biotinylation at 12 days *in vitro*. *A*, surface expression of GluA1 was reduced in neurons from $APP_{Sw,Ind}$ mice but was restored partially by DAPT (*upper blot*). The total amount of GluA1 was not affected by $APP_{Sw,Ind}$ expression (*middle blot*). DAPT treatment shows light reduction in protein expression (*lower blot*, GAPDH from total extract). *B*, quantification of treated primary neurons. *Bars* represent mean ± S.E. ($n = 3$ embryos/genotype). ***, $p < 0.001$ WT *versus* $APP_{Sw,Ind}$; #, $p < 0.01$ $APP_{Sw,Ind}$ *versus* $APP_{Sw,Ind}$ plus DAPT.

phosphatase, calcineurin, may be involved in endocytosis of AMPAR (13, 17, 19). Our results show that increase in $[Ca^{2+}]_i$, induced by oAβ, is dependent on ionotropic glutamate receptors and necessary for oAβ-mediated internalization of GluA1. Moreover, we have observed that the increase in $[Ca^{2+}]_i$ drives the activation of calcineurin and the subsequent dephosphorylation of Ser-845 and internalization of GluA1. Our results do provide a direct relationship between oAβ, rise in $[Ca^{2+}]_i$, activation of calcineurin, and reduction of phospho-Ser-845, a pathway that causes AMPAR removal from extrasynaptic and synaptic sites.

These results are consistent with reports showing that oAβ facilitates LTD (19, 47). The oAβ-mediated decrease in GluA1 in the cell surface may be part of the primary stages of the mechanism by which oAβ facilitates/induces LTD, inhibits LTP, and causes synapse failure. A recent study has suggested that induction of LTP needs transient incorporation of GluA1 homomers at perisynaptic sites (48). This incorporation is associated with phosphorylation of GluA1 Ser-845 (12, 49), which prevents endocytosis (11, 27) and lysosomal degradation of GluA1 (9). To test the relevance of oAβ modulation of GluA1 phosphorylation and turnover on synaptic plasticity events, we took advantage of a recent described protocol of cLTP in cultured neurons (12, 40, 50). In these previous studies an LTD-

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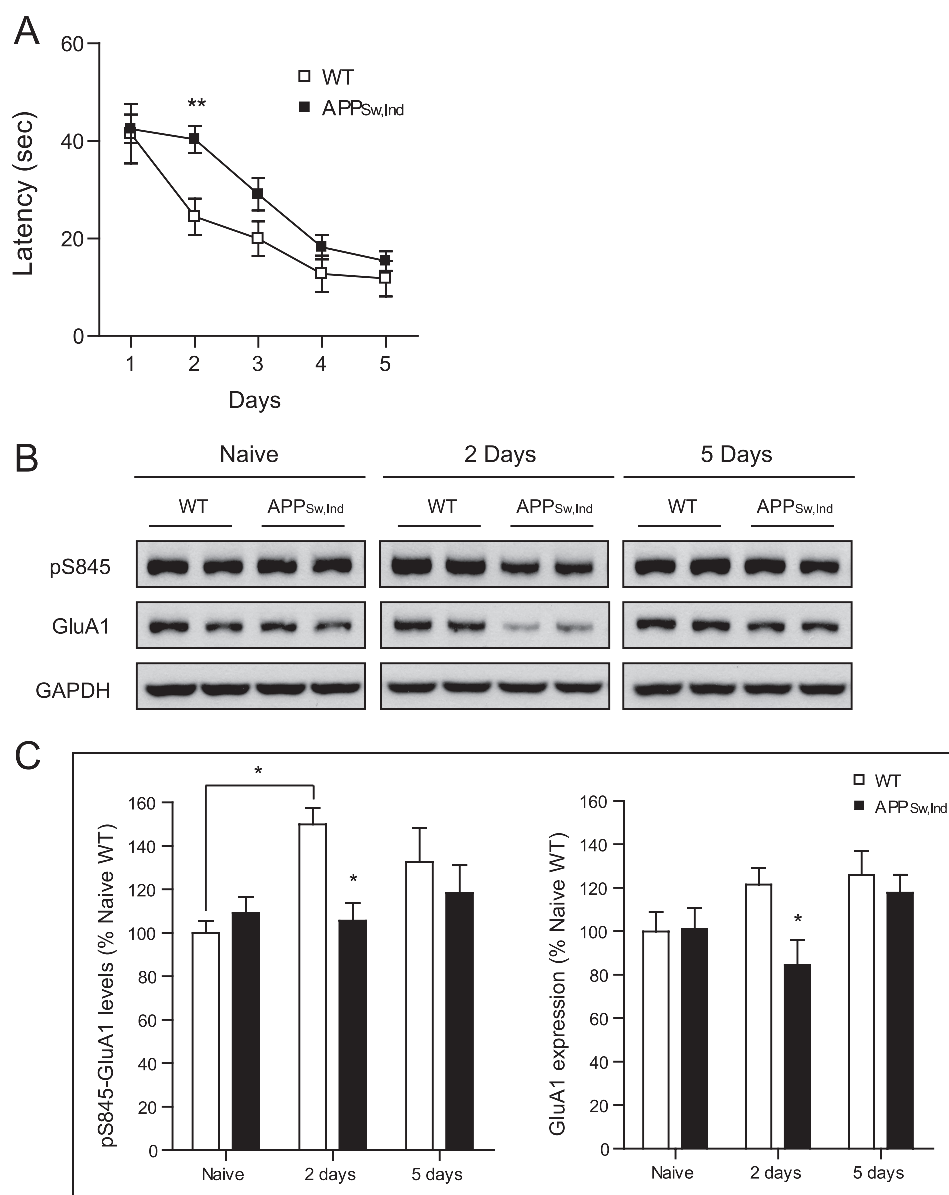

FIGURE 7. **$APP_{Sw,Ind}$ mice show lower levels of phosphorylated GluA1 at Ser-845 at 6 months of age.** *A*, $APP_{Sw,Ind}$ transgenic mice display learning deficits in the Morris water maze. Six-month-old littermate $APP_{Sw,Ind}$ and non-transgenic control mice ($n = 4$ WT/$n = 6$ $APP_{Sw,Ind}$ genotype) were trained in the Morris water maze for 5 days. $APP_{Sw,Ind}$ mice learned the task at 5 days, but they required significantly longer latencies to locate the platform after 2 days of training (two-way analysis of variance; latencies: genotype effect, $F_{(1)} = 10.71$; day effect, $F_{(4)} = 24.38$; $p < 0.0001$). Data represent the mean ± S.E. **, $p < 0.01$. *B*, representative immunoblots of hippocampal total protein extract from naive, 2 days and 5 days after Morris water maze task mice. Extracts were probed with the indicated antibodies. *C*, densitometric quantification of changes expressed as mean ± S.E. (naive wild type is indicated as 100%). *, $p < 0.05$.

like stimulation of NMDAR produces a decrease in phospho-Ser-845 and surface expression of GluA1, whereas stimulation with forskolin and rolipram recovered phosphor-Ser-845 and GluA1 surface expression. Our data show that oAβ prevents forskolin/rolipram-mediated increase of phosphor-Ser-845 and cell surface GluA1 levels, which supports recent evidence showing that oAβ is able to block LTP (6, 18, 51).

Which are the mechanisms involved in oAβ-induced blockade of cLTP? It is possible that oAβ affects PKA activity (52) or causes changes in the interactions of AMPAR with scaffolding proteins (53). This would reduce the stability of the receptor with synaptic partners at the post-synaptic membrane (54–56). Further experiments are needed to explore the eventual effect of oAβ on scaffolding proteins and its role in the regulation of AMPAR trafficking. Another possibility may involve the altered stoichiometry of AMPAR by oAβ. It is known that GluA1 delivery to synaptic terminals is a key event in LTP maintenance, whereas an increase in GluA2 and a decrease in GluA1 synaptic levels would favor the induction and maintenance of LTD (8, 57). Here, we provide evidence that oAβ affects the total amount of GluA2 associated to GluA1. This oAβ-mediated decrease in the interaction of GluA1 and GluA2, together with the decrease in membrane delivery of GluA1, causes a decrease in the ratio GluA2/GluA1 ratio affecting subunit composition and functionality of AMPARs at the synapses and facilitating LTD *versus* LTP.

A similar effect on GluA1 phosphorylation and recycling was also observed when endogenous Aβ was naturally secreted in

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neuronal cultures. Primary neuronal cultures of a genetic model of AD, expressing the human mutant $APP_{Sw,Ind}$ (24), accumulate Aβ in the media producing a reduction in cell surface AMPAR, which is reversed by a γ-secretase inhibitor. Because $APP_{Sw,Ind}$ mice develop early synaptic deficits associated with increased Aβ level (26), we analyzed changes in phosphorylated AMPAR subunits at initial pathological and memory stages (24, 25). As previously described (26), 6-month-old $APP_{Sw,Ind}$ transgenic mice displayed spatial learning deficits in Morris water maze during the first days of training. Our present results show a strong correlation between the learning deficits and reduced GluA1 phosphorylation at Ser-845 in $APP_{Sw,Ind}$ mice. Thus, we suggest that initial learning and memory deficits in these transgenic mice may result from impaired AMPAR trafficking and be caused by a calcineurin-dependent reduction in the levels of phosphorylated GluA1 at Ser-845. This mechanism is complementary to the calcineurin-dependent modulation of activity-dependent gene transcription required for learning through the cAMP-response element-binding protein coactivator CRTC1 that we have observed previously (26).

In conclusion, we suggest a direct relationship between soluble oligomers of amyloid-β present on early stages of AD. We have demonstrated that oAβ induces the loss of AMPAR in the cell surface that could be related to early memory deficits observed in an experimental model of AD. In addition, we provide evidence that oAβ increase $[Ca^{2+}]_i$ influx through ionotropic glutamate receptors, leading to calcineurin activation and dephosphorylation and internalization of AMPAR. We suggest that this mechanism is linked to early hippocampal-dependent memory deficits observed in AD.

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