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β-Amyloid Enhances Glial Glutamate Uptake Activity and Attenuates Synaptic Efficacy*

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Although amyloid β-protein (Aβ) has long been implicated in the pathogenesis of Alzheimer's disease, little is known about the mechanism by which Aβ causes dementia. Aβ leads to neuronal cell death *in vivo* and *in vitro*, but recent evidence suggests that the property of the amnesic characteristic of Alzheimer's disease can be explained by a malfunction of synapses rather than a loss of neurons. Here we show that prolonged treatment with Aβ augments the glutamate clearance ability of cultured astrocytes and induces a dramatic decrease in glutamatergic synaptic activity of neurons cocultured with the astrocytes. Biotinylation assay revealed that the enhancement of glutamate uptake activity was associated with an increase in cell-surface expression of GLAST, a subtype of glial glutamate transporters, without apparent changes in the total amount of GLAST. This phenomenon was blocked efficiently by actin-disrupting agents. Thus, Aβ-induced actin-dependent GLAST redistribution and relevant synaptic malfunction may be a cellular basis for the amnesia of Alzheimer's disease.

Amyloid β-protein (Aβ),[1] a peptide with 40–42 residues, is a main element of senile plaque, a hallmark of Alzheimer's disease (AD) (1, 2), and is accumulated highly in the forebrain of AD patients, as well as transgenic mice overexpressing mutant β-amyloid precursor protein (βAPP), which develop AD-like pathology (3, 4). Although numerous studies showed that exogenously applied or endogenously produced Aβ leads to neuronal cell death, the amnesic feature of AD cannot be explained by the neuronal loss alone (5). Indeed, accumulating evidence indicates that Aβ induces severe impairment of excitatory neurotransmission in the hippocampus (6–8) and thereby may cause memory deficits (9). In mutant βAPP transgenic mice, such synaptic malfunction often appears in advance of Aβ plaque formation (10, 11), and cognitive deterioration is also observed without apparent neurodegeneration (4, 12). Aβ-induced synaptic deterioration rather than neuronal loss is, therefore, likely to be a main cause of early AD dementia (5, 13). However, the mechanisms by which Aβ causes such synaptic malfunction remain to be elucidated.

Excitatory neurotransmission is tightly regulated by a rapid clearance of the neurotransmitter glutamate from the extracellular milieu through Na^+-dependent L-glutamate transporters that are expressed on astrocytes, *i.e.* GLAST and GLT-1 (14, 15). We therefore investigated the effect of Aβ on glutamate uptake activity in cultured cortical astrocytes. Here we show for the first time that Aβ ending at 42 residues (Aβ (1–42)) induces an increase in the activity of GLAST. This work further demonstrates that Aβ (1–42) stimulates actin-dependent GLAST redistribution from subcellular compartment to the cell surface. Such up-regulation of GLAST function may attenuate glutamatergic synaptic efficacy.

EXPERIMENTAL PROCEDURES

Materials—Chemically synthesized Aβ (1–40) and Aβ (1–42) were gifts from Dr. T. Shirasawa (Department of Molecular Genetics, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). The Aβs were purified in basic conditions to avoid aggregation, with the reverse-phase HPLC so that 50 pmol of each of these molecules gave a single and sharp peak on HPLC. Their purity and amino acid composition were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (16). Affinity-purified rabbit anti-GLAST and GLT-1 primary antibodies were gifts from Dr. K. Tanaka (Tokyo Medical Dental University, Tokyo, Japan). The specificity of these antibodies was reported previously (17, 18). L-[^{3}H]Glutamate and fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody were purchased from Amersham Biosciences. Actinomycin D, dihydrokainate (DHK), immobilized avidin, peroxidase-conjugated anti-rabbit IgG antibody, LY294002, nifedipine, threo-β-hydroxy aspartate (THA), and wortmannin were obtained from Sigma. Cycloheximide, cytochalasin D, latrunculin A, and thapsigargin were obtained from Wako Chemicals (Osaka, Japan). H-7, propidium iodide, sulfo-*N*-hydroxysuccinimide-biotin, U-126, and genistein were obtained from Calbiochem, Molecular Probes (Eugene, OR), Pierce, Promega (Madison, WI), and Research Biochemicals (Natick, MA), respectively.

Astrocyte Cultures—Cortical astrocytes were prepared from postnatal 2-day-old rat pups (SLC, Shizuoka, Japan) as described previously (19). Cortical hemispheres were trypsinized (0.25%) and plated in Eagle's minimal essential medium with 10% fetal bovine serum. The medium was exchanged every 3–4 days, and on reaching confluence the cells were trypsinized and replated once. The confluent cultures were treated with a serum-free medium for 24 h and used for experiments. In these cultures, more than 97% of cells were astrocytes, and <1% were microglial cell, as assessed by the astrocyte-specific marker GFAP and the microglial marker OX-42, respectively (data not shown). The number of microglia was not changed significantly by Aβ treatment.

Neuron Cultures—Cultures of embryonic neurons were prepared from E18 rat cerebral cortex (SLC) as described previously (20). For plating on a monolayer of astrocytes, cells were suspended in Neurobasal (Invitrogen) containing 10% fetal bovine serum and plated at 500 cells/mm^2. After 24 h, cells were maintained further with serum-free

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[1] The abbreviations used are: Aβ, amyloid β-protein; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; HPLC, high pressure liquid chromatography; DHK, dihydrokainate; THA, threo-β-hydroxy aspartate; sEPSC, spontaneous excitatory postsynaptic current; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; ANOVA, analysis of variance.

Neurobasal supplemented with 2% B27 (Invitrogen). Experiments were performed at day 7 *in vitro*.

Electrophysiological Recordings—Whole-cell voltage clamp (−70 mV) recordings were obtained from cultured hippocampal neurons. Recording solutions contained the following (in mM): 147 NaCl, 3 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, 25 μM D-2-amino-5-phosphonopentanoic acid, and 10 μM picrotoxin, adjusted to pH 7.4. Patch recording pipettes (6 megohms) were filled with intracellular solutions containing the following (in mM): 120 $CsMeSO_3$, 20 CsCl, 1 EGTA, 0.4 NaGTP, 4 MgATP, 5 QX314, and 10 HEPES, pH 7.3, with CsOH at 35 °C. Whole-cell recordings were made with Axopatch 200B amplifiers, digitized at 10 KHz by DIGIDATA 1320A interface, and acquisition and analysis were performed with the pCLAMP8 (Axon Instruments, Foster City, CA). Neurons with series resistances in the range of 8 to 17 megohms were selected for analyses. Spontaneous excitatory postsynaptic currents (sEPSCs) were obtained by randomly selecting intervals of 200 s from the stored data for each neuron. The non-NMDA receptor antagonist CNQX blocked sEPSC completely (data not shown).

Glutamate Uptake—L-[^{3}H]Glutamate uptake of astrocytes was measured as described (19). Briefly, cultures were washed for 30 min with a modified Hanks' balanced salt solution and exposed to a combination of 0.1 μCi/ml [^{3}H]glutamate and 10 μM unlabeled glutamate for 7 min. Uptake was terminated by ice-cold Hanks' solution. Astrocytes were lysed in 0.5 N NaOH. Aliquots were taken for scintillation counting and for protein assays. Because Aβ aggregates spontaneously, the total amount of proteins was increased corresponding to the doses of Aβ. Because the number of astrocytes per well was relatively constant (data not shown), uptake rates were normalized per well (not per unit weight protein).

Biotinylation—Biotinylation of cell surface proteins was performed as described by Davis *et al.* (21) and Duan *et al.* (22) with some modifications. After drug treatment, the astrocyte cultures were rinsed with phosphate-buffered saline (PBS), incubated in sulfo-NHS-biotin solution (1.5 mg/ml in PBS) for 20 min at 4 °C. The cultures were washed twice with PBS containing 100 mM glycine to stop the reaction. After 45 min of incubation with the glycine-containing PBS at 4 °C, the cells were lysed in 300 μl/well of lysis buffer with protease inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 250 μM phenylmethylsulfonyl fluoride, 1 mg/ml trypsin inhibitor, and 1 mM iodoacetamide) for 1 h at 4 °C and then centrifuged at 16,000 *g* for 15 min at 4 °C to remove debris. Before the lysate was incubated with avidin-conjugated beads, the aliquot was taken for Western blot analysis as the "total cell lysate" fraction. The remaining lysates (150 μl) were incubated with equal volumes of avidin beads slurry and centrifuged at 16,000 × *g* for 15 min, and the supernatants were taken for Western blot analysis as the "intracellular" fraction. The pellets were washed four times with the lysis buffer with the protease inhibitors and resuspended in 300 μl of Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 5% 2-mercaptoethnol) and then treated for 30 min at 70 °C. After centrifugation at 16,000 × *g* for 15 min, the supernatants were taken as the "biotinylated (cell surface)" fraction. All three samples for Western blot analysis were diluted to be the same aliquot and frozen at −20 °C until analysis. The protein samples were loaded on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blotted with anti-GLAST or GLT-1 antibody (1:1000) and then with the peroxidase-conjugated anti-rabbit IgG (1:5000). Immunoreactive proteins were visualized with an enhanced chemiluminescence kit (PerkinElmer Life Sciences).

Immunocytochemistry—After the treatment with Aβ, the astrocytes cultures in 35-mm dishes were washed twice with PBS and fixed with 4% paraformaldehyde for 5 min, permeabilized with 0.25% Triton X-100 for 5 min, and blocked with 2% horse serum for 30 min. The cultures were incubated with anti-GLAST or GLT-1 antibody (1:2500) overnight at 4 °C and then with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:5000) and 5 μg/ml propidium iodide for 1 h at room temperature. The dishes were broken and settled on glass coverslips upside down. The fluorescence images were obtained with a laser scanning confocal system Micro Radiance 1000 (Bio-Rad).

RESULTS

Aβ Attenuates Glutamatergic Neurotransmission in Neuroglial Cocultures—The initial set of experiments was designed to examine the effect of Aβ on synaptic transmission in primary cultures of cortical neurons. After day 7 *in vitro* neurons were exposed to 20 μM Aβ (1–42) for 12 h, and sEPSCs were recorded by whole-cell patch clamp techniques. Aβ-treated neurons exhibited a slight but significant decrease in both the mean amplitude and the frequency of sEPSCs (Fig. 1). This result is the first evidence that Aβ attenuates neuronal activity in culture.

In the brain, however, neurons are surrounded by a larger number of astrocytes, which render physical and physiological supports for neurons (23). To measure the Aβ effect under more physiological conditions, neurons were plated onto the monolayer of confluent astrocytes and processed for the same experimental treatment. In this coculture system, a similar decrease in sEPSC amplitude and frequency was produced by Aβ treatment, but surprisingly, the detrimental effect of Aβ was much larger in the presence of astrocytes (Fig. 1*C*).

Immunohistochemical staining for microtubule-associated protein-2 and glial fibrillary acidic protein revealed that the survival of neurons or astrocytes was unaffected by the exposure to Aβ; the number of surviving cells was 79.7 ± 4.7 (neurons) and 374.1 ± 13.4 (astrocytes) per mm^2 in control cultures and 72.5 ± 3.3 (neurons) and 394.0 ± 15.5 (astrocytes) per mm^2 in Aβ-treated cultures (means ± S.E. of 8–11 cultures). Lactate dehydrogenase (LDH) assay also indicated that Aβ did not increase the activity of LDH released from astrocyte cultures; the percentages of released LDH to the total cellular LDH are 18.7 ± 5.4% in control cultures and 16.8 ± 4.8% in Aβ-treated cultures ($n = 4$). Similarly, Western blot analysis showed that glial expression of actin was unchanged by Aβ treatment (see Fig. 4*C*). Propidium iodide-labeled nuclei displayed no aberration in Aβ-treated astrocytes (see Fig. 5, *C* and *D*). All these results indicate that Aβ treatment did not affect the cell viability. Therefore, the result that Aβ-induced synaptic malfunction was aggravated by the presence of astrocytes suggests that the Aβ effect is mediated, at least in part, by an alteration of astrocytic physiological functions.

Because one of the major roles of astrocytes is to terminate neurotransmission by the uptake of extracellular glutamate through high affinity glutamate transporters, our data suggest that Aβ enhances astrocytic glutamate uptake activity. To address this possibility, sEPSCs were recorded at a low temperature, because hypothermal conditions can attenuate efficiently the activity of glial glutamate transporters (24, 25). A significant difference in the Aβ effect between neuron-enriched cultures and neuroglial cocultures was no longer observed at a lower temperature (24 °C). We further attempted to determine whether the Aβ effect is blocked by THA, a potent inhibitor of glial glutamate transporters, but this inhibitor *per se* induced the swelling of Aβ-treated neurons and disturbed successful whole cell recordings. Nonetheless, the result at a low temperature implies Aβ-induced alteration in glutamate transporter activity. Thus, the following experiments have focused on the effect of Aβ on the glutamate clearance ability of astrocytes.

Aβ Facilitates GLAST-mediated Glutamate Uptake—Glutamate transport activity in pure cultures of cortical astrocytes was measured as uptake activity of L-[^{3}H]glutamate. Baseline uptake activity was hindered completely in Na^+-free medium and abolished by THA in a concentration-dependent manner (Fig. 2*A*). These data indicate that the uptake activity was mediated by Na^+-dependent secondary active transport via glutamate transporters. The uptake was unaffected by even a high concentration of DHK, a selective GLT-1 inhibitor (Fig. 2*A*), which suggests that GLAST is a predominant glutamate transporter in our cultures. Consistent with this, Western blot analysis could not detect apparent immunoreactivity for GLT-1 in our cultures (data not shown; see also Refs. 19 and 26). Thus we consider that this culture system is useful in investigating the molecular behavior of GLAST, one of the major glutamate

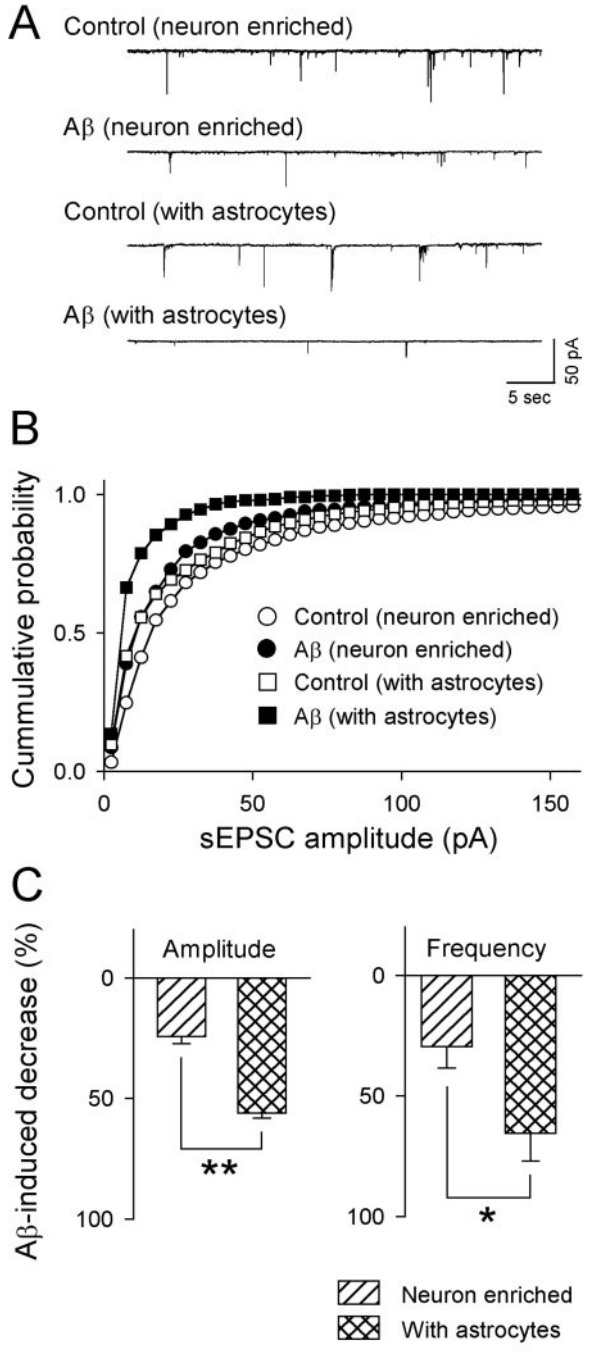

FIG. 1. **Aβ attenuates synaptic responses of cortical neurons growing on astrocyte monolayers.** Neuron-enriched/astrocyte-poor cultures (*neuron enriched*) or cocultures of neurons and astrocytes (*with astrocytes*) were treated with vehicle (*Control*) or 20 μM Aβ for 12 h. *A*, representative traces of sEPSCs. *B*, Aβ caused a significant leftward shift of the cumulative probability histogram in both neuron-enriched cultures and cocultures (each $p < 0.01$, Kolmogorov-Smirnov test). Aβ did not change series resistances: 11.5 ± 0.9 megohms in control neurons and 12.2 ± 0.5 megohms in Aβ-treated neurons. *C*, summary of the suppressive effect of Aβ on sEPSC amplitude and frequency. Each value in the ordinates was obtained by averaging the percentage changes in mean amplitude or event frequency. The Aβ effect on spontaneous synaptic activities was more severe in neuroglial cocultures than in neuron-enriched cultures. Baseline sEPSC amplitude was 37.0 ± 1.5 (neuron enriched) and 27.5 ± 1.2 pA (with astrocytes). Baseline frequency was 1.60 ± 0.26 (neuron enriched) and 1.10 ± 0.30 Hz (with astrocytes). Thus, the amplitude and frequency were both attenuated in the presence of astrocytes. The effect of this astrocyte was abolished completely by THA (35.6 ± 2.5 pA of amplitude and 1.58 ± 0.31 Hz of frequency in THA), suggesting that the basal activity of astrocytic glutamate transporters decreases synaptic efficacy. This idea is consistent with many previous reports (24, 54–57) showing that glutamate transporters regulate basal synaptic transmission. We also examined the effect on miniature EPSCs, which were recorded in the presence of 1 μM tetrodotoxin to prevent spontaneous spike activity. The Aβ-induced decrease in the amplitude, but not frequency, of miniature EPSCs was enhanced by culturing neurons with astrocytes (data not shown), which is in accordance with a study (24) that THA increases the size, but not frequency, of events. Glial glutamate transporters are, therefore, likely to regulate synaptic activity but not spike generation. *, $p < 0.05$; **, $p < 0.01$; Student's *t* test. Data are means ± S.E. of 8–10 neurons from three independent experiments.

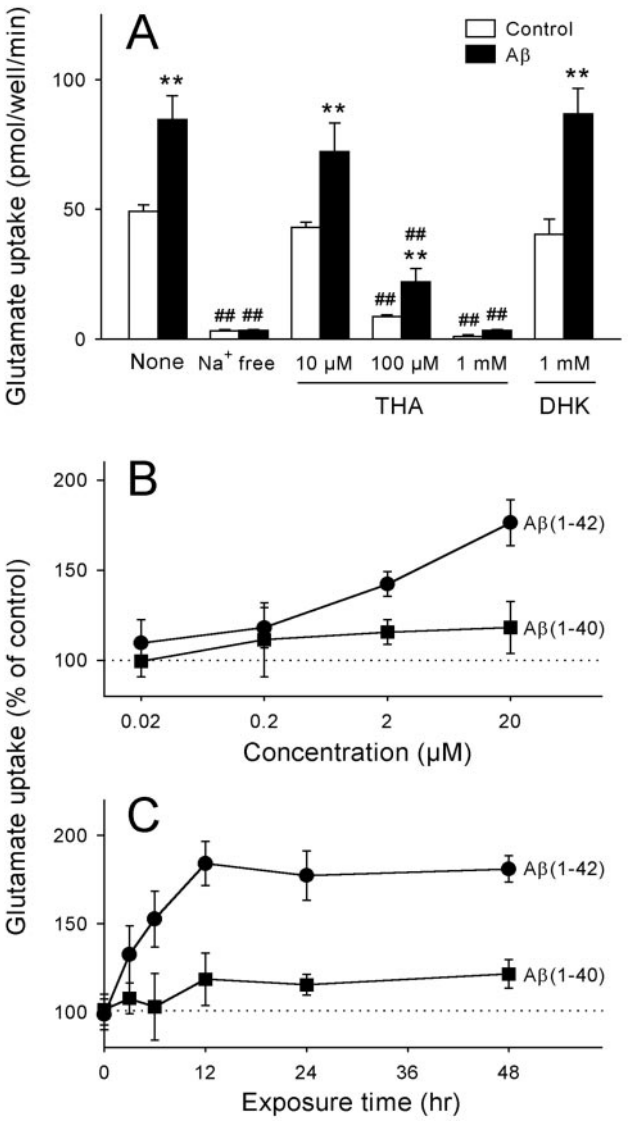

FIG. 2. **Aβ (1–42) enhances THA-sensitive glutamate uptake activity in a concentration- and time-dependent manner.** *A*, confluent cultures of astrocytes were treated with 20 μM Aβ (1–42) for 48 h, and L-[^{3}H]glutamate uptake activity was measured in normal or Na^+-free medium or in the presence of THA or DHK. **, $p < 0.01$ *versus* Control; ##, $p < 0.01$ *versus* None; Tukey's test after ANOVA. *B*, glutamate uptake activities were measured after a 48-h treatment with Aβ (1–40) or Aβ (1–42) at concentrations ranging from 0.02 to 20 μM. *C*, uptake activities were measured 0, 3, 6, 12, 24, or 48 h after the incubation with 20 μM Aβ (1–40) or 20 μM Aβ (1–42). The relative activity is expressed as a percentage of baseline uptake of untreated astrocytes. THA-sensitive glutamate uptake activity was significantly augmented 3 h after Aβ (1–42) treatment ($p < 0.05$) and reached the saturation state within 12 h ($p < 0.01$), but little effect was observed for Aβ (1–40). Data are means ± S.E. of four different cultures.

transporters of the adult forebrain (15, 27). Incidentally, when astrocytes were cocultured with neurons for 7 days, the uptake activity was unchanged: 47.9 ± 7.8 pmol/well/min in pure as-

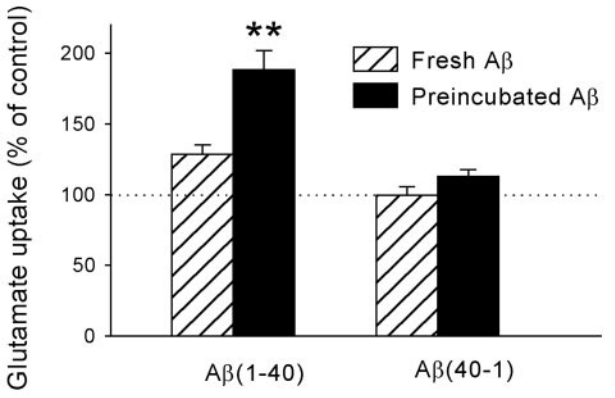

FIG. 3. **Aggregated Aβ (1–40) causes an increase in astrocytic glutamate uptake activity.** Immediately after being solubilized, Aβ (1–40) or Aβ (40–1) was applied to cultured astrocytes at 20 μM for 48 h (*Fresh Aβ*). After the solubilization, the Aβ was incubated at 37 °C for 7 days to allow spontaneous aggregation and applied to astrocytes at 20 μM for 48 h (*Preincubated Aβ*). The glutamate uptake activity was enhanced by preincubated Aβ (1–40) but not by fresh Aβ (1–40), fresh Aβ (40–1), or preincubated Aβ (40–1). The ordinate indicates a percentage of the uptake activity in control astrocytes. **, $p < 0.01$ *versus* control; Tukey's test after ANOVA. Data are means ± S.E. of four independent experiments.

trocyte cultures and 58.9 ± 11.8 pmol/well/min in cocultures with neurons ($p > 0.1$, Student's t test; means ± S.E. of four cases). These results suggest that neuronal contribution to the total activity of glutamate uptake assumed in the experiments of Fig. 1 is substantially low as compared with glial transport activity and that neurons do not cause a change in GLAST activity in astrocytes.

As predicted by our electrophysiological data, continuous application of 20 μM Aβ (1–42) for 48 h induced a significant increase in the rate of glutamate uptake (Fig. 2*A*). This enhancement was inhibited efficiently by THA but not by DHK (Fig. 2*A*), which suggests that the augmented uptake activity was unlikely because of the emergence of GLT-1 activity and that it was totally attributable to the enhancement of GLAST activity.

The Aβ (1–42)-induced increase in glutamate uptake activity showed a concentration dependence in the range of 0.02 to 20 μM (Fig. 2*B*). More than 20 μM Aβ (1–42) severely deteriorated the viability of astrocytes (data not shown). The time dependence of the Aβ effect was investigated at a concentration of 20 μM. The facilitation of uptake was observed 3 h after exposure to Aβ and reached apparent steady state after 12 h (Fig. 2*C*).

The shorter form Aβ (1–40), another type of endogenous Aβ, was virtually ineffective (Fig. 2, *B* and *C*). Although the difference in sequence between Aβ (1–42) and Aβ (1–40) is only two residues of C terminus, Aβ (1–42) aggregates more rapidly than Aβ (1–40) (28). Like Aβ (1–42), Aβ (25–35), a biologically active, hydrophobic fragment of Aβ (29), is also highly prone to aggregation (30). This subfragment could also reproduce the effect of Aβ (1–42) (data not shown). Because it is generally believed that aggregated Aβ is responsible for AD progression (1, 2), fresh Aβ (1–40) was incubated at 37 °C for 7 days to allow aggregation (31) and then applied to astrocyte cultures. The preincubated Aβ (1–40) enhanced efficiently glutamate transport activity up to a level comparable with Aβ (1–42) (Fig. 3). The control peptide Aβ (40–1), a reverse-sequence peptide that is stable and does not form aggregates, showed no effect even after preincubation (Fig. 3). These results suggest that the aggregation of Aβ is essential for the enhancement of glutamate uptake.

Aβ Stimulates the Cellular Trafficking of GLAST—Eadie-Hofstee plots of the uptake activity showed that 20 μM Aβ (1–42) produced a significant increase in the V_{max} value from 126.0 ± 6.8 to 202.0 ± 8.3 pmol/well/min with a minimal change in the K_m value (Fig. 4*A*), suggesting that Aβ (1–42) causes an increase in functional GLAST proteins. To determine whether Aβ-stimulated transport requires *de novo* mRNA/protein synthesis, we examined the effects of the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide. However, neither of these inhibitors affected the activity of glutamate uptake of intact or Aβ (1–42)-treated astrocytes (Fig. 4*B*), which suggests that Aβ increases the activity of GLAST without mRNA/protein synthesis. Indeed, Western blot analysis revealed that the Aβ (1–42) treatment induced no apparent change in the total amount of GLAST (Fig. 4*C*). This is consistent with the report showing that the expression level of EAAT1, a human GLAST homologue, is not altered in AD brain (32).

Because the membrane trafficking system is known to regulate the activity of some transporters, *e.g.* the neuronal glutamate transporter EAAC1 expressed in C6 glioma (21), serotonin transporters expressed in HEK293 cells (33), the γ-aminobutyric acid transporter GAT1 expressed in *Xenopus* oocytes (34), and dopamine transporters expressed in PC12 cells (35), it is also possible that the Aβ effect is achieved by an increase in GLAST proteins on the cell surface. This possibility was addressed by a membrane-impermeant biotinylation assay. Biotinylated, cell surface protein fractions were separated from nonbiotinylated, intracellular protein fractions by using avidin-conjugated beads. The expression of GLAST in these two fractions was assessed by Western blot analysis (Fig. 4*C*). In Aβ (1–42)-treated astrocytes, GLAST expression increased in the biotinylated (cell surface) fraction and decreased complementarily in the nonbiotinylated (intracellular) fraction. These results indicate that Aβ (1–42) caused GLAST translocation from the intracellular compartment to the plasma membrane.

The cellular distribution of GLAST in Aβ-treated astrocytes was examined further by immunohistochemical staining (Fig. 5). The nuclei were labeled with propidium iodide to distinguish each cell. Aβ (1–42) caused apparent clustering of GLAST immunoreactivity along the edge of the soma and also slightly in the cytoplasmic part, whereas in control astrocytes, GLAST was distributed throughout the cytoplasm (Fig. 5). Although Brera *et al.* (36) reported that long term treatment with Aβ leads to cell death of astrocytes, we found no evidence for shrinkage or degeneration of propidium iodide-labeled nucleus at least after a 48-h treatment with 20 μM Aβ (1–42). Therefore, the possibility that Aβ (1–42)-evoked GLAST redistribution is merely because of cell damage could be ruled out.

Aβ Induces Actin-dependent GLAST Redistribution—To determine whether Aβ-induced increase in glutamate uptake is mediated by GLAST translocation, we examined the effect of cytochalasin D and latrunculin A, inhibitors of actin polymerization, which is the cellular event known to be essential for subcellular membrane trafficking (37). The inhibitors attenuated significantly Aβ-induced up-regulation of glutamate uptake without affecting the baseline activity of control astrocytes (Fig. 6). The microtubule disrupter colchicine had no influence on the Aβ (1–42)-stimulated transport (data not shown). These data suggest that the Aβ effect on glutamate uptake activity is mediated by GLAST redistribution dependent on actin rearrangement.

Finally, we attempted a series of pharmacological investigations to clarify the signaling pathway underlying Aβ-induced increase in GLAST activity. EAAC1 translocation is regulated by protein kinase C (21, 37). Because GLAST possesses multiple phosphorylation sites for protein kinase C (38), we tested the effect of H-7, an inhibitor of protein kinase C and A. How-

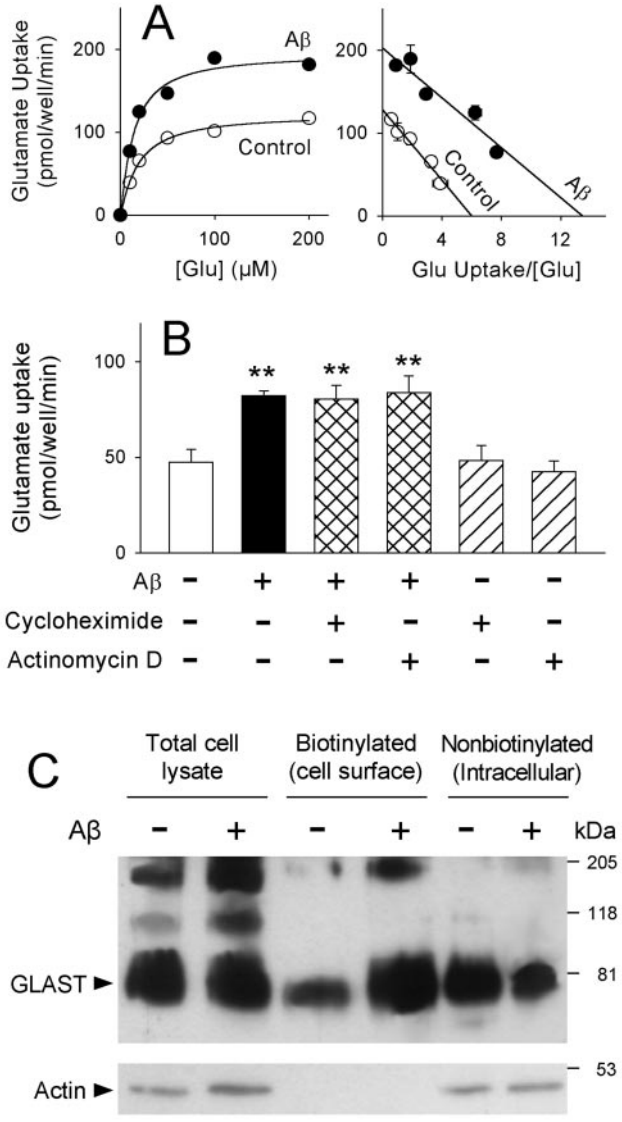

FIG. 4. **Aβ-induced GLAST translocation to the plasma membrane.** *A*, a concentration dependence of glutamate transport activity was examined in the astrocytes treated with 20 μM Aβ (1–42) for 48 h (*left panel*). The data were plotted in an Eadie-Hofstee format, and the V_{max} and K_m values were calculated by a linear regression analysis (*right panel*). The average V_{max} values were 126.0 ± 6.8 nmol/well/min (*Control*) and 202.0 ± 8.3 nmol/well/min (*Aβ*). The average K_m values were 19.1 ± 4.0 μM (*Control*) and 14.2 ± 1.6 μM (*Aβ*). Aβ (1–42) induced a substantial increase in the V_{max} (but not K_m) value ($p < 0.01$). *B*, Aβ (1–42) (20 μM) was coapplied with 1 μM actinomycin D or 10 μM cycloheximide for 48 h. Neither inhibitor affected the facilitatory effect of Aβ, which suggests that the up-regulation of glutamate uptake is not mediated by *de novo* synthesis of mRNA or protein. Data are means ± S.E. of four cases. *C*, representative immunoblot of the effect of Aβ on the expressions of GLAST (*top panel*) and actin (*bottom panel*) in the total cell lysate, biotinylated (cell surface), and nonbiotinylated (intracellular) fractions. Cell surface proteins were labeled with membrane-impermeable biotin. Anti-GLAST antibody recognized a protein with a molecular mass of ~64 kDa (monomer) and also its putative dimer and trimer (58). Treatment with 20 μM Aβ (1–42) for 48 h did not alter the total amount of GLAST but caused an increase in biotinylated GLAST and a compensatory decrease in nonbiotinylated GLAST. The immunoreactivity for actin, an index of intracellular proteins, was not changed in any fractions. These results indicate that Aβ induced the membrane trafficking of GLAST. Experiments were repeated with at least four different cultures, producing the same results. We did not quantify the density of immunoreactive bands, because the edge of each band was somewhat unclear, probably because of GLAST glycosylation (59) and random biotinylation.

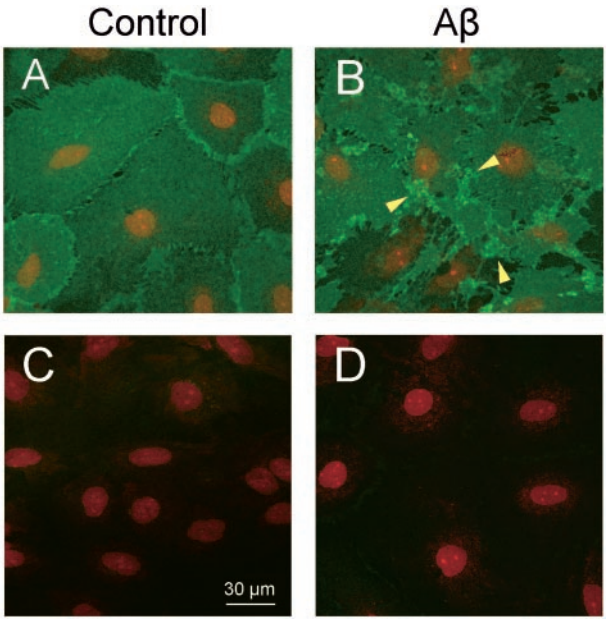

FIG. 5. **Aβ alters the cellular distribution of GLAST immunoreactivity in astrocytes.** Astrocytes were cultured in the absence (*A* and *C*) or presence (*B* and *D*) of 20 μM Aβ (1–42) for 48 h and then immunostained for GLAST (*green*). Propidium iodide (*red*) was used for counterstaining. *Panels C* and *D* show nonspecific signals of the secondary IgG-fluorescein isothiocyanate in the absence of anti-GLAST antibody. Aβ-treated astrocytes displayed cluster-like GLAST spots at the outer margins of the cell body (*arrowheads*). Similar results were obtained in every such experiment conducted ($n = 5$).

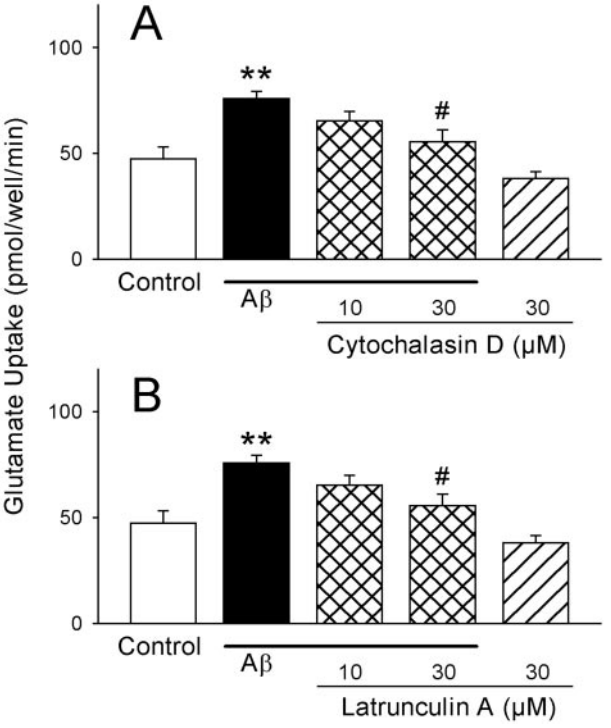

FIG. 6. **Inhibitors of actin polymerization abolished Aβ-induced enhancement of glutamate uptake activity.** Cytochalasin D (*A*) or latrunculin A (*B*) was coapplied with 20 μM Aβ (1–42) for 6 h. Both inhibitors attenuated significantly Aβ-induced enhancement of glutamate uptake. **, $p < 0.01$ *versus* Control; #, $p < 0.01$ *versus* Aβ alone; Tukey's test after ANOVA. Data are means ± S.E. of four independent experiments.

ever, 300 μM H-7 failed to prevent the Aβ (1–42) effect (the relative uptake activity to 20 μM Aβ (1–42) alone, 99.6 ± 4.1%; means ± S.E. of four cases). Although phosphatidylinositol

3-kinase is also involved in EAAC1 trafficking (21), the inhibitor LY294002 (30 μM) or wortmannin (100 nM) did not affect the Aβ (1–42)-stimulated uptake (106.0 ± 4.2 and 106.0 ± 9.4%, respectively). Likewise, we found that the effect of Aβ (1–42) was blocked by none of the drugs tested, *i.e.* the tyrosine kinase inhibitor genistein (30 μM, 103.0 ± 4.4%) or herbimycin A (10 μM, 96.6 ± 6.6%), the inhibitor of mitogen-activated protein kinase U 0126 (300 nM, 109.0 ± 8.8%), the inhibitor of microsomal Ca^{2+}-ATPase thapsigargin (1 μM, 108.0 ± 6.9%), the L-type calcium channel blocker nifedipine (100 μM, 103.0 ± 6.5%), the disrupter of synaptic vesicle-associated protein botulinum toxin C (100 nM, 101.0 ± 0.8%), the Na^+/K^+-ATPase inhibitor ouabain (1 mM, 93.5 ± 7.6%), or the antioxidant Trolox (300 μM, 104.0 ± 3.8%). The validity of concentrations of each agent was certified by our recent study (19, 39). Thus, Aβ (1–42)-induced GLAST translocation appears to be independent of classically known signaling pathways.

DISCUSSION

AD is the most common form of dementia in elderly individuals and is associated with a progressive, neurodestructive process of the human neocortex, which is characterized by senile plaques containing Aβ (1, 2). Although abnormal Aβ (1–42) accumulation has been implicated as an early and critical event in the etiology and pathogenesis of AD (40), the mechanism by which Aβ causes dementia has not been understood fully. One possible mechanism is that Aβ induces neuronal loss or enhances the vulnerability of neurons to excitotoxicity. Contrary to this simple scheme, however, recent computational analyses of a neural associative memory model indicated that neuronal loss cannot account, by itself, for the property of the amnesic characteristic of AD but rather that a malfunction of synapses, without an associated loss of neurons, can explain all the features of AD (41, 42). In support of this view, a quantitative morphometric analysis using cerebral cortical biopsy tissues from AD patients implied that a major loss of synapses at an early stage of AD forms a fundamental part of the pathological process (43). Furthermore, Aβ potently inhibited high K^+-evoked acetylcholine release from hippocampal slices independently of apparent neurotoxicity (44, 45). Therefore, Aβ-induced cell death may be less important for AD dementia than the selective impairment of synaptic function (5, 13). The present study has shown that Aβ induced a decrease in synaptic activities of cortical neurons without apparent cell death. Interestingly, the detrimental effect of Aβ was more severe when neurons were cocultured with astrocytes. Because the astrocyte-induced increase in the Aβ effect was abolished at a low temperature and, because Aβ stimulated the activity of the astrocytic glutamate transporter GLAST, we believe that Aβ-induced synaptic malfunction is attributable, at least in part, to a functional change in GLAST, *i.e.* the abnormal redistribution of GLAST. These findings are compelling evidence that Aβ alters the physiological property of neural functions without neuronal cell loss. Interestingly, recent evidence shows that GLAST immunoreactivity is evident in pyramidal cells in the cortex of AD patients (32, 46) and mutant βAPP-overexpressed mice (47). It is also possible that a similar GLAST translocation occurs in neurons, contributing to AD pathogenesis.

Previous studies showed that the fragment Aβ (25–35) induces a decrease in glutamate uptake of rat-cultured astrocytes when applied at a high concentration of 100 μM (48, 49). Our study indicated, however, that at less than 20 μM concentration, Aβ (25–35) caused a substantial increase in glutamate uptake. In transgenic mice expressing mutant βAPP, the concentration of Aβ in the brain is not more than the low micromolar range (1 to 4 μM), and such low concentrations are sufficient to cause marked impairment in learning and memory (50). Thus, we speculate that our results represent a pathophysiological action of Aβ and that the Aβ effect at higher doses merely reflects a physical damage to cells. In cultured microglia, indeed, an electrophysiological study suggested that chronic treatment with 20 μM Aβ (25–35) enhances glutamate transport current (51). This supports strongly our findings, although we determined neither the biochemical feature of glutamate transporters nor the effect on synaptic function.

Actin reorganization appears to be involved in GLAST trafficking in Aβ-treated astrocytes, but our pharmacological approach could not determine intracellular signaling pathways underlying the Aβ effect. Some signaling pathways including protein kinase C and phosphatidylinositol 3-kinase have been suggested to mediate the cellular translocation of other types of transporters (21, 33–35). However, none of them seems to be associated with Aβ-induced GLAST redistribution. Duan *et al.* (22) reported that glutamate itself induces rapid up-regulation of GLAST expression at the astrocyte cell surface, but they also failed to identify relevant signal transduction mechanisms. Very recently, several intracellular proteins were shown to interact with the neuronal glutamate transporters EAAC1 and EAAT4 (52, 53). Identifying adaptor molecules of GLAST would be helpful to clarify biochemical targets of Aβ and the signaling pathways responsible for cellular translocation of the transporter.

In summary, we have shown for the first time that Aβ (1–42) stimulates actin-dependent up-regulation of cell-surface expression of GLAST in cultured astrocytes and attenuates synaptic function of cultured neurons. These findings provide new insights into the targets of Aβ. Elucidating the mechanisms underlying the modulation of glial glutamate transporters may lead to a novel therapeutic strategy for AD.

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