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SHORT COMMUNICATION

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# β-Amyloid prevents excitotoxicity via recruitment of glial glutamate transporters

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**Abstract** Amyloid β-protein (Aβ), a putative pathogenic endotoxin involved in Alzheimer's disease, induces redistribution of glutamate transporters in astrocytes and promotes their pump activity. Because the transporters are assumed to protect neurons against excitotoxicity by removing extracellular glutamate, we hypothesized that Aβ alters the vulnerability of neurons to glutamate. Cerebrocortical neuron-astroglial co-cultures were exposed to glutamate, the concentration of which was selected so that only 20% of the neurons exhibited degeneration. When cultures were pre-treated with Aβ, exposure to the same "mild" glutamate concentration failed to damage neurons. The Aβ-induced protection was abolished by a glial glutamate transporter inhibitor. Thus, Aβ can alleviate excitotoxicity through glutamate transporter activity. The present results may challenge prevailing concepts that Aβ-induced neuron loss causes Alzheimer's dementia and also provide practical insights into neuro-glial interactions in glutamate toxicity.

**Keywords** Amyloid beta-protein · Cell death · Amino acid transport system · Astrocytes · Glutamic acid · Alzheimer's disease

## Introduction

Glutamate is a major neurotransmitter used at most excitatory synapses in the mammalian central nervous system. Its extracellular concentration is normally kept at a low level by $Na^+$-dependent L-glutamate transporters expressed in astrocytes, i.e. L-glutamate/L-aspartate transporter GLAST and L-glutamate transporter-1 GLT-1 (Kanai et al. 1993). Because excessive concentrations of glutamate trigger cell death, a phenomenon termed excitotoxicity (Oleny 1989), the ability of astrocytes to remove glutamate rapidly from the extracellular milieu is probably of importance in protecting neurons against glutamate excitotoxicity. Functional changes in these glutamate transporters could therefore be linked directly to the vulnerability of neurons. In practice, however, little is known about the specific role of the astrocytic transporters in neuronal excitotoxicity.

In the brains of Alzheimer's disease (AD) patients, amyloid β-protein (Aβ), a peptide with 42 residues, accumulates in senile plaques (Small and McLean 1999). Aβ appears to be pathogenic in AD dementia because transgenic mice over-expressing mutant β-amyloid precursor protein found in familial AD display spontaneous Aβ accumulation in micromolar concentrations and develop AD-like cognitive deterioration (Chapman et al. 1999; Schenk et al. 1999; Chen et al. 2000). Likewise, prolonged infusion of exogenous Aβ into the rat brains can replicate severe deficits in learning and memory (Nitta et al. 1994; Stéphan et al. 2001).

We and other groups have shown that Aβ causes neuronal cell death and also enhances the vulnerability of neurons to excitotoxicity (Forloni et al. 1993; Yankner et al. 1990; Mattson et al. 1992). Because these observations are in accordance with a common feature seen in the AD brains, i.e. medial temporal atrophy, neuron loss has long been believed to be the most probable cause of AD dementia. However, most of these studies have used relatively high doses of Aβ and excitotoxins to examine such toxicity. Because Aβ is markedly hydrophobic, its action in such excessive doses may merely reflect direct physical damage to cells. The effect of Aβ at pathologically relevant, lower concentrations is still ill defined. Interestingly, our previous study has indicated that at such low concentrations (2–20 μM), Aβ induces a substantial increase in glutamate uptake activity of astrocytes by translocating GLAST to the plasma membrane (Ikegaya et al. 2002). Considering the functional roles of glutamate transporters, these data would appear to be inconsistent with the contempo-

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rary belief that Aβ augments the susceptibility of neurons to glutamate toxicity. To clarify the Aβ effect under more pathophysiologically plausible conditions, therefore, the present study sought to determine how "mild" excitotoxicity is affected by Aβ (20 μM). We found that Aβ alleviates excitotoxicity through glutamate transporter activity. This work may challenge prevailing concepts on Aβ-induced neuron loss.

## Materials and methods

Highly pure Aβ(1–42) was a gift from Dr. T. Shirasawa (Department of Molecular Genetics, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). Experiments were performed according to the Japanese Pharmacological Society's guide for the care and use of laboratory animals.

Cortical astrocytes were prepared from 2-day postnatal Wistar rat pups (SLC, Shizuoka, Japan). Cortical hemispheres were dissected out and dissociated by 0.25% trypsinization. The cell suspension was centrifuged at 250 *g* for 5 min, the pellet resuspended in Eagle's MEM (Nissui Pharmaceuticals, Tokyo, Japan) with 10% fetal bovine serum (Cell Culture Technologies, Cleveland, Ohio, USA) and transferred into 75-cm$^2$ culture flasks at a density of 10,000 cells/mm$^2$. Cultures were maintained in a humidified, 5% $CO_2$ incubator at 37 °C. The medium was exchanged every 3–4 days. After reaching confluency, the cultures were detached from the flask with trypsin/EDTA solution and replated onto 48-well culture plates at 200 cells/mm$^2$. In these cultures, more than 95% of cells were astrocytes, as assessed by the astrocyte-specific marker glial fibrilliary acidic protein (GFAP, see Matsuura et al. 2002). At day 12 in vitro, neurons from cortical hemispheres were plated onto the astrocyte monolayer. Briefly, the cortices were dissected from embryonic 18-day-old Wistar rat (SLC) and dispersed with trypsin. Cells were suspended in Neurobasal (Life Technologies, Gaithersburg, Md., USA) containing 10% fetal bovine serum and plated at 500 cells/mm$^2$. The medium was changed to serum-free Neurobasal supplemented with 2% B27 (Life Technologies) 24 h after plating. Experiments were performed after 7 days. To visualize neurons growing on astrocyte monolayers, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.25% Triton X-100, then immuno-stained with an antibody against microtubule-associated protein-2 (MAP2) (1:4,000) (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by HRP-diaminobenzidine (ABC kit, 1:200, Vector Laboratories, Burlingame, Calif., USA). The number of cells bearing MAP2-positive processes was regarded as the number of surviving neurons.

L-Glutamate concentrations were measured using a colorimetric method (Abe et al. 2000). The method is designed to take advantage of the fact that glutamate degradation by glutamate dehydrogenase is accompanied by conversion of NAD to NADH, which can thereby reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT formazan by the support of L-methoxyphenazine methosulphate (MPMS), an electron coupling agent. The substrate mixture (1 ml) was prepared as follows: 20 U glutamate dehydrogenase (EC 1.4.1.3; Wako, Osaka, Tokyo) and 2.5 mg NAD (Sigma, St. Louis, Mo., USA) were dissolved in 0.9 ml 0.2 M Tris-HCl buffer (pH 8.2) with 0.1% (v/v) Triton X-100, then 0.1 ml of MTT stock solution (2.5 mg/ml in phosphate-buffered saline, pH 7.4) (Sigma) and 1 μl MPMS stock solution (100 mM in phosphate-buffered saline, pH 7.4) (Sigma) were added. A 50-μl aliquot of culture supernatant was transferred to another 96-well culture plate and mixed with 50 μl substrate mixture. The reaction (10 min at 37 °C) was stopped by adding 100 μl of a solution containing 50% dimethylformamide and 20% sodium dodecylsulphate (pH 4.7). The absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm. The standard curve was constructed in each assay using cell-free culture medium containing a known concentration of glutamate. The concentration of L-glutamate in samples was estimated from the standard curve.

The significance of differences between means was determined by two-way repeated-measures ANOVA and post hoc Tukey's test for multiple pair-wise comparisons. Differences were considered significant if $P<0.05$. Unless otherwise specified, all data are given as means±SEM from four independent experiments (each five wells)

## Results

In neuron-enriched cultures, Aβ is known to increase the vulnerability to excitotoxicity (Mattson et al. 1992). In the brain, however, neurons are surrounded by a much larger number of astrocytes that render physical and physiological support for neurons. Therefore, our experiments were designed to assess the Aβ effect in a neuron-glial co-culture system (Fig. 1A).

Cell mixtures prepared from rat cerebral cortices were plated onto the monolayer of rat cortical astrocytes at a density of 500 cells/mm$^2$. Under serum-free conditions, the number of surviving neurons stabilized by 7 days in culture (data not shown), the density being 79.7±4.7 neurons/mm$^2$. The cultures were then exposed to glutamate, aspartate, or *N*-methyl-D-aspartate (NMDA) at 0.1–1,000 μM for 30 min and the number of surviving neurons counted

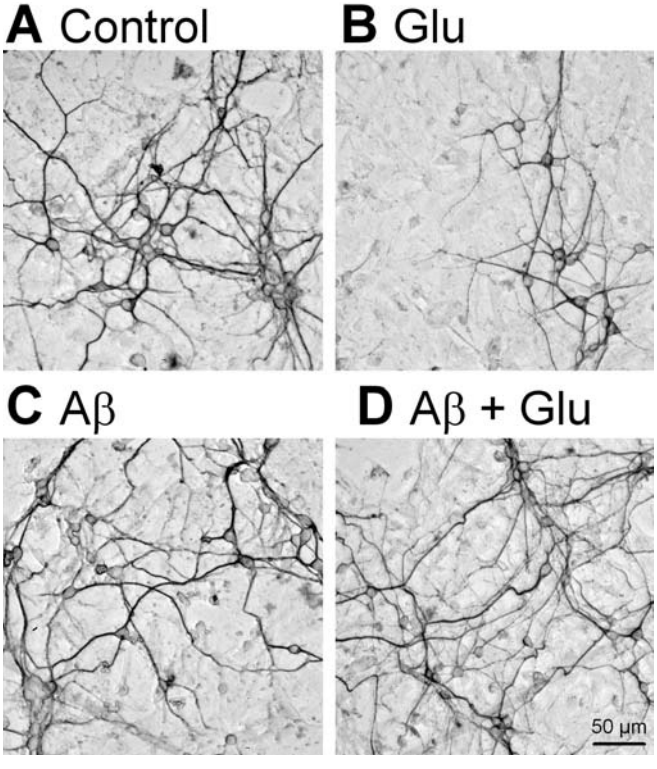


**Fig. 1A–D** Pre-treatment with β amyloid (*Aβ*) alleviates glutamate-elicited excitotoxicity in cortical neurons growing on astrocyte monolayers. Representative photomicrographs of 7-day-in-vitro neurons preincubated in the absence (**A**, **B**) or presence (**C**, **D**) of 20 μM Aβ(1–42) for 6 h and then exposed to 10 μM glutamate (*Glu*) for 30 min (**B**, **D**). After a further 24 h incubation in normal culture medium, neurons were immunostained for the viability marker microtubule-associated protein-2 (MAP2)

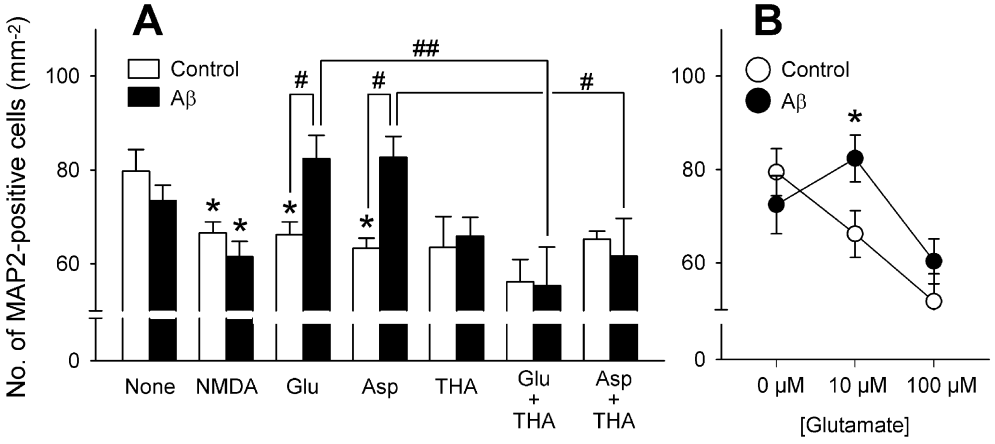


**Fig. 2A, B** Summary of *N*-methyl-D-aspartate (*NMDA*)- or glutamate-induced cytotoxicity in Aβ-pre-treated neurons. **A** After being incubated in the absence (*open columns*) or presence (*closed columns*) of 20 μM Aβ(1–42) for 6 h, cultures were exposed to either NMDA, glutamate or aspartate (*Asp*) (each 10 μM) for 30 min, in the presence (30 min) or absence of the glial glutamate transport inhibitor L-(–)-threo-3-hydroxyaspartate (*THA*, 0.1–1 mM), as indicated. Means±SEM of four independent experiments per group; $^{*}P<0.05$ versus None, $^{\#}P<0.05$, $^{\#\#}P<0.01$: Tukey's test after ANOVA. **B** Glutamate concentration dependency of the Aβ effect. Means±SEM, $n$=3–6 independent experiments per group; $^{*}P<0.05$ vs. Control, Tukey's test after ANOVA

after 24 h. All the toxins at 10 μM and above significantly decreased the number of viable (MAP2-positive) neurons (data not shown; for glutamate, see Fig. 2B). We therefore employed the threshold dose of 10 μM to assess excitotoxicity in the following experiments; at this concentration, glutamate, aspartate and NMDA decreased the number of surviving neurons by 17.0±3.4%, 19.0±3.8% and 16.5±3.4%, respectively ($P<0.05$) (Figs. 1B and 2).

We next examined the effect of pre-treating the co-cultures with 20 μM Aβ(1–42) for 6 h on this threshold excitotoxicity. The Aβ pre-treatment per se did not affect the viability of astrocytes (see Ikegaya et al. 2002) or the number of neurons (Fig. 1C and 2A) whereas higher concentrations of Aβ evoked severe neurotoxicity (data not shown). In the Aβ-treated cultures, however, subsequent 30-min exposure to 10 μM glutamate no longer induced neuronal death (Fig. 1D and 2). Similar results were obtained with 10 μM aspartate. In contrast, NMDA-induced neurodegeneration was unaffected by pre-treatment with Aβ (Fig. 2A).

We have reported previously that Aβ facilitates the glutamate clearance ability of astrocytes (Ikegaya et al. 2002). Because aspartate, but not NMDA, can be taken up by astroglial glutamate transporters (Brew and Attwell, 1987), the above effect of Aβ may reflect Aβ-mediated regulation of glutamate clearance activity in astrocytes. To address this possibility, we investigated the effect of L-(–)-threo-3-hydroxyaspartate (THA), an inhibitor of glial glutamate transporters, on Aβ-induced improvement of neuron survival. THA alone showed a tendency to cause neurotoxicity but the effect was not significant (Fig. 2A). THA abolished the Aβ-induced rescue from the toxicity of either glutamate or aspartate (Fig. 2A).

In our previous study, Aβ increased glutamate uptake activity of cultured astrocytes by 80% from approximately 0.13 to 0.23 fmol/min/cell (Ikegaya et al. 2002), but we did not establish whether this increased uptake actually reduced the concentration of glutamate in the medium. In the present study we measured the glutamate concentration in the culture media directly. By 30 min after addition of 10 μM glutamate to 200 μl of medium, the glutamate level in control neuro-astrocyte co-cultures was had fallen to 6.84±0.90 μM (mean±SD of five wells). In cultures pre-treated with 20 μM Aβ(1–42) for 6 h, the glutamate was reduced further to 4.68±0.91 μM (four wells). Thus, the final concentration of glutamate was significantly less in Aβ-treated cultures ($P<0.01$, Student's $t$-test). It is possible, therefore, that the near-threshold excitotoxic glutamate concentration established above was raised in Aβ-treated cultures. Consistent with this is the finding that Aβ could not rescue the neurons from the toxic effect of glutamate at a higher concentration (100 μM).

## Discussion

By employing relatively low Aβ and excitotoxin concentrations, we have shown for the first time that Aβ can attenuate the degree of glutamate-induced excitotoxicity in neuro-glial co-cultures. The Aβ-induced amelioration was not observed for the neurotoxicity induced by the non-transportable agonist NMDA and appeared to require glial glutamate transporter activity.

Medial temporal atrophy is a common feature of AD brains (Small and McLean, 1999) and a large body of experimental evidence has demonstrated that Aβ evokes neuron death (Forloni et al. 1993; Yankner et al. 1990; Mattson et al. 1992). However, recent computational analyses have revealed that neuron loss alone cannot account for all aspects of the amnestic characteristic of AD. Conversely a malfunction of synapses, without an associated loss of neurons, can explain all the features of AD (Ruppin and Reggia 1995; Horn et al. 1996). In support of this view, a quantitative morphometric analysis using temporal and

frontal cortical biopsy material obtained within a few years of the onset of clinical AD has implied that a massive decrease in the number of synapses per neuron is a fundamental part of the pathological process (Davies et al. 1987). In transgenic mice over-expressing mutant Aβ precursor protein, synaptic malfunction often appears in advance of Aβ plaque formation (Hsia et al. 1999; Larson et al. 1999; Kamenetz et al. 2003) and cognitive deterioration occurs in the absence of any clinical signs of histological brain injury (Chen et al. 2000; Moechars et al. 1999). Consistent with this, exogenously applied Aβ severely impairs excitatory neurotransmission and synaptic plasticity in the hippocampus (Lambert et al. 1996; Fitzjohn et al.2001), which may be ascribed partly to aberrant GLAST activity (Ikegaya et al. 2002). Such synaptic malfunction may be associated with memory deficits (Stéphan et al. 2001). Therefore, Aβ-induced cell death is probably less important for earliest amnesic symptoms than is synaptic impairment (for review, see Small et al. 2001; Selkoe 2002). Evidence also indicates that even at the end of the disease, synapse loss, but not numbers of plaques or tangles, degree of neuronal perikaryal loss or extent of cortical gliosis, is the major correlate of cognitive impairment (Terry et al. 1991). In this regard, the present study stands by the idea that AD dementia is not associated with neuron loss at least during an early phase of AD. Aβ-mediated up-regulation of glial glutamate transporters may rather represent a cellular defence against excitotoxicity.

In addition to its implications for AD, our results are significant with respect to neuro-glial interaction via glial glutamate transporter activity. The importance of the glial ability of glutamate clearance has been discussed intensively over the last few decades. Nonetheless, there has been no direct evidence for the extent to which glutamate uptake by glial cells may actually influence neuronal excitotoxicity. By utilizing Aβ as an investigative tool, we have shown that changes of the uptake activity can actually change extracellular glutamate concentrations such that neuronal vulnerability is altered. To our knowledge, this is the first indication for the relationship between the ability of glial glutamate uptake and its effect on excitotoxicity-relevant glutamate concentrations. The present study may hence provide tangible insights into dynamic interactions between neurons and glial cells.

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