Development/Plasticity/Repair

Glutamate and Amyloid β-Protein Rapidly Inhibit Fast Axonal Transport in Cultured Rat Hippocampal Neurons by Different Mechanisms

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Impairment of axonal transport leads to neurodegeneration and synapse loss. Glutamate and amyloid β-protein (Aβ) have critical roles in the pathogenesis of Alzheimer's disease (AD). Here we show that both agents rapidly inhibit fast axonal transport in cultured rat hippocampal neurons. The effect of glutamate (100 μM), but not of Aβ_{25-35} (20 μM), was reversible, was mimicked by NMDA or AMPA, and was blocked by NMDA and AMPA antagonists and by removal of extracellular Ca^{2+}. The effect of Aβ_{25-35} was progressive and irreversible, was prevented by the actin-depolymerizing agent latrunculin B, and was mimicked by the actin-polymerizing agent jasplakinolide. Aβ_{25-35} induced intracellular actin aggregation, which was prevented by latrunculin B. Aβ_{31-35} but not Aβ_{15-20} exerted effects similar to those of Aβ_{25-35}. Full-length Aβ_{1-42} incubated for 7 d, which specifically contained 30–100 kDa molecular weight assemblies, also caused an inhibition of axonal transport associated with intracellular actin aggregation, whereas freshly dissolved Aβ_{1-40}, incubated Aβ_{1-40}, and fresh Aβ_{1-42} had no effect. These results suggest that glutamate inhibits axonal transport via activation of NMDA and AMPA receptors and Ca^{2+} influx, whereas Aβ exerts its inhibitory effect via actin polymerization and aggregation. The ability of Aβ to inhibit axonal transport seems to require active amino acid residues, which is probably present in the 31–35 sequence. Full-length Aβ may be effective when it represents a structure in which these active residues can access the cell membrane. Our results may provide insight into the early pathogenetic mechanisms of AD.

Key words: axonal transport; glutamate; amyloid β-protein; actin; Ca^{2+}; cultured hippocampal neurons

Introduction

Impairment of axonal transport leads to neuronal degeneration (Moretto and Sabri, 1988; Yonekawa et al., 1998), and thus it may be an early event in the pathogenesis of some neurodegenerative diseases (Brimijoin, 1982; Warita et al., 1999; Williamson and Cleveland, 1999). In Alzheimer's disease (AD) as well as other dementing disorders, a widespread loss of synapses occurs in the brain, and synaptic injury is an early event during the progression of AD (Masliah et al., 1989; Masliah, 1995). One of critical causes of synapse loss is deficiency in axonal transport (Cull, 1975). Actually, axonal transport is impaired in the brain of AD patients (Suzuki and Terry, 1967; Younkin et al., 1986; Richard et al., 1989; Praprotnik et al., 1996).

In neurodegenerative diseases including AD, excessive glutamate in extracellular space is recognized as an excitotoxin, playing a role in the development of the diseases (Arias et al., 1998; Lancelot and Beal, 1998). Amyloid β-protein (Aβ), which accumulates in senile plaques in brains of AD patients, is also the toxic agent that causes neuronal degeneration and cell death and is profoundly involved in the pathogenesis of AD (Yankner et al.,

1990; Mattson et al., 1992; Kowall, 1994); however, the influence of these agents on axonal transport has rarely been studied. Here, we investigated the effects of glutamate and Aβ_{25-35}, an active fragment of Aβ, on fast axonal transport of membrane-bound particles in cultured rat hippocampal neurons using video-enhanced microscopy. Our data suggest that glutamate and Aβ_{25-35} rapidly inhibit fast axonal transport. The effect of glutamate is mediated by activation of NMDA and AMPA receptors and subsequent Ca^{2+} influx, whereas the effect of Aβ_{25-35} occurs via polymerization and aggregation of intracellular actin. Furthermore, we indicate that specific residues and structures are required for the naturally occurring Aβ to inhibit axonal transport.

Materials and Methods

Cell culture. The experimental protocol was approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine. Hippocampi were removed from fetal Wistar rats on day 16 of gestation, and were immediately immersed in 4°C Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) containing 0.5 mM L-glutamine (Wako, Osaka, Japan). These hippocampal tissues were dissected and then incubated for 15 min at 37°C in Ca^{2+}, Mg^{2+}-free PBS (Invitrogen) containing 0.2 mg/ml DL-cysteine hydrochloride (Sigma, St. Louis, MO), 0.2 mg/ml albumin bovine serum (Sigma), 5 mg/ml D-(+)-glucose (Wako), 0.55 mg/ml papain (Worthington Biochemicals, Freehold, NJ), and 0.01% DNase (Sigma). Then fetal calf serum was added (30% v/v of PBS)

to inhibit enzyme activity. Cells were isolated from the tissues using fire-polished pipettes (0.2–0.5 mm inner diameter). Subsequently, the isolated cells were plated onto polylysine-coated glass coverslips (30 × 40 mm, 50 μm thickness) and cultured for 7 d in serum-free Neurobasal medium (Invitrogen) containing 0.5 mM L-glutamine (Invitrogen) and 0.04% B-27 supplement (Wako) at 37°C in a humidified 5% CO_2 atmosphere.

Experimental preparations. The coverslip on which cells were cultured was attached with waterproof tape to the underside of a 0.5-mm-thick stainless-steel chamber (50 × 80 mm) with a lozenge-shaped hole (25 × 35 mm). The volume of the chamber was ~0.45 ml. The topside of the chamber was covered with another coverslip, leaving small openings on both sides to perfuse cells with a solution. The culture medium was then replaced with HEPES-buffered salt solution (see below; 37°C). The chamber was mounted onto the stage of an inverted Zeiss Axiomat microscope equipped with an oil-immersed Plan-Apochromat 63× objective (Carl Zeiss, Oberkochen, Germany). The stage was maintained at 37°C by a thermocontroller. The drug-containing solution (3 ml) was injected into one side opening using a Pasteur pipette, and the solution spilling from the other side opening was removed by an infusion pump.

Solutions and drugs. The composition of HEPES-buffered salt solution, pH 7.4, was (in mM): 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 25 D-(+)-glucose (all from Wako). Ca^{2+}-free solution consisted of (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 25 D-(+)-glucose, and 2 EGTA (Wako). Amyloid β-protein fragments 25–35 ($Aβ_{25-35}$; Sigma) and 31–35 ($Aβ_{31-35}$; Sigma) were dissolved in distilled water to a concentration of 2 or 10 mM. Fragments 1–40 ($Aβ_{1-40}$; Sigma) and 1–42 ($Aβ_{1-42}$; Sigma), and acetyl fragment 15–20 amide ($Aβ_{15-20}$ amide; Sigma) were initially dissolved in 10 mM NaOH, because they are less soluble in water, and then diluted with distilled water to a concentration of 0.5 mM for $Aβ_{1-40}$ and $Aβ_{1-42}$ and 1 mM for $Aβ_{15-20}$ amide. Simultaneously, the pH of these solutions was adjusted to 7.4 using either NaOH or HCl. Then these solutions were further diluted at concentrations indicated in Results with HEPES-buffered salt solution and used for experiments on axonal transport and rhodamine–phalloidin stainings. The solutions (0.5 mM) of $Aβ_{1-40}$ and $Aβ_{1-42}$ incubated for 7 d at room temperature were also used after dilution with HEPES-buffered salt solution. The solutions of 1 mM $Aβ_{15-20}$ amide, 2 mM $Aβ_{31-35}$, 2 mM $Aβ_{25-35}$, 0.5 mM $Aβ_{1-40}$, and 0.5 mM $Aβ_{1-42}$ were subjected to SDS-PAGE. L-glutamic acid hydrochloride (glutamate; Wako), NMDA (Sigma), AMPA (Sigma), and (±)-MK-801 (Sigma) were dissolved directly in HEPES-buffered salt solution. CNQX (Sigma) and latrunculin B (Biomol, Plymouth Meeting, PA) were each dissolved in dimethyl sulfoxide (DMSO; Wako), and jasplakinolide (Molecular Probes, Eugene, OR) was dissolved in methanol. These organic solutions were then diluted with HEPES-buffered solution. The concentration of DMSO and methanol was 0.01%, and at this concentration these organic solvents had no effect on axonal transport.

Video-enhanced microscopy system. Nomarski images acquired by an inverted microscope were transformed into video images by a video camera (Harpicon, Hamamatsu Photonics, Hamamatsu, Japan) and a cam-
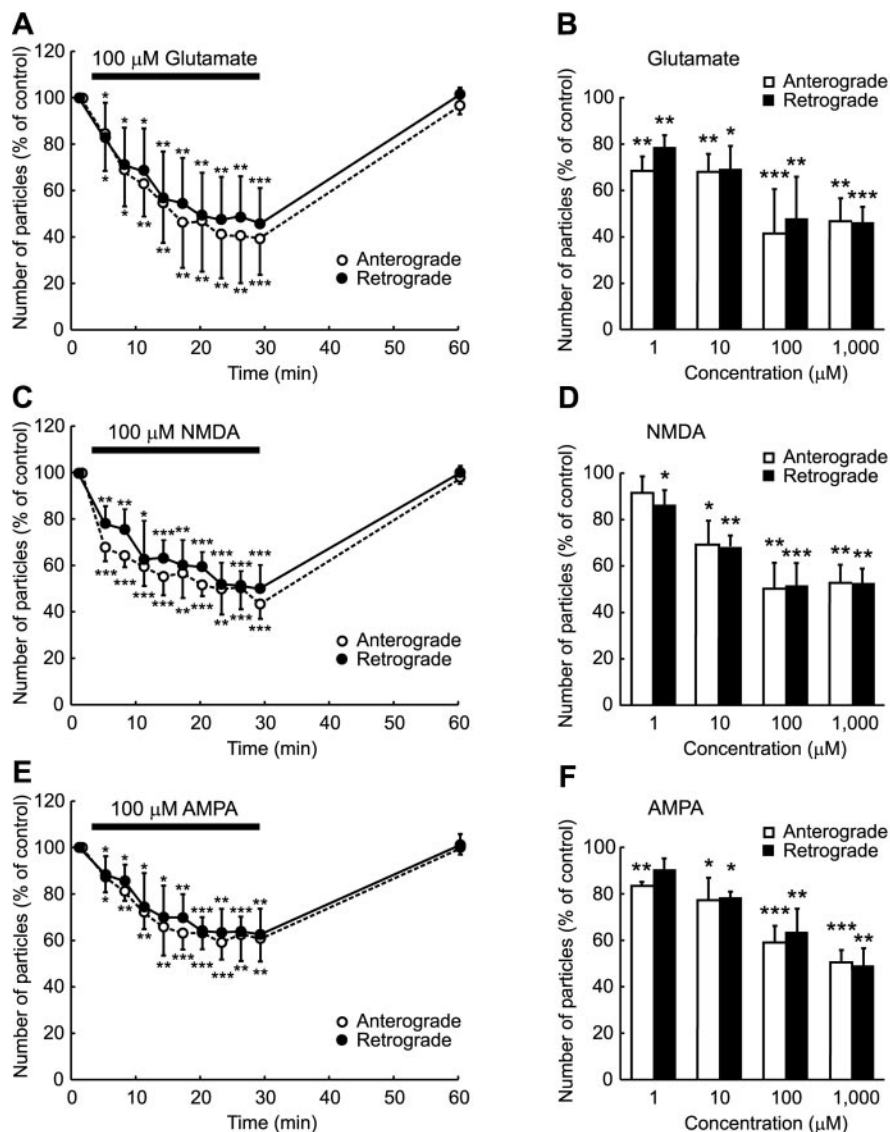
Figure 1. Effects of glutamate and its agonists on the number of particles transported in neurites of cultured rat hippocampal neurons. *A, C, E,* Percentage changes in the number of transported particles of control (the value before application) induced by 26 min application of 100 μM glutamate (*A*), 100 μM NMDA (*C*), and 100 μM AMPA (*E*). Note that the number of transported particles was rapidly reduced by these drugs and restored by removal of the drugs. *B, D, F,* Concentration dependence of reduction in the number of transported particles induced by glutamate (*B*), NMDA (*D*), and AMPA (*F*). The value obtained at 20 min after each application of the drugs is expressed as a percentage of control (the value before application). Data in all panels are expressed as mean ± SD of five neurons. Error bars represent SD. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$ compared with control (100%).

era controller (C2741, Hamamatsu Photonics). The video images were digitalized and enhanced by a video image enhancement system (DVS-20, Hamamatsu Photonics), displayed in real-time on a video monitor (C1864, Hamamatsu Photonics), and stored on a video recorder (PVW-2800, Sony, Tokyo, Japan). This processing provided a 10,000-fold final magnification on the video monitor.

Analysis of axonal transport. Axonal transport was analyzed from the video replay. The numbers of particles (diameter ≥50 nm) moving toward the axon terminal (anterograde) and back to the cell body (retrograde) were counted before, during, and after application of the drug. In the control extracellular medium (HEPES-buffered salt solution, pH 7.4, 37°C), the mean numbers of particles (per minute) transported in anterograde and retrograde directions were 59.7 ± 19.7 (mean ± SD; $n = 105$) and 57.5 ± 19.7 ($n = 105$), respectively. Averaged data in each experiment are expressed as mean (± SD) percentage of the control value that was obtained before the drug application. ANOVA was used to evaluate the statistical significance of fluctuations over time. Differences
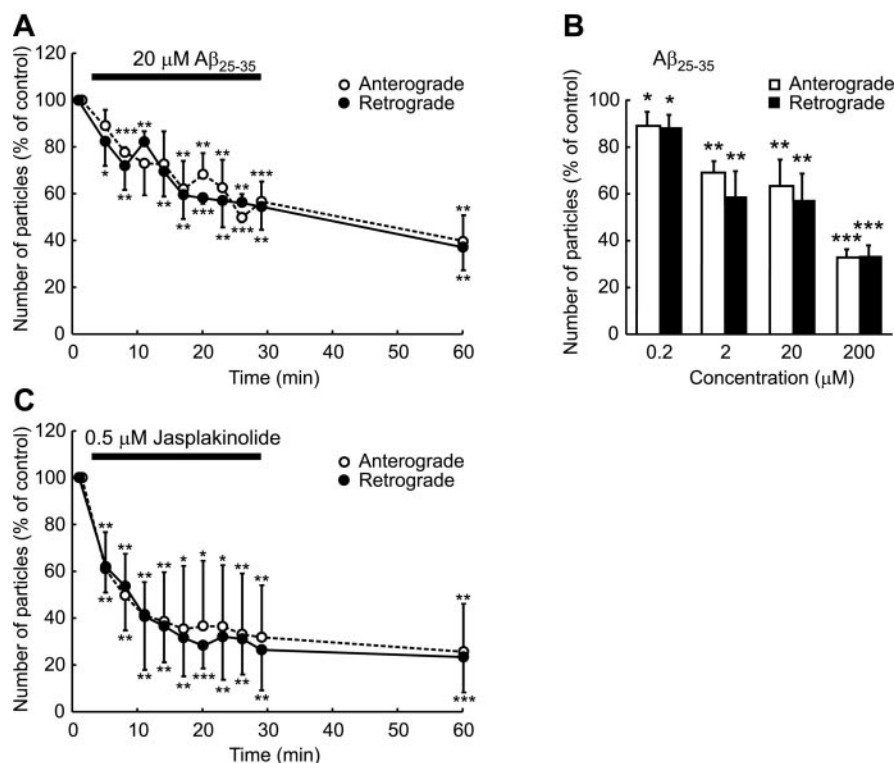
Figure 2. Effects of Aβ$_{25-35}$ and jasplakinolide, an actin-polymerizing agent, on the number of particles transported in neurites of cultured rat hippocampal neurons. *A, C,* Percentage changes in the number of transported particles of control (the value before application) induced by 26 min application of 20 μM Aβ$_{25-35}$ (*A*) and 0.5 μM jasplakinolide (*C*). Note that the number of transported particles was rapidly and progressively reduced by both drugs and not restored after removal of the drugs. *B,* Concentration dependence of reduction in the number of transported particles induced by Aβ$_{25-35}$. The value obtained at 20 min after each application of various concentrations of Aβ$_{25-35}$ is expressed as a percentage of control (the value before application). Data in all panels are expressed as mean ± SD of five neurons. Error bars represent SD. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$ compared with control (100%).

between control and test conditions were examined for statistical significance using the Student's paired *t* test.

Rhodamine–phalloidin staining. Hippocampal cells grown on coverslips were treated for 30 min with drugs (glutamate, Aβ fragments, latrunculin B, and a combination of Aβ$_{25-35}$ and latrunculin B) dissolved in a HEPES-buffered solution. Then, cells were fixed with 4% paraformaldehyde for 3 min at room temperature. After fixation, they were washed three times with 0.025 M PBS containing 0.3% Triton X-100 (PBST). The cells were incubated for 30 min at room temperature in 5 U/ml rhodamine–phalloidin (Molecular Probes) dissolved in PBST and washed three times with PBS. The stained cells were examined with a Zeiss Axiovert 135 TV microscope equipped with a 546 nm excitation filter and a 590 nm emission filter.

SDS-PAGE. Ten micrograms of each Aβ in a solution was mixed with Tris-HCl SDS sample buffer, loaded onto 5–20% gradient acrylamide gel (ATTO, Tokyo, Japan), and electrophoresed. Proteins were visualized with SimplyBlue SafeStain (Invitrogen).

Results
Effects of glutamate on axonal transport
Video-enhanced microscopy displayed movement of particles in neurites of cultured rat hippocampal neurons. Continuous application (26 min) of 100 μM glutamate significantly reduced the number of particles moving in anterograde and retrograde directions (Fig. 1*A*). The number began to decrease within 2 min and reached a plateau at 40–50% of the number before the application (control) 20 min after the start of application. The decreased number was reversed to the control level after a 30 min washout

of glutamate. A higher concentration (1000 μM) of glutamate produced similar effects. The number of particles transported in both directions was decreased to 40–50% of control by 1000 μM glutamate (Fig. 1*B*) and was restored to ~85% of control 30 min after removal of the drug (data not shown; *n* = 5). The effects of glutamate were concentration dependent from 1 to 1000 μM (Fig. 1*B*). Application of NMDA at a concentration of 100 μM reduced the number of particles (~50% of control) transported in anterograde and retrograde directions in a similar manner to 100 μM glutamate (Fig. 1*C,D*). AMPA at a concentration of 100 μM also rapidly decreased the number of particles to ~60% of control in both directions (Fig. 1*E,F*). The inhibitory effects induced by each 100 μM NMDA and 100 μM AMPA were reversed to control levels 30 min after washout of the drugs (Fig. 1*C,E*). The effects of NMDA and AMPA were concentration dependent from 1 to 1000 μM (Fig. 1*D,F*). Because both NMDA and AMPA mimicked the effects of glutamate, these types of receptors could possibly mediate the effect of glutamate on axonal transport. Video-enhanced microscopy also revealed the progressive formation of membrane blebs in neurites during 26 min application of glutamate at concentrations ≥100 μM (see Fig. 3*A*). The size of each bleb was decreased considerably after washout for 30 min.

Effects of Aβ$_{25-35}$ on axonal transport
Application of Aβ$_{25-35}$ at a concentration of 20 μM resulted in a rapid and progressive decrease in the number of particles transported in anterograde and retrograde directions during 26 min application (Fig. 2*A*). The effect of Aβ$_{25-35}$ was concentration dependent from 0.2 to 200 μM (Fig. 2*B*). Even after washout, the decreasing effect of 20 μM Aβ$_{25-35}$ was progressive and irreversible (Fig. 2*A*), although the effect induced by a lower concentration, 2 μM, of Aβ$_{25-35}$ applied for 26 min was restored to the control level within 30 min after washout (data not shown; *n* = 5). We also found that neurites were shrunken by exposure to 20 μM Aβ$_{25-35}$, and longitudinal microtubules in neurites became clearly visible (Fig. 3*B*). This phenomenon was irreversible even after washout. These morphological features were extremely different from those observed in glutamate-treated neurons (Fig. 3*A*).

Effects of glutamate and Aβ$_{25-35}$ under various conditions
It has been reported that Aβ acts on glutamate receptors (Calligaro et al., 1993; Cowburn et al., 1994, 1997; Le et al., 1995) and that both glutamate and Aβ exert their toxic effects by Ca^{2+} influx (Joseph and Han, 1992; Mattson et al., 1992; Hartley et al., 1993; Weiss et al., 1994; Le et al., 1995; Limbrick et al., 2001). Aβ disrupts the intracellular actin network (Davis et al., 1999), and actin depolymerization prevents neurotoxicity of glutamate and Aβ$_{25-35}$ (Furukawa and Mattson, 1995; Furukawa et al., 1995).

Actin can interact with microtubules, which are the main tracks for fast axonal transport (Nagele et al., 1988; Bearer and Reese, 1999). The above experiments indicated that glutamate and $A\beta_{25-35}$ induced morphological changes in neurites, implicating the possible involvement of the cytoskeleton. Therefore, to identify the mechanisms mediating the effects of glutamate and $A\beta_{25-35}$, we investigated the effects of these two agents in the presence of glutamate receptor antagonists or the actin filament depolymerizer, or in the absence of extracellular Ca^{2+}.

As shown in Figure 4A, the inhibitory effect of 100 μM glutamate was completely blocked by pretreatment with both the NMDA receptor antagonist MK-801 (20 μM) and the AMPA receptor antagonist CNQX (100 μM), confirming the involvement of NMDA and AMPA receptors in the effect of glutamate on axonal transport. In contrast, the effect of 20 μM $A\beta_{25-35}$ was not blocked by pretreatment with a combination of 20 μM MK-801 and 100 μM CNQX (Fig. 4B). In Ca^{2+}-free extracellular medium (with 2 mM EGTA), the inhibitory effect of 100 μM glutamate was abolished completely (Fig. 4C). Similarly, the effects of NMDA (100 μM) and AMPA (100 μM) were each blocked in the absence of extracellular Ca^{2+} (data not shown; $n = 5$ each). On the contrary, the effect of 20 μM $A\beta_{25-35}$ remained in the absence of extracellular Ca^{2+} (Fig. 4D). Nevertheless, the effect of 20 μM $A\beta_{25-35}$ was abolished by pretreatment with 5 μM latrunculin B, an actin filament depolymerizer (Fig. 4F). The presence of latrunculin B (5 μM) did not block the decreasing effect of glutamate (Fig. 4E), although the decreased axonal transport tended to recover at the end of the glutamate application period (Fig. 4E). The actin-polymerizing agent jasplakinolide (0.5 μM) mimicked the effect of 20 μM $A\beta_{25-35}$ (Fig. 2C). From these results, the inhibitory effect of glutamate on axonal transport is mediated mainly by Ca^{2+} influx after activation of NMDA and AMPA receptors. In contrast, the inhibitory effect of $A\beta_{25-35}$ is likely caused by actin polymerization but not by glutamate receptor activation or Ca^{2+} influx. In these experiments, application of 20 μM MK-801 plus 100 μM CNQX (Fig. 4A,B) and of 5 μM latrunculin B (Fig. 4E,F) did not significantly affect axonal transport, although in 2 of 10 neurons treated with latrunculin B, axonal transport in both anterograde and retrograde directions increased to >10% above the control value. In such latrunculin B-sensitive neurons, polymerized or aggregated actin originally present might be depolymerized and dissolved by latrunculin B, allowing axonal transport to increase slightly. We also confirmed that axonal transport was not changed by Ca^{2+}-free solution over a 2 hr experimental period.
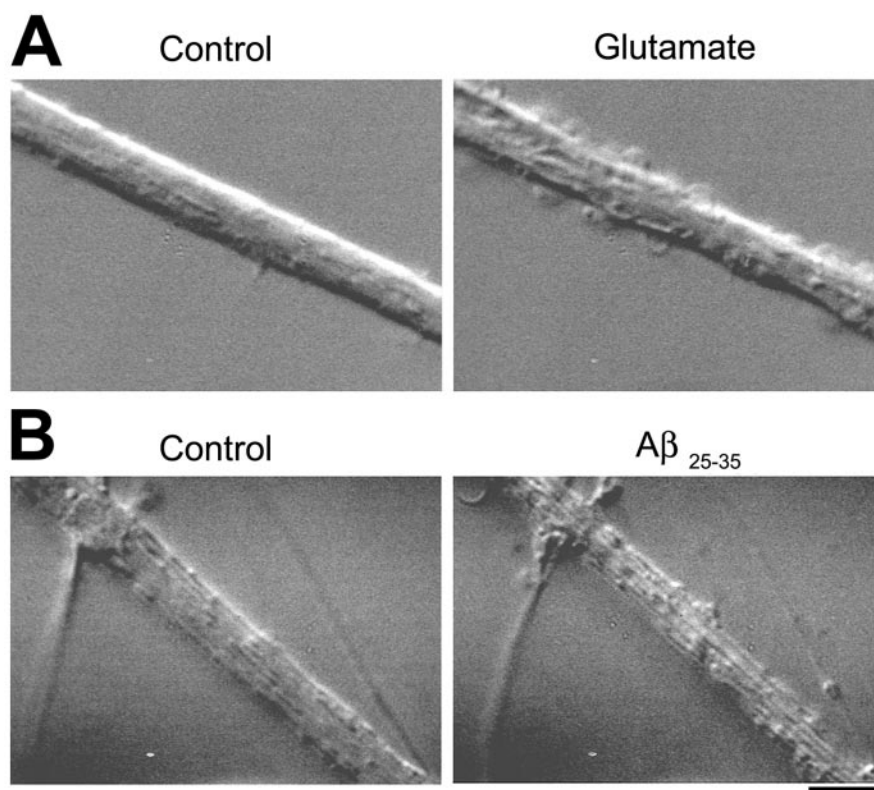

Figure 3. Morphological changes induced by glutamate and $A\beta_{25-35}$ during video-enhanced microscopic recordings. Video images of neurites were acquired before (Control) and 20 min after treatment with 100 μM glutamate (A), and before (Control) and 20 min after treatment with 20 μM $A\beta_{25-35}$ (B). Note that the membrane in the neurite was blebbing in glutamate-treated neurons, whereas the neurite was shrunken and microtubules were clearly visible in the $A\beta_{25-35}$-treated neuron. Scale bar, 2 μm.

latrunculin B (5 μM), and a combination of $A\beta_{25-35}$ (20 μM) and latrunculin B (5 μM), all dissolved in HEPES-buffered salt solution. The effects of washout (for 30 min) after exposure to $A\beta_{25-35}$ (20 μM) were also examined. Control neurons (treated with HEPES-buffered solution alone) revealed a reticular distribution of actin filaments in cell bodies and neurites (Fig. 5A). In glutamate-treated neurons, the distribution of actin filaments was diffuse and the staining was more intense, in particular in the periphery of the cell body, than in control neurons (Fig. 5B), indicating an increase in fragmented actin filaments. Neurons treated with $A\beta_{25-35}$ showed characteristic features of actin cytoskeleton (Fig. 5C). These neurons were stained intensely, with brightly stained aggregates of actin filaments in both cell bodies and neurites, indicating hyperpolymerization of actin filaments. Interestingly, formation of filopodia, which results from polymerization of actin filaments (Yamada et al., 1970, 1971; Oldenbourg et al., 2000), was observed frequently in $A\beta_{25-35}$-treated neurons. The same features were observed in neurons that were washed for 30 min after treatment with $A\beta_{25-35}$ (Fig. 5D). In contrast, staining in latrunculin B-treated neurons was weak and diffuse in both cell bodies and neurites (Fig. 5E), indicating dissolution of actin filaments. Simultaneous treatment with latrunculin B and $A\beta_{25-35}$ abolished the characteristic features observed in neurons treated with $A\beta_{25-35}$ alone (Fig. 5F).

Rhodamine–phalloidin staining of the actin cytoskeleton
Cultured hippocampal neurons were stained with rhodamine–phalloidin, which specifically binds to filamentous actin, after treatment for 30 min with glutamate (100 μM), $A\beta_{25-35}$ (20 μM),

Effects of various Aβ fragments
Aβ molecules found mainly in senile plaques are $A\beta_{1-40}$ and $A\beta_{1-42}$ (Iwatsubo et al., 1994). Recent studies have shown that $A\beta_{1-40}$ and $A\beta_{1-42}$ assemblies, soluble oligomers and fibril
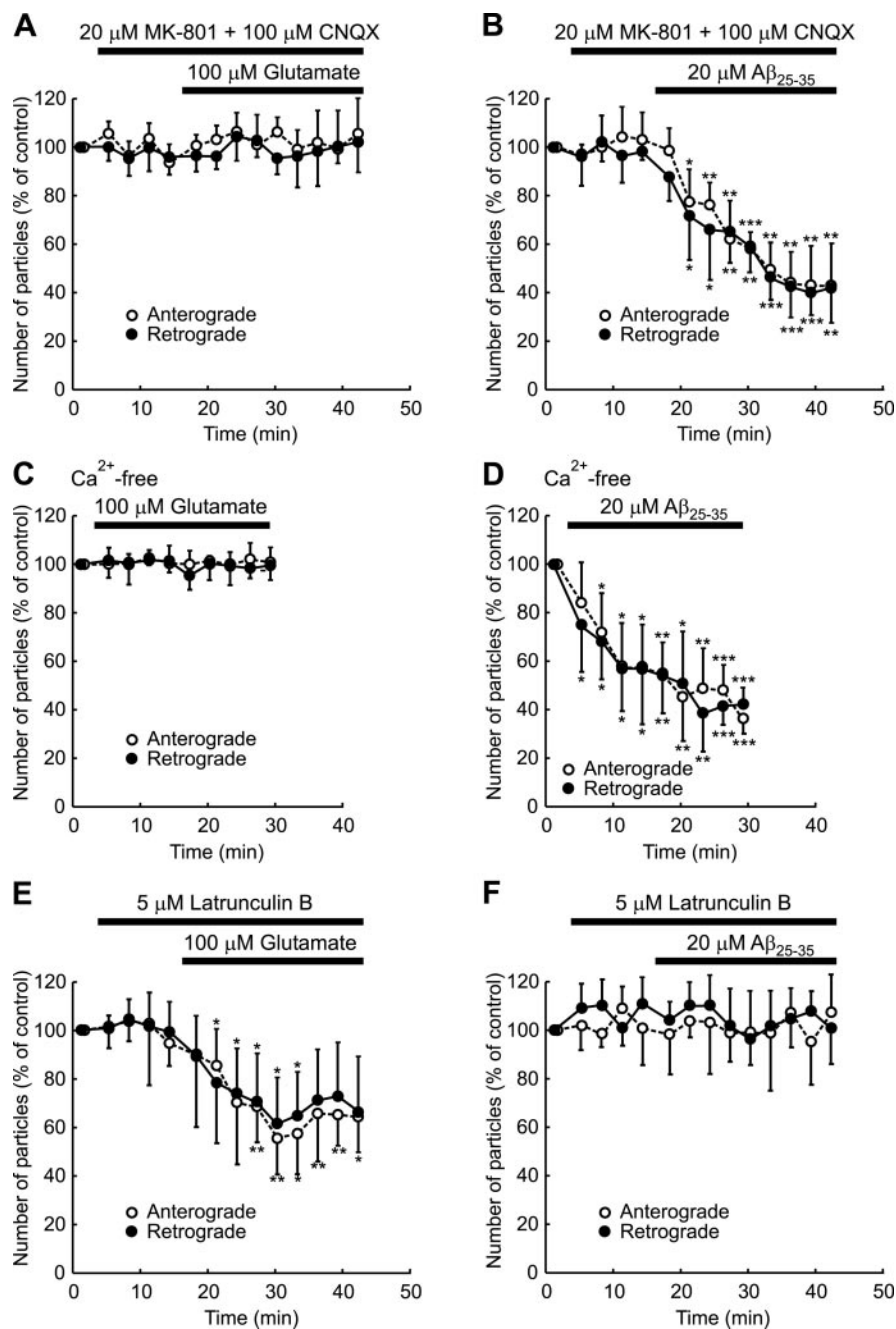
Figure 4. Effects of glutamate and Aβ$_{25-35}$ under various conditions. Data represent percentage changes in the number of transported particles of control (the value before application). Glutamate (100 μM) (A, C, E) and Aβ$_{25-35}$ (20 μM) (B, D, F) were applied after treatment with a combination of 20 μM MK-801, an NMDA receptor antagonist, and 100 μM CNQX, an AMPA receptor antagonist (A, B), in Ca^{2+}-free solution (C, D), and after treatment with 5 μM latrunculin B, an actin depolymerizer (E, F). Each data point indicates the mean ± SD of the values obtained from five neurons. Error bars represent SD. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$ compared with control (100%).

intermediates (protofibrils), have potent neurotoxic activities (Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 1999). Because Aβ is secreted from brain cells as a monomeric form (Haass et al., 1992), the process toward the assembly is necessary for neurotoxicity of Aβ$_{1-40}$ and Aβ$_{1-42}$. Hydrophobic residues of Aβ are correlated with fibril formation (Li et al., 1999; Petkova et al., 2002). Thus, we further examined the effects of the hydrophobic Aβ fragments, Aβ$_{15-20}$ amide and Aβ$_{31-35}$, and full-length Aβ$_{1-40}$ and Aβ$_{1-42}$, to identify residues and structures contributing to the inhibition of axonal transport. Aβ fragments were used

immediately after dissolution (fresh condition). Because Aβ$_{1-40}$ and Aβ$_{1-42}$ assemble to form large structures during incubation (Pike et al., 1991; Kayed et al., 2003), the effects of these agents incubated for 7 d were also investigated. Light microscopic screening and SDS-PAGE were performed simultaneously to examine the solubility and assembly state of Aβ fragments in solution (Fig. 6A–E). The results are summarized in Table 1. Aβ$_{31-35}$ (20 μM) but not Aβ$_{15-20}$ amide (20 μM) induced intracellular actin aggregation and inhibition of axonal transport similar to that of Aβ$_{25-35}$. Thus, the active site in Aβ is probably present in residues 31–35. Among fresh and incubated full-length Aβs, only incubated Aβ$_{1-42}$ (20 μM) was effective. In contrast, fresh Aβ$_{1-40}$ (20 μM), incubated Aβ$_{1-40}$ (20 μM), or fresh Aβ$_{1-42}$ (20 μM) had no effect. SDS-PAGE analysis revealed that Aβ$_{15-20}$ amide and Aβ$_{31-35}$ were present in low molecular weight forms (<1 kDa) (Fig. 6C). Aβ$_{25-35}$ was composed of both low molecular weight molecules (~1 kDa) and aggregates that did not enter the SDS-PAGE gel (Fig. 6C), and this fragment formed precipitates detected by light microscopy (Fig. 6A); that is, in these shorter fragments, there was no correlation between their structures and activities. Fresh Aβ$_{1-40}$ was primarily monomeric (Fig. 6D), whereas incubated Aβ$_{1-40}$ additionally produced aggregates (Fig. 6E) and precipitates (Fig. 6B). Fresh Aβ$_{1-42}$ contained monomers, pentamers, and hexamers (Fig. 6D), whereas incubated Aβ$_{1-42}$ contained 30–100 kDa high molecular weight forms in addition to the lower molecular forms observed in fresh Aβ$_{1-42}$ (Fig. 6E). Thus, among various states of full-length Aβ observed in this experiment, only high molecular weight assemblies (30–100 kDa) of Aβ$_{1-42}$ appear to participate in the inhibition of axonal transport, whereas lower molecular weight molecules (monomers, pentamers, and hexamers) of Aβ$_{1-40}$/Aβ$_{1-42}$ or aggregates and precipitates of Aβ$_{1-40}$ do not.

Discussion
Inhibitory effects of glutamate and Aβ on axonal transport
We show evidence that glutamate and Aβ$_{25-35}$ inhibit anterograde and retrograde fast axonal transport in cultured rat hippocampal neurons. Only a few previous reports have described the effects of these agents on axonal transport. One of them has shown that glutamate inhibits axonal transport of neurofilaments (Ackerley et al., 2000). Neurofilaments, carried within axons by slow axonal transport, have recently been found to be transported by similar mechanisms to fast axonal transport, microtubule-based and kinesin and dynein motors-dependent

mechanisms, with a long rest period (Roy et al., 2000; Wang et al., 2000). One more report has demonstrated, using immunohistochemistry, that human $A\beta_{1-42}$ applied *in vivo* inhibits fast axonal transport of acetylcholinesterase, amyloid precursor protein, vesicular acetylcholine transporter, and synaptophysin in the sciatic nerve of the rat (Kasa et al., 2000). Therefore, the present and previous studies strongly suggest that both glutamate and Aβ are agents that inhibit fast axonal transport. We also found that the response of axonal transport to glutamate and $A\beta_{25-35}$ was rapid, being detected within a few minutes after the start of stimulation. Thus, axonal transport may be one of the early targets for toxicity of these two agents.

The effect of glutamate, even when applied at high concentrations (≥100 μM) for 26 min, was reversed after removal of glutamate. These exposure concentrations and duration are sufficient to cause cell death in cultured neurons 24 hr later (Hartley et al., 1993). Despite this, the effect of glutamate on axonal transport was reversible, probably by receptor-mediated mechanisms as described below. In contrast, the effect of $A\beta_{25-35}$ at 20 μM, a concentration able to kill neuronal cells several days later (Yankner et al., 1990; Weiss et al., 1994), was irreversible and progressive even after washout. Similar prolongation and irreversibility of the effects of Aβ have been reported previously. Aβ, at various concentrations, induces a prolonged activation of pro-apoptotic markers, caspase 3 and annexin V binding (A. R. White et al., 2001), an irreversible inward cation current (Furukawa et al., 1994; Sanderson et al., 1997), and a prolonged glutamate-induced $[Ca^{2+}]_i$ increase even after removal of glutamate (Scorziello et al., 1996) in neuronal or neuron-like cells. In astrocytes, Aβ irreversibly produces a reactive morphological change (Kato et al., 1997). Thus, Aβ can cause prolonged and irreversible changes in some cellular structures and functions including axonal transport. The present study using rhodamine–phalloidin revealed that 20 μM $A\beta_{25-35}$-induced changes in the actin cytoskeleton were irreversible after removal of $A\beta_{25-35}$. Therefore, persistent intracellular structural changes induced by $A\beta_{25-35}$ may result in an irreversible impairment of axonal transport.

Different mechanisms of inhibition of axonal transport by glutamate and Aβ

Glutamate can trigger Ca^{2+} influx after activation of NMDA and AMPA receptors to cause excitotoxicity (Frandsen and Schousboe, 1993; Hartley et al., 1993). In agreement with this, we find that the inhibitory effect of glutamate on axonal transport is mediated by activation of
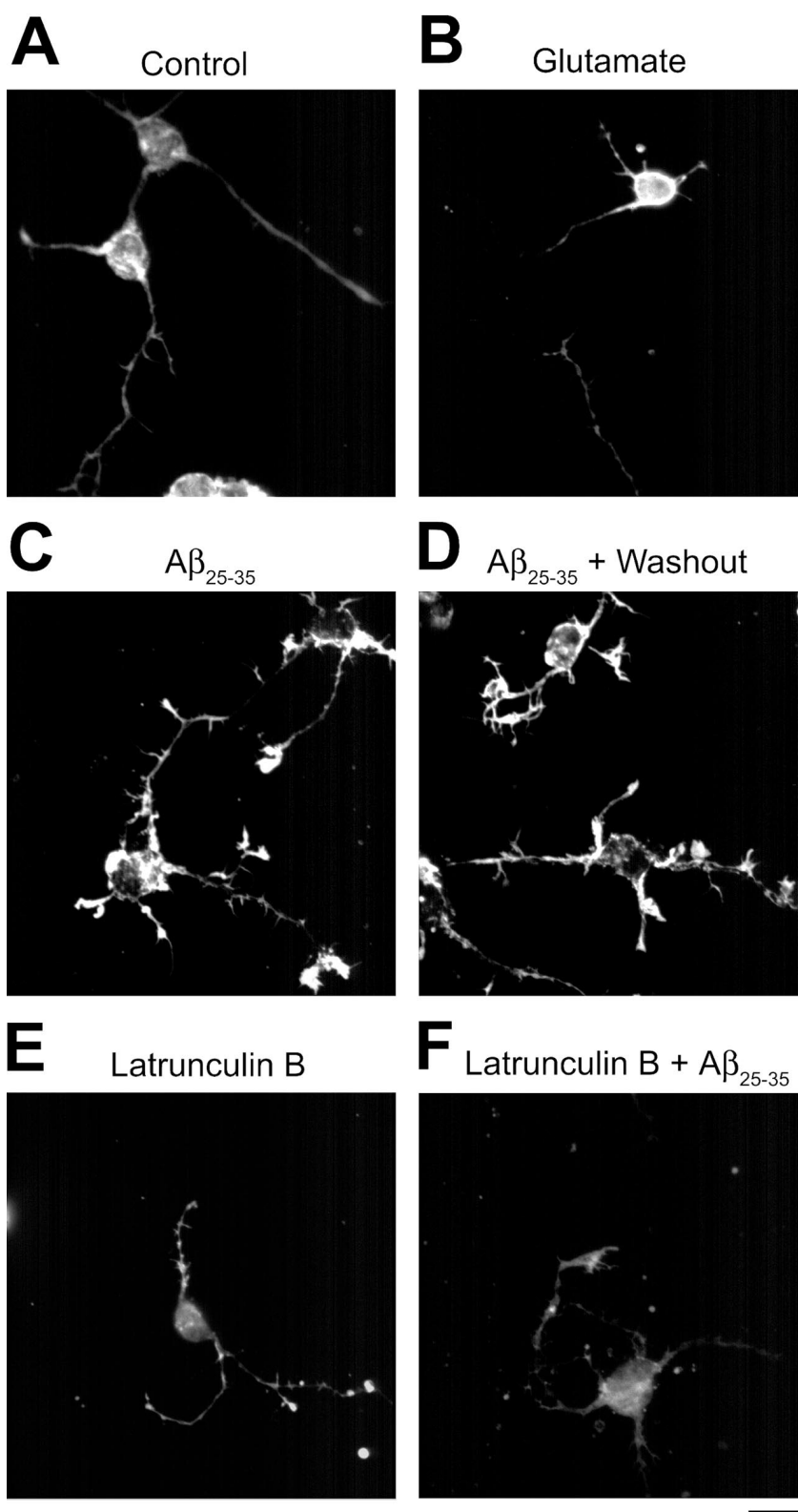
Figure 5. Rhodamine–phalloidin staining of actin filaments in cultured hippocampal neurons. Neurons were stained with rhodamine–phalloidin after treatment of drugs dissolved in HEPES-buffered solution. *A*, Control (treatment with HEPES-buffered solution alone for 30 min). *B*, Treatment with 100 μM glutamate for 30 min. *C*, Treatment with 20 μM $A\beta_{25-35}$ for 30 min. *D*, Treatment with 20 μM $A\beta_{25-35}$ for 30 min followed by washout for 30 min. *E*, Treatment with 5 μM latrunculin B for 30 min. *F*, Simultaneous treatment with 20 μM $A\beta_{25-35}$ and 5 μM latrunculin B for 30 min. Scale bar, 20 μm.
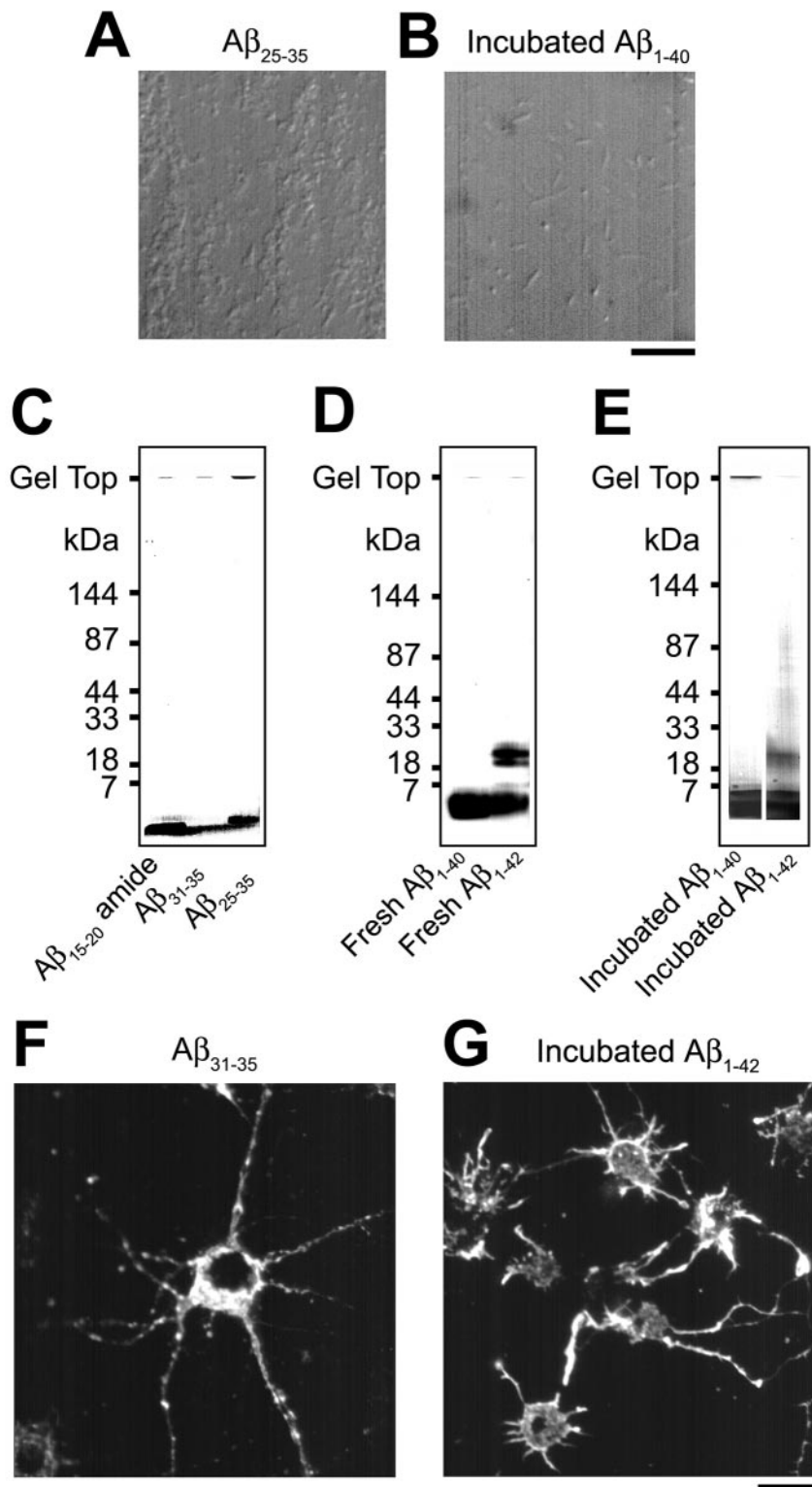
Figure 6. Photographs representing properties of various Aβ fragments. A, B, Precipitates detected under light micros-copy in Aβ$_{25-35}$ solution (A) and in incubated Aβ$_{1-40}$ solution (B). Aβ$_{25-35}$ produced cotton-like precipitates (A), and incubated Aβ$_{1-40}$ formed fiber-like or amorphous precipitates (B). Scale bar, 10 μm. C–E, SDS-PAGE profiles of various Aβ fragments. F, G, Rhodamine–phalloidin staining of intracellular actin filaments in hippocampal neurons treated with Aβ$_{31-35}$ (F) and with incubated Aβ$_{1-42}$ (G). Aβ$_{31-35}$ (20 μM) and incubated Aβ$_{1-42}$ (20 μM) formed intracellular actin aggregates. Scale bar, 20 μm.

both NMDA and AMPA receptors and subsequent Ca^{2+} influx. In contrast, the effect of Aβ$_{25-35}$ is unlikely to be mediated by NMDA or AMPA receptors, or by Ca^{2+} influx. This seems to be inconsistent with the previously reported mechanisms of Aβ-induced neurodegeneration and cell death. It has been proposed that Aβ has an affinity for the agonist recognition sites of the NMDA receptor (Cowburn et al., 1994; 1997) and increases intracellular Ca^{2+} through an influx of extracellular Ca^{2+} in cultured neurons (Joseph and Han, 1992; Weiss et al., 1994). Toxicity of Aβ can be inhibited by the NMDA receptor antagonist (Le et al., 1995) and by Ca^{2+} channel blockers (Weiss et al., 1994). On the contrary, our proposed mechanisms are compatible with some other previous investigations. Cell death induced by Aβ is not mediated by intracellular early Ca^{2+} accumulation, because Aβ inhibits spontaneous Ca^{2+} oscillation and does not affect high K^+- and glutamate-evoked Ca^{2+} increase (Gao et al., 1998). The neurotoxic effect of Aβ is not blocked by glutamate receptor antagonists (Busciglio et al., 1993; Weiss et al., 1994; Gray and Patel, 1995) or by Ca^{2+} channel blockers (Whitson and Appel, 1995). Collectively considered, Aβ can trigger multiple signaling cascades leading to neuronal cell damage, and one or some of which may be specific for the impairment of axonal transport. Here we found that the inhibitory effect of Aβ$_{25-35}$ on axonal transport was abolished by the actin-depolymerizing agent latrunculin B. In addition, the actin-polymerizing agent jasplakinolide mimicked the effect of Aβ$_{25-35}$, and Aβ$_{25-35}$ induced aggregation of intracellular actin. Because aggregation of actin is supposed to result from actin polymerization (Lee et al., 1998; Fabian et al., 1999; Bai et al., 2002), our results suggest that the inhibitory effect of Aβ$_{25-35}$ on axonal transport occurs via polymerization and subsequent aggregation of actin. Neurite shrinkage and filopodia formation induced by Aβ$_{25-35}$, revealed by the present morphological investigation, may also be explained as being caused by polymerization of actin filaments. Actin polymerization dynamics involves changes in cell shape and size. Filopodia are formed by polymerization of actin (Yamada et al., 1970, 1971; Oldenbourg et al., 2000). Such filopodia formations may be involved in the previously reported neurotrophic action of Aβ (Whitson et al., 1990; Yankner et al., 1990; Koo et al., 1993), which appears to contribute to aberrant sprouting of neurites around senile plaques in AD (Whitson et al., 1990; Koo et al., 1993). Furthermore, in support of our conclusion, Aβ has been suggested to cause disruption of the intracellular actin network (Davis et al., 1999), which results from actin

Table 1. Properties and effects of various Aβ fragments

Aβ fragment	Precipitates detected under light microscopy	Structural properties analyzed by SDS-PAGE	Intracellular actin aggregation	Effects on axonal transport
Aβ$_{15-20}$ amide	−	Low molecular weight forms (<1 kDa)	−	No effect
Aβ$_{31-35}$	−	Low molecular weight forms (<1 kDa)	+	Inhibitory
Aβ$_{25-35}$	+	Low molecular weight forms (~1 kDa) Aggregates	+	Inhibitory
Fresh Aβ$_{1-40}$	−	Monomers	−	No effect
Incubated Aβ$_{1-40}$	+	Monomers Aggregates	−	No effect
Fresh Aβ$_{1-42}$	−	Monomers Pentamers Hexamers	−	No effect
Incubated Aβ$_{1-42}$	−	Monomers Pentamers Hexamers High molecular weight forms (30–100 kDa)	+	Inhibitory

Aβ fragments dissolved in HEPES-buffered salt solution, pH 7.4, at a concentration of 20 μM were used for light microscopic analysis, intracellular actin observation with rhodamine–phalloidin, and axonal transport study under video-enhanced microscopy. Ten micrograms of each Aβ fragment dissolved in an aqueous solution were subjected to SDS-PAGE. +, Detected; −, not detected.

polymerization (Senderowicz et al., 1995; Fabian et al., 1999; S. R. White et al., 2001) as well as depolymerization. Hirano bodies, one of pathologic features found frequently in neurites adjacent to a senile plaque in the AD brain, include abundant actin polymers (Goldman, 1983; Galloway et al., 1987). Depolymerization of actin attenuates Aβ neurotoxicity (Furukawa and Mattson, 1995). Thus, actin polymerization appears to relate to some neurotoxic effects of Aβ. Certainly, our results are strongly supported by recent evidence that Aβ induces actin polymerization to form stress fibers in SN1 cells, a septal neuronal cell line, and in cultured mouse hippocampal neurons (Song et al., 2002). Presently, it is not known how extracellular Aβ polymerizes intracellular actin; however, because Aβ forms pores on cell membrane (Furukawa et al., 1994; Sanderson et al., 1997; Kawahara and Kuroda, 2000) and activates intracellular cascades that cause actin polymerization, such as the p38 mitogen-activated protein kinase (MAPK) signaling pathway (Song et al., 2002), Aβ could interact with intracellular actin.

Axonal transport can be modified by the dynamics of the cytoskeleton, including actin filaments. A number of observations indicate the participation of actin in fast axonal transport of organelles. Organelles can be transported axonally along actin filaments (Kuznetsov et al., 1992; Morris and Hollenbeck, 1995). Microtubule-based tracks for fast axonal organelle transport include actin filaments (Bearer and Reese, 1999), and actin is a component of granular, microtubule-associated cross-bridges that connect to other structures, including other microtubules, neurofilaments, organelles, and plasma membrane (Nagele et al., 1988). Changes in intracellular actin states have been shown to modify axonal transport (Goldberg et al., 1980; Goldberg, 1982; Brady et al., 1984; Kuznetsov et al., 1992; Morris and Hollenbeck, 1995; Ligon and Steward, 2000). Several early studies suggest that some actin-depolymerizing agents such as cytochalasins, DNase I, and gelsolin seem to inhibit fast axonal transport (Goldberg et al., 1980; Isenberg et al., 1980; Goldberg, 1982; Brady et al., 1984; Nemhauser and Goldberg, 1985; Kuznetsov et al., 1992), which appears to be inconsistent with the present results indicating that polymerization and aggregation of actin inhibit axonal transport; however, some of these agents aggregate actin, on the other hand. Indeed, fast axonal transport of mitochondria is impeded by cytochalasin D-induced actin aggregates but not affected by latrunculin B (Ligon and Steward, 2000). Nemhauser and Goldberg (1985) have described the possibility of the secondary effects of

DNase I on axonal transport, i.e., a substantial part of the inhibition of fast axonal transport caused by DNase I is attributable to depolymerization of microtubules, or DNase I induces a decrease in microtubules by reducing actin filaments. Indeed, polymerization of actin with jasplakinolide inhibits intracellular motility in non-neuronal cells, HL60 cells, and monocytes (Fabian et al., 1999). These previous studies support our proposal that actin polymerization and aggregation induced by Aβ inhibit fast axonal transport.

Aβ residues and structures contributing to the inhibition of axonal transport

Furthermore, we investigated various Aβ fragments including Aβ$_{1-40}$ and Aβ$_{1-42}$, the main fragments found in senile plaques in the human AD brain. We obtained the results that Aβ$_{31-35}$ induced aggregation of actin filaments and inhibition of axonal transport similarly to Aβ$_{25-35}$, whereas Aβ$_{15-20}$ amide had no effects. Thus, the active site in full-length Aβ may reside in fragments 31–35, as suggested previously (Yan et al., 1999; Qi and Qiao, 2001; Bond et al., 2003). In these shorter fragments, there was no correlation between structures and activities. In contrast, in full-length Aβ, the activity seems to depend on its structure. Our results indicated that incubated Aβ$_{1-42}$ produced an inhibition of axonal transport and actin aggregation, whereas fresh Aβ$_{1-40}$, incubated Aβ$_{1-40}$, or fresh Aβ$_{1-42}$ had no effect. Because incubated Aβ$_{1-42}$ but not fresh Aβ$_{1-42}$ contained 30–100 kDa molecules corresponding to higher oligomers or protofibrils (or protofilaments), these structures of Aβ$_{1-42}$ may participate in the impairment of axonal transport. Our data also indicate that Aβ$_{1-40}$ in monomers, aggregates, or participates has no apparent effect, although the possibility remains that Aβ$_{1-40}$ in other states is effective. These results are consistent with the current concept that soluble oligomers and protofibrils of full-length Aβ are neurotoxic (Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 1999; Dahlgren et al., 2002; Kim et al., 2003). Furthermore, on the basis of our results, we postulate that residues 31–35 may be the key to understanding the structure dependence of the activity of full-length Aβ. We propose that Aβ is effective when it represents a structure in which the active site can access the cell membrane. This proposal is supported by the recent x-ray diffraction study by Bond et al. (2003). They suggest that residues 31–35 are exposed at the surface of oligomeric Aβ and interact with the cell membrane. Assembly of full-length Aβ can be driven by the for-

mation of β-sheet structures where hydrophobic residues 17–20 are in contact with hydrophobic residues 31–35 (Lee et al., 1995; Li et al., 1999). Monomers of $A\beta_{1-40}$ and $A\beta_{1-42}$ exist as random extended chain (Zeng et al., 2001) or α-helix (Barrow and Zagorski, 1991; Coles et al., 1998; Zeng et al., 2001), and dimer and small oligomers can form a β-sheet (Barrow and Zagorski, 1991; Chaney et al., 1998) or an irregular structure that is not α-helix or β-structure (Huang et al., 2000). Higher oligomers form β-sheet (Huang et al., 2000) and appear to combine with each other to build protofibrils (or protofilaments), and then protofibrils (or protofilaments) associate into larger fibrils (Chaney et al., 1998; Walsh et al., 1999; Serpell, 2000; Petkova et al., 2002; Bitan et al., 2003). Because the results of the present study as well as other recent studies (Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 1999; Dahlgren et al., 2002) indicate that full-length Aβ assemblies, such as higher oligomers and protofibrils, are toxic, the β-sheet formation seems to contribute to toxicity in full-length Aβ. In much larger assemblies such as fibrils, however, hydrophobic β-sheets that contain residues 31–35 are associated laterally, and then the β-sheet structures are positioned inside the Aβ assembly or are shielded by hydrophilic residues (Chaney et al., 1998; Petkova et al., 2002). In such states, toxic 31–35 residues can no longer access the cell membrane, and therefore Aβ may become ineffective; however, further structural evidence is needed to confirm this speculation.

In summary, glutamate and $A\beta_{25-35}$ rapidly inhibit anterograde and retrograde fast axonal transport in cultured hippocampal neurons. The effects of glutamate are mediated by activation of NMDA and AMPA receptors and subsequent Ca^{2+} influx, which is different from the inhibitory mechanisms of $A\beta_{25-35}$. The latter is likely mediated by actin polymerization and aggregation. The ability of Aβ to exert these effects seems to require specific amino acid residues and structures. These results may provide insight into the early pathogenetic mechanisms of AD.

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