Internalized Antibodies to the Aβ Domain of APP Reduce Neuronal Aβ and Protect against Synaptic Alterations*§

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Immunotherapy against β-amyloid peptide (Aβ) is a leading therapeutic direction for Alzheimer disease (AD). Experimental studies in transgenic mouse models of AD have demonstrated that Aβ immunization reduces Aβ plaque pathology and improves cognitive function. However, the biological mechanisms by which Aβ antibodies reduce amyloid accumulation in the brain remain unclear. We provide evidence that treatment of AD mutant neuroblastoma cells or primary neurons with Aβ antibodies decreases levels of intracellular Aβ. Antibody-mediated reduction in cellular Aβ appears to require that the antibody binds to the extracellular Aβ domain of the amyloid precursor protein (APP) and be internalized. In addition, treatment with Aβ antibodies protects against synaptic alterations that occur in APP mutant neurons.

Active immunization for β-amyloid peptide (Aβ) has been demonstrated to reduce Aβ plaques and improve cognitive function in transgenic mouse models of Alzheimer disease (AD) (1–4). In human AD patients actively immunized with Aβ, subjects with high antibody titers appeared to have slowed cognitive deterioration and reduced plaque burden, although 6% of subjects developed meningoencephalitis (5–7). Passive immunotherapy in mouse models of AD has provided similar benefits to those seen with active immunization (4). The mechanisms by which Aβ antibodies reduce Aβ plaque pathology in the brain remain unclear (8). Data suggest roles for antibody-mediated microglial activation and Aβ efflux from the brain in the reduction of Aβ (9, 10). Interestingly, intracerebral injection of Aβ antibody reduced levels of both extracellular and intracellular Aβ in a triple transgenic (3×Tg) mouse carrying mutations in amyloid precursor protein (APP), presenilin 1 and tau (11), and reduction of intraneuronal Aβ was the better correlate with cognitive improvement (12). How Aβ antibodies reduce intracellular Aβ is not known. Increasing evidence supports that intraneuronal Aβ accumulation is important in the pathogenesis of AD. Intraneuronal Aβ accumulation has been reported in transgenic mouse models of cerebral amyloidosis (13–19), human AD (20–22) and Down syndrome (21, 23–25). Moreover, cultured neurons from APP mutant transgenic mice develop subcellular Aβ accumulation and synaptic alterations that parallel those observed in vivo in the brain with β-amyloidosis (26, 27).

We now report that Aβ antibodies decrease levels of intracellular Aβ in culture and provide evidence that antibody binding to the Aβ domain of APP and internalization of the antibody/APP complex appear to be required to reduce intracellular Aβ. Moreover, Aβ antibody treatment protects against synaptic alterations that occur in APP mutant neurons in culture.

**EXPERIMENTAL PROCEDURES**

* cDNA Constructs—Wild-type (wt) and mutant (K44E) dynamin-1 cDNA containing GFP were previously described (28). Cells were transfected overnight using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Anti-GFP antibody was obtained from Upstate Biotechnology.

§ Cell Culture and Treatments—Primary neuronal cultures from Tg2576 mice (29), and littermates were prepared as described (26). Primary neurons were used at 19 days in vitro (DIV). Mouse N2a neuroblastoma cells either untransfected (N2a) or stably transfected with the 670/671 Swedish mutation human APP (Sw-N2a) were grown as described previously (30). Mouse N2a neuroblastoma cells stably transfected with the C-terminal fragment of human APP C99 (C99-N2a) were previously described (31). Chloroquine (Sigma, 100 μM) and NH4Cl (Sigma, 50 mM) were added to cells 1 h prior to treatment and kept in culture during 3 h antibody incubation. The γ-secretase inhibitor N-[(3,5-difluorophenacetyl)-1-allyl]-S-phenylglycine t-buty l ester (DAPT; Calbiochem) was diluted in culture media to 250 nM and then added to cells for 3 h.
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**Antibody Treatment**—Several well characterized Aβ antibodies were used: monoclonal 6E10 (human Aβ residues 5–10; IgG1) and 4G8 (Aβ residues 17–24; IgG2a) (Signet Laboratories), G2-11 (Aβ42 C terminus; IgG1; Genetix Co.), anti-Aβ42 (Aβ42 C terminus; Chemicon), polyclonal anti-Aβ40 (Aβ40 C terminus; Chemicon), and polyclonal APP-ab2 (human Aβ residues 1–10; Labvision NeoMarkers). Other antibodies used were: monoclonal P2-1 (specific for the N terminal of human APP; IgG1; Affinity BioReagents) and mouse anti-human IgG (Jackson ImmunoResearch). We added fresh medium to cells just prior to each treatment. Final antibody concentration in all treatments was 2 μg/ml. Antibodies were added to the culture for different time points, as indicated. Treatment for 24 h with Aβ antibody 6E10 did not induce neuronal death, evaluated using the TUNEL labeling method and lactate dehydrogenase (LDH) assay (see below). For internalization studies, neurons were kept at 4 °C for 45 min with the indicated antibody. Cells were then either immediately fixed (0 min) or incubated at 37 °C for 10, 30, 60, or 180 min and then fixed in 4% paraformaldehyde in phosphate buffer (PB).

**Cell Death Assay**—19 DIV Tg2576 neurons grown on poly-d-lysine precoated coverslips were incubated 24 h with or without 6E10 antibody. Neurons were washed twice with PB saline (PBS) and fixed in 4% paraformaldehyde. The TUNEL staining kit (Roche Applied Science) was used to stain apoptotic neurons according to the manufacturer’s instructions. Nuclei were stained with the Hoechst stain. Counts of TUNEL positive nuclei and total nuclei were performed with Metamorph (Universal Imaging Co.) on 6–10 fields per coverslip at 20× magnification. The ratio between TUNEL-positive nuclei and Hoechst-positive nuclei was calculated. LDH kit (Sigma) was used to evaluate whether antibody treatment was inducing cell damage after incubation for 24 h with or without Aβ antibodies. Levels of LDH were measured in the media according to the manufacturer’s instructions.

**Aβ Immunoprecipitation and Detection**—Primary neurons and Sw-N2a cells were washed twice, harvested in ice-cold PBS, and centrifuged. Cell lysates were treated with 6% SDS containing 10 μl/ml of β-mercaptoethanol, sonicated, and then heated at 95 °C for 6 min. After centrifugation, supernatants were either loaded directly (neuron lysates) into 10–20% Tricine gels (Invitrogen) for Aβ detection or immunoprecipitated (N2a cells) overnight at 4 °C with 4G8 antibody (in 190 mM NaCl, 50 mM Tris-HCl pH 8.3, 6 mM EDTA and 2.5% Triton X-100). The latter were then incubated with rabbit anti-mouse secondary antibody (Cappell) together with protein A-Sepharose beads (GE Healthcare) for 2 h at 4 °C. Samples were subjected to electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were boiled in PBS for 5 min and immunoblotted as described (26). The immunoreaction was visualized by a chemiluminescence system (Pierce). Band intensities were quantified using Scion Image software. The area under the band peak and above the baseline was quantified.

To determine secreted APPβ levels, media were centrifuged 5 min at 1,000 × g to pellet cellular debris. 1 ml of supernatant was collected and incubated with an antibody against secreted APPβ (Signet) overnight at 4 °C (in 190 mM NaCl, 50 mM Tris-HCl, pH 8.3, 6 mM EDTA, and 2.5% Triton X-100). Samples were incubated with rabbit anti-mouse secondary antibody together with protein A-Sepharose beads for 2 h at 4 °C. The same media were further used to immunoprecipitate secreted APPα fragments with antibody 6E10 in the same conditions described for secreted APPβ. Western blot analyses were performed as described above using 22C11 (Roche Applied Science) as primary antibody.

**ELISA Analyses**—19 DIV Tg2576 primary neurons or Sw-N2a cells were incubated for 24 h in the presence or absence of antibody 6E10 or 4G8 and collected as described previously. Concentrations of Aβ1-40 and Aβ1-42 were measured by using the respective ELISA kits (BIOSOURCE) according to manufacturer’s instructions.

**Biochemical Measurements of Surface APP**—19 DIV primary neurons or Sw-N2a cells were incubated for 3 h with antibody 6E10. After two washes with PBS containing 1 mM CaCl2 and 0.5 mM MgCl2 (PBS-Ca-Mg), cells were plated on ice to block endocytosis and incubated with PBS-Ca-Mg containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 20 min. Cultures were rinsed in ice-cold culture medium to quench the biotin reaction. Cultures were lysed in 200 μl of 3% SDS. The homogenates were centrifuged at 14,000 × g for 15 min at 4 °C. Fifteen microliters of the supernatant were removed to measure total protein levels; the remaining supernatant was incubated with 100 μl of Neutravidin agarose (Pierce) overnight at 4 °C. Samples were then washed three times with a buffer containing: 150 mM NaCl, 10 mM Tris-HCl, pH 8.3, 5 mM EDTA, 0.1% Triton X-100, 0.01% bovine serum albumin, and protease inhibitor mixture (Roche Applied Science). Bound proteins were resuspended in 30 μl of SDS sample buffer and boiled. Quantitative Western blots were performed on both total and biotinylated (surface) proteins using APP N-terminal antibody 22C11 and tubulin antibody (Sigma). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) and captured on autoradiography film (Amersham Biosciences Hyperfilm ECL). Digital images, produced by densitometric scans of autoradiographs on a ScanMaker 8700 (Microtek) were quantified using NIH Image 1.63 software. The surface/total APP ratio was calculated for each culture.

**Degradation Assay for APP and C99**—Sw-N2a or C99-N2a cells were treated with biotin as described above. After cells were rinsed in ice-cold culture medium to quench the biotin reaction, fresh ice-cold medium containing 6E10 antibody (2 μg/ml) was added. Cells were kept on ice for 5 min to allow the antibody recognition of surface APP or C99. Cells were then placed at 37 °C for 45 min. After treatment, cells were collected and lysed as described for the assay of surface APP, above. Western blot analyses of full-length APP were performed using 22C11 antibody; Western blot analyses of C99 peptides were performed using 6E10 antibody.

**Immunofluorescence**—Cells were grown on poly-d-lysine-coated coverslips (Fisher). After antibody treatment, cells were washed in ice-cold PBS and fixed for immunofluorescence, as described previously (27). The following antibodies were used: Aβ42, synapsin I, and PSD-95 (Chemicon), APP 369 (anti-C terminus of APP, (30), EEA1 (BD Transduction), Tsg101 (Genetix), and Lamp2 (Zymed Laboratories Inc.). Fluores-
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cent secondary antibodies were either Alexa-488 or Alexa-546 (Molecular Probes) or Cy2- or Cy3-conjugated (Jackson ImmunoResearch). To determine antibody uptake, cells were incubated with secondary antibodies with or without prior permeabilization with 0.1% saponin. Cells were viewed with an Olympus Optical IX-70 microscope equipped with an ORCA-ER CCD camera (Hamamatsu Photonics) and a 60×, 1.4 NA plan apochromat objective. Metamorph software was used for quantitative analysis. To quantify Aβ immunofluorescence, 5–10 neurons or Sw-N2a cells were randomly picked from each of three independent experiments, and average intensities were measured in selected areas. To quantify Aβ staining in Sw-N2a cells after transfection with the dynamin-1 cDNA containing GFP, we only considered cells that were transfected (GFP-positive). For quantification of 6E10 antibody internalization and Aβ42 staining, intensity threshold was set using Metamorph 6.1 so that fluorescence of neurons was above background fluorescence. Total fluorescence per 30 μm of a thresholded neurite was automatically quantified. PSD-95 puncta were quantified as described previously (27). One or two coverslips from each culture were analyzed, 5–10 neurons per coverslip. From each neuron, 3–5 neuritic segments 30 μm in length were selected from areas where single puncta could be outlined. Images were thresholded so that only the brightest puncta, with intensity at least twice that of the neuritic shaft, were outlined. Using the integrated morphometric analysis feature in Metamorph, puncta density was automatically measured.

Confocal Microscopy—Immunofluorescence Aβ antibody internalization and intracellular Aβ reduction were examined by confocal microscopy using an Axiovert 100 M inverted microscope equipped with an LSM 510 laser scanning unit and a 63 × 1.4 NA plan apochromat objective (Carl Zeiss, Inc.), Ar488, HeNe1543 nm lasers, and LP560 and BP505–530. Optical sections were acquired at 0.7 μm thickness.

Live Cell Microscopy of Antibody Uptake—Cells were imaged using an Olympus Optical IX-70 microscope equipped with an ORCA-ER CCD camera, a 60×, 1.4 NA plan apochromat objective and a 37 °C heated chamber. Images were obtained using a Hamamatsu Orca ER digital camera. N2a cells were transfected with human APP-YFP for 3–4 h. Cells were incubated for 10 min on ice with Alexa-555-conjugated 6E10 antibody (6E10 was labeled using an Alexa Fluor 555 Monoclonal Antibody Labeling Kit, Invitrogen) in live imaging solution (120 mM NaCl, 3 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM glucose, 10 mM Hepes). Cells were washed twice in ice cold live imaging solution before imaging. Frames were automatically and sequentially acquired every 20 s with the FITC and Rhodamine filters using Metamorph.

Metabolic Labeling—Primary neurons were plated in 10-cm dishes. 7-Day-old cultures after 30-min starvation in cysteine/methionine-free medium (Invitrogen) were pulsed in fresh cysteine/methionine free medium with 1 mM [35S]methionine/cysteine (PerkinElmer Life Sciences) in the presence or absence of 2 μg/ml Aβ antibody 6E10 for 20–30 min and then chased in Neurobasal medium (Invitrogen) for 15 min, 45 min, or for media studies, 90 min. Media were centrifuged for 10 min transferred to clean tubes and immunoprecipitated with Aβ antibody 4G8. Cells were collected in ice-cold PBS and lysed. Samples were immunoprecipitated with Aβ antibody 4G8. 35S signal was visualized using a phosphorimager system (Hewlett Packard Cyclone).

**RESULTS**

Treatment with monoclonal Aβ antibodies (6E10, 4G8; Fig. 1A) reduced levels of Aβ in primary neurons at 19 DIV derived from Tg2576 mice harboring the Swedish mutant human APP and in N2a neuroblastoma cells stably transfected with AD Swedish mutant human APP (Sw-N2a) (Fig. 1, B–G). Specifically, treatment of cultured Tg2576 neurons with 2 μg/ml of Aβ antibodies for 24 h resulted in a 36 ± 12% (6E10) and 30 ± 11% (4G8) decrease in levels of Aβ as quantified by Western blot (Fig. 1B, E). In contrast to these N-terminal to mid Aβ domain/APP antibodies, treatment of neurons with G2-11, a C-terminal-specific Aβ42 antibody (Fig. 1A), did not induce changes in intracellular levels of Aβ (Fig. 1E), suggesting that binding to the exposed extracellular domain of Aβ was required for Aβ antibody-mediated reduction in intracellular Aβ. Similar reductions in levels of Aβ after treatment with Aβ antibodies were evident by ELISA analyses, where reduction of both Aβ40 and Aβ42 was observed with either 6E10 or 4G8 treatment (Fig. 1D). To further confirm these biochemical results, we also evaluated intracellular Aβ immunofluorescence (32) following Aβ antibody treatment, which revealed a reduction in intracellular Aβ42 immunofluorescence in cultured Tg2576 neurons as shown by confocal imaging (Fig. 1C). Treatment for 24 h with Aβ antibody 6E10 did not alter neuronal morphology or induce neuronal death as evaluated using the TUNEL labeling method or the LDH assay (supplemental Fig. S1). Western blot analyses demonstrated that incubation of Sw-N2a cells with Aβ antibodies had similar reductions in levels of Aβ. Specifically, 24 h of treatment with Aβ antibodies 6E10 or 4G8 reduced levels of intracellular Aβ by 51 ± 10% and 54 ± 11%, respectively, compared with untreated controls (Fig. 1F). Since these Aβ antibodies bind Aβ peptides and the Aβ domain within APP, we also treated Sw-N2a cells with a monoclonal antibody directed against the N-terminal ectodomain of human APP (antibody P2-1, Fig. 1A); this treatment did not reduce levels of intracellular Aβ (Fig. 1F). ELISA analyses confirmed the reductions of both Aβ40 and Aβ42 after 24 h treatment with Aβ antibodies 6E10 or 4G8 in Sw-N2a cells (Fig. 1G).

To investigate the mechanism whereby Aβ antibodies reduced intracellular Aβ, we examined whether the binding of cell surface APP by Aβ antibodies might be involved. Sw-N2a or untransfected N2a cells were first incubated with 6E10 or 4G8 for 24 h and then fixed, rinsed and stained with a fluorescent secondary antibody without cell permeabilization. The human-specific antibody 6E10 stained the cell surface of Sw-N2a cells...
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**FIGURE 1.** Aβ antibody treatment reduces cellular levels of Aβ. A, sequences in APP/Aβ recognized by the antibodies used in this study (not drawn to scale). G2-11 and anti-Aβ40 are specific for the C terminus of Aβ42 and Aβ40 peptides, respectively. 6E10 (human-specific), APP-ab2 (human-specific), and 4G8 are specific for the extracellular Aβ domain of APP. P2-1 (human-specific) and 369 are specific to the N- and C-terminal regions of APP, respectively. B, treatment with Aβ antibodies reduces levels of intracellular Aβ. Incubation of Tg2576 neurons with the indicated antibodies (2 μg/ml) for 24 h, directed at the extracellular portion of the Aβ domain within APP, reduces intraneuronal Aβ. Levels of full-length APP were unchanged. C, representative images of Aβ42 immunofluorescence. Tg2576 neurons treated for 24 h with Aβ antibody 6E10 revealed reduced Aβ42 immunofluorescence compared with untreated control Tg2576 neurons. Scale bar: 10 μm. D, ELISA analysis revealed reduction of Aβ40 and Aβ42 in Tg2576 neuron cell lysates after 24-h incubation with either antibody 6E10 or 4G8 (n = 3; *, p < 0.05; **, p < 0.01). E, Tg2576 neurons were treated with the indicated antibodies (2 μg/ml) for 24 h (n = 9 for 6E10 and 4G8; n = 3 for G2-11) and neuron cell lysates were then analyzed by Western blot. Aβ levels were reduced only by antibodies 6E10 and 4G8 but not G2-11, directed at the C terminus of Aβ42. Densitometric quantitation of Western blots performed on Tg2675 neuronal lysates is expressed as relative amount of Aβ in treated compared with untreated cells (*, p < 0.05; **, p < 0.01). F, immunoprecipitation followed by Western blot analysis on Sw-N2a cells also revealed reductions in levels of Aβ after 24-h incubation with antibodies against Aβ (6E10, 4G8) but not by an antibody against the APP ectodomain (P2-1). Densitometric quantitation is expressed as relative amount of Aβ in treated compared with untreated cells (n = 4; **, p < 0.01). G, ELISA analysis confirmed reduction of intraneuronal Aβ (Aβ40 and Aβ42) in Sw-N2a cells after 24-h incubation with either antibody 6E10 or 4G8 (n = 4; *, p < 0.05; **, p < 0.01).
body was applied without permeabilization (Fig. 2A) and antibody internalization when secondary antibody was applied after permeabilization (Fig. 2B). To better delineate Aβ antibody uptake in Sw-N2a cells and cultured Tg2576 neurons, we analyzed Z stack confocal images that more clearly reveal the intracellular localization of antibody (supplemental Fig. S4, A and B). These results supported that recognition of antibodies to the extracellular exposed Aβ domain of cell surface APP was required for antibody internalization.

To investigate whether Aβ antibodies remain associated with APP after internalization, live cell imaging was done on N2a cells transiently transfected with APP-YFP and treated with fluorescently conjugated Aβ antibody 6E10 (Fig. 2C and supplemental movie S1). As indicated by the arrows, internalized fluorescent 6E10 co-localized with APP-YFP within the same vesicle transported in the retrograde direction within a neurite. Internalized Aβ antibody 6E10 also co-localized with APP (369) in neuronal processes in fixed neurons (supplemental Fig. S5). To determine whether there was a correlation between antibody internalization and reduction in Aβ, we performed a time course of incubation with 6E10 (10 min, 1 h, or 7 h) in Tg2576 neurons, which revealed a progressive accumulation of Aβ antibody in neurons, especially evident within neuronal processes (Fig. 2, D and F). Quantification of Aβ42 immunofluorescence in neuronal processes after antibody treatment revealed a reduction of intraneuronal Aβ42 over time, which inversely correlated with antibody uptake in these neurites (Fig. 2, E and F). Internalized Aβ antibody 6E10 did not co-localize with intracellular Aβ42 in neurites after 10 min, at which time 6E10 would be expected to localize in early endosomes. However, at 1 h some co-localization of 6E10 with Aβ42 was evident (Fig. 2E), consistent with a late endosomal localization at this time (32).

To investigate whether treatment with Aβ antibody promoted internalization of full-length APP from the plasma membrane, we used biotin to specifically label APP at the cell surface of Tg2576 neurons. After 3-h incubation, there was a 40 ± 14% reduction in surface levels of full-length APP in antibody 6E10-treated compared with untreated neurons (n = 5; p < 0.05), consistent with increased APP internalization upon Aβ antibody binding (Fig. 3A).

To determine whether endocytosis of the Aβ antibody/APP complex upon Aβ antibody treatment was required to reduce intracellular Aβ, Sw-N2a cells were transfected with either wild-type GFP-dynamin (wtDyn) or the dominant negative K44E mutant GFP-dynamin (DynK44E) cDNA, which blocks endocytosis (28). Sw-N2a cells transfected with the wtDyn construct and incubated for 3 h with Aβ antibody 6E10 displayed a similar pattern of antibody internalization to the untransfected Sw-N2a cells, while cells transfected with the dominant negative DynK44E construct revealed only surface staining and no internalization of the antibody (Fig. 3B). In Sw-N2a cells transfected with wtDyn, treatment with 6E10 reduced levels of intracellular Aβ by 61 ± 1% compared with untreated cells (n = 3, p < 0.01). In contrast, in Sw-N2a cells transfected with DynK44E, 6E10 treatment did not reduce levels of intracellular Aβ (Fig. 3C). These results were confirmed by immunofluorescence experiments, which revealed a 41 ± 8% decrease in cellular Aβ42 in wtDyn transfected cells after 6E10 treatment, whereas antibody treatment did not reduce levels of Aβ42 in DynK44E transfected Sw-N2a cells (Fig. 3D). The overall reduction in Aβ levels upon transfection with DynK44E (Fig. 3, C and D) confirms recent work (33) and is consistent with previous studies indicating that APP internalization is important for Aβ generation (34, 35). At the same time, the reduction in Aβ generation by the dynamin mutant also limits the interpretation of the data obtained with regards to effects of Aβ antibody treatment.

We used antibody co-localization studies to examine whether internalization of the Aβ antibody/APP complex followed the classical endocytic pathway for cell surface receptors. Since antibodies against subcellular markers are mostly mouse monoclonal antibodies, APP mutant neurons were incubated with a 6E10-like rabbit Aβ antibody, APP-ab2, for different time points (0, 10, 30, 60, 180 min) and then co-stained with markers for subcellular compartments. After 10-min incubation, Aβ antibody was internalized within neurites and showed a punctate pattern that co-localized with the early endosomal marker EE1 (supplemental Fig. S6). In contrast, after 30-min incubation, Aβ antibody co-localization was more pronounced with the late endosomal/multivesicular body marker Tsg101. At later time points (60 and 180 min), internalized APP-ab2 co-stained with the late endosomal/lysosomal marker Lamp2 within processes and cell bodies (supplemental Fig. S6).

It has been reported that cell surface binding by antibodies can promote internalization and degradation of receptors in lysosomes (36). Antibody binding to the N terminus of cell surface APP was previously used to follow APP internalization to endosomal-lysosomal compartments (37, 38). To investigate whether Aβ antibody binding targeted internalized APP to the degradative pathway, we carried out biotin surface labeling on Sw-N2a cells at 4 °C followed by incubation of cells at 37 °C in the presence or absence of Aβ antibody 6E10 or the APP N- terminal antibody P2-1. After 15-min incubation there was no significant difference in levels of biotinylated APP within cells with 6E10 or P2-1 treatment (supplemental Fig. S7). In contrast after 45-min incubation with antibody 6E10, levels of biotinylated APP decreased by 45 ± 10% compared with untreated control cells (Fig. 4A, n = 3; p < 0.01), suggesting either increased degradation and/or increased secretase cleavage of APP. Although P2-1 did not reduce levels of Aβ (Fig. 1E), it did decrease levels of biotinylated APP. However, the magnitude of the effect (28 ± 9% compared with control) was less than that for 6E10 (Fig. 4A, n = 3, p < 0.05).

To further investigate the mechanism whereby Aβ antibodies reduce intracellular Aβ, we assessed Aβ generation at earlier time points of treatment using metabolic labeling. Primary neurons were pulsed for 20 min in [35S]methionine/cysteine containing media and then chased for 15 min and 45 min in the presence or absence of 6E10. Although there was no significant change in levels of newly generated intraneuronal Aβ at 15 min (not shown), there was a significant 30 ± 9% reduction in levels of [35S]methionine/cysteine-labeled intraneuronal Aβ at 45 min (Fig. 4B). Another explanation for the reduction of intracellular Aβ levels after Aβ antibody treatment could have been an increase of Aβ secretion. To investigate this hypothesis, we
Internalized Aβ Antibodies Reduce Neuronal Aβ generation and/or increased degradation of newly generated Aβ. Therefore, we examined the effect of Aβ antibody treatment on the generation of Aβ by β- and γ-secretases. To assess whether Aβ antibody decreased β-site amyloid cleaving enzyme (BACE) processing of APP (39), we measured the amount of the N-terminal APP fragment secreted after β-site cleavage of full-length APP (sAPPβ) using an antibody specific for sAPPβ. Remarkably, there was a 41 ± 3.5% increase in levels of secreted sAPPβ (n = 4; p < 0.05; Fig. 4C and supplemental Fig. S8B, upper panel) in Aβ antibody 6E10 treated (3 h) compared with untreated Sw-N2a cells. We also measured levels of N-terminal APP secreted after α-site cleavage of full-length APP (αAPPα). There was a trend for reduced levels of αAPPα in the media of 6E10 treated compared with untreated Sw-N2a cells, although this did not reach significance (supplemental Fig. S8B, lower panel). To further investigate the effect of Aβ antibody treatment on APP processing, we measured levels of APP C-terminal fragments (CTFs) after BACE cleavage (βCTFs or C99) or α-secretase cleavage (αCTFs or C83) in Tg2576 neurons treated for 24 h with Aβ antibody 6E10. In 6E10 treated neurons, there was a 92 ± 34% increase of βCTFs compared with untreated neurons (supplemental Fig. S8C, middle and lower panels). In contrast, there was a trend for decreased levels of αCTFs which did not reach significance (supplemental Fig. S8C, lower panel). The lack of increase in αCTFs in the presence of increased βCTFs argues against a generalized inhibition of γ-secretase.

pulsed primary neurons for 30 min in [35S]methionine/cysteine containing media and then chased for 90 min in the presence or absence of 6E10. In the media of 6E10-treated neurons, there was a trend for decreased levels of secreted Aβ compared with untreated cells which did not reach significance (supplemental Fig. S8A) suggesting that antibody treatment either reduced

FIGURE 3. APP endocytosis is promoted by Aβ antibody binding and is required for reduction of cellular Aβ. A, Tg2576 neurons (19 DIV) were treated with Aβ antibody 6E10 for 3 h and then incubated on ice with biotin to label surface APP. Western blot analysis revealed decreased levels of surface full-length APP in Aβ antibody 6E10-treated compared with untreated control neurons. Total levels of APP were unchanged in cell lysates. B, Sw-N2a cells transiently transfected with wild-type GFP-dynamin (wtDyn) or dominant negative GFP-dynamin (DynK44E) revealed internalization of Aβ antibody 6E10 after 3 h treatment in both untransfected control cells (unttransf) and wtDyn-transfected cells but not in DynK44E-transfected cells, which revealed a surface pattern of staining. C and D, in Sw-N2a cells transfected with wtDyn, treatment with 6E10 reduced levels of intracellular Aβ by 61 ± 1% compared with untreated cells. In contrast, in Sw-N2a cells transfected with DynK44E, 6E10 treatment did not reduce levels of intracellular Aβ. Aβ was measured by Western blotting (C) and Aβ42 immuno-fluorescence (D). Corresponding quantitation was expressed as a relative amount of Aβ in treated compared with untreated cells (n = 3; *, p < 0.05; **, p < 0.01; scale bar: 10 μm).
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**FIGURE 4.** Aβ antibody induced reduction of Aβ does not act via β- or γ-secretase inhibition and requires the late endosomal/lysosomal system. **A,** levels of internalized biotinylated full-length APP in Sw-N2a cells treated on ice with 6E10 or P2-1 antibody, followed by 45-min incubation at 37 °C, were reduced compared with untreated Sw-N2a cells (n = 3). Densitometric quantitation is expressed as a ratio of biotinylated APP to total APP in treated compared with untreated cells. **B,** metabolic labeling of primary neurons pulsed for 20 min with [35S]methionine and chased for 45 min in the presence or absence of antibody 6E10. In the presence of antibody 6E10 (or P2-1 antibody, followed by 45-min incubation at 37 °C, were reduced compared with untreated Sw-N2a cells (**C**). Levels of internalized biotinylated APP in Sw-N2a cells treated on ice with 6E10 or P2-1 antibody, followed by 45-min incubation at 37 °C, were reduced compared with untreated controls (**D**). Densitometric quantitation of sAPPβ in treated compared with untreated cells is shown. **E,** C99-N2a cells were treated with antibody 6E10 for 3 h did not decrease γ-secretase processing of APP but rather showed a trend for decrease (Fig. 4D). Had 6E10 decreased γ-secretase activity, C99 levels should have increased, as was seen when γ-secretase was inhibited with DAPT (Fig. 4D). To examine the effect of Aβ antibody treatment with inhibition of γ-secretase activity, we treated Sw-N2a cells for 3 h with γ-secretase inhibitor DAPT in the presence or absence of 6E10. Levels of βCTFs were increased by 19 ± 6% in DAPT and 6E10 treated cells compared with those treated with DAPT alone (supplemental Fig. S9A). This result is consistent with the data demonstrating that Aβ antibody treatment increased levels of sAPPβ and βCTF (Fig. 4C). Performing the same experiment on C99-N2a cells, which precludes β-cleavage, there was a trend for a decrease, which did not reach significance, in levels of C99 after 3 h of treatment with DAPT and 6E10 compared with DAPT alone (supplemental Fig. S9B). Since the Aβ antibody mediated reduction of Aβ did not appear to result from decreased secretase cleavage of APP, and since internalized Aβ antibodies trafficked to late endo-
Some/lysosomes, we considered that Aβ antibodies promote the late endosomal/lysosomal degradation of APP and APP-derived products, such as C99 and Aβ. To investigate whether Aβ antibody treatment induced the degradation of C99, we carried out biotin labeling on C99-N2a cells at 4 °C followed by incubation of cells at 37 °C in the presence or absence of Aβ antibody 6E10. After 45-min incubation with antibody 6E10, levels of biotinylated C99 decreased by 57 ± 7% (Fig. 4E), consistent with Aβ antibody induced degradation of C99. To further investigate whether the late endosomal/lysosomal system is involved in the Aβ antibody mediated reduction of Aβ, we tested whether inhibition of late endosomal/lysosomal function would interfere with the ability of Aβ antibodies to reduce intracellular Aβ. Indeed, incubation of Sw-N2a cells with 6E10 (3 h) in the presence of the lysosomal inhibitor chloroquine (40) prevented and/or counterbalanced the Aβ antibody-induced reduction of intracellular Aβ (Fig. 4F; similar results were obtained using ammonium chloride; data not shown).

Synaptic dysfunction is considered to be the earliest neurobiological alteration in AD (41, 42), and reduction of intraneuronal Aβ was the best Aβ-correlate of cognitive improvement in an AD mouse model (12). Therefore, we examined whether reduction of intraneuronal Aβ by Aβ antibodies could protect against the synaptic alterations that we previously described in APP mutant neurons in culture (27). We confirmed that the number of PSD-95 puncta (an important scaffold protein of the post-synaptic density) was reduced in Tg2576 neurons (supplemental Fig. S12). Treatment of wild-type neurons with Aβ antibody 6E10 (or Aβ antibody 4G8; supplemental Fig. S10) for 24 h restored the number of PSD-95 puncta to 96 ± 6% of wild-type levels (Fig. 5B; for an additional representative figure, see supplemental Fig. S11). In contrast, treatment with a C terminus-specific Aβ42 antibody (Chem42) was unable to restore PSD-95 puncta in Tg2576 neurons (supplemental Fig. S12). Treatment of wild-type neurons with Aβ antibody 6E10 had no effect on levels of PSD-95 puncta (data not shown).

**DISCUSSION**

Aβ immunotherapy remains an exciting therapeutic direction for AD, although the biological mechanism(s) whereby Aβ antibodies reduce Aβ in brain and improve cognitive function in AD mouse models are incompletely understood. Several hypotheses have been proposed. The “sink hypothesis” suggests that peripherally administrated Aβ antibodies can reduce levels of Aβ in the plasma and drive efflux of Aβ from the brain, where it is more concentrated, to the periphery (9). Another hypothesis is based on evidence that peripherally administrated Aβ antibodies can cross the blood brain barrier and enter the central nervous system (4), where Aβ antibodies can mediate degradation of Aβ aggregates by inflammatory cell activation (4, 43). However, evidence also indicates that Fc-mediated antibody-directed microglial activation is not necessary to reduce Aβ plaques, since Aβ immunotherapy was effective in FcRγchain knock-out/APP mutant transgenic mice (44) and F(ab’)2 fragments reduced plaque pathology in Tg2576 mice (45). This suggests that other mechanism(s) may be occurring as well. Recent evidence and our data suggest an additional scenario where Aβ antibodies reduce intracellular Aβ.

Intraneuronal Aβ accumulation is increasingly being linked with early, preplaque electrophysiological, synaptic, and pathological abnormalities (46). For example, Aβ42 accumulation and oligomerization were associated with ultrastructural pathology within distal processes and synapses prior to, and in areas devoid of, plaques in AD transgenic mice and human AD brain (17, 26). Aβ antibodies were shown to reduce intracellular Aβ in triple transgenic mice (11), and this reduction in intracellular Aβ was the best correlate of cognitive improvement (12). The biological mechanism by which Aβ antibodies reduced intracellular Aβ was unclear. Interestingly, antibodies
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gainst the cleavage site of BACE on APP (47) and intrabodies against Aβ (48) were suggested as new cellular therapeutic strategies for AD.

We provide evidence that antibodies against Aβ, previously shown by multiple groups to reduce plaque pathology in vivo (8), reduce intracellular levels of Aβ in cultured neurons by binding to the extracellular Aβ domain and protect against synaptic alterations in APP mutant neurons. We demonstrate that Aβ antibodies directed to the N-terminal to mid-domain of Aβ can be specifically endocytosed after binding at the cell surface. Our results could be important in why passive immunization is especially effective when using antibodies directed at the N-terminal region of Aβ (4, 11, 12, 49, 50).

A potential mechanism by which Aβ antibodies reduced intracellular Aβ could have been inhibition of β- or γ-secretase activities. We did not observe such inhibition. Specifically, the increase in sAPPβ and, in C99-N2a cells, the lack of an increase in C99 with Aβ antibody treatment argued against β- or γ-secretase inhibition, respectively. In fact, the increase of C99 in cells expressing full-length APP in conjunction with increased sAPPβ secretion suggests that the internalization of full-length APP induced by Aβ antibody treatment actually promotes BACE cleavage. Another possible mechanism for the antibody-mediated reduction in Aβ could have been from increased clearance of intracellular to extracellular Aβ, although the lack of an increase in levels of extracellular Aβ argues against this possibility. In fact, there was a trend for decreased Aβ secretion with Aβ antibody treatment that did not reach significance. Another potential mechanism for Aβ antibody-mediated clearance of intracellular Aβ is by enhancing cellular degradation after antibody binding to the Aβ domain of cell surface APP. Since Aβ antibodies directed to the C terminus of Aβ did not reduce intracellular Aβ, we hypothesized that Aβ antibodies act by binding to full-length APP and/or APP CTFs and not on potentially surface-associated Aβ in our experiments. The binding and internalization of antibodies to the ectodomain of cell surface APP was previously used to study APP internalization and trafficking and was employed to follow APP to endosomal-lysosomal compartments (37, 38, 51). Recent studies are increasingly suggesting an important role of endosomes in APP processing; for example, the majority of APP transported down axons by fast axonal transport was reported to be full-length, supporting endosomal secretase cleavage in neurons (52). Interestingly, alterations in the endosomal-lysosomal system are among the earliest changes described in AD and Down syndrome brains (53). Our data provide evidence for involvement of the endosomal/lysosomal system in intraneuronal Aβ clearance after Aβ antibody treatment. Co-localization of endocytosed Aβ antibody with Aβ42 was evident at 1-h incubation, when the internalized antibody co-stained with late endosomal/lysosomal markers and not at 10 min when internalized antibody co-localized with early endosomes. Our results are most consistent with Aβ antibody-induced increased internalization of APP from the cell surface to early endosomes, followed by increased β-cleavage (elevated C99) and then enhancement of C99 trafficking to the late endosomal lysosomal pathway for degradation. We hypothesize that enhanced trafficking of Aβ antibody bound C99 through late endosomes where γ-secretase components have been localized (54, 55) limits β-cleavage and thereby also increased Aβ secretion. That inhibition of late endosomes/lysosomes with chloroquine or ammonium chloride prevented Aβ clearance after Aβ antibody treatment supports the involvement of the endosomal-lysosomal system in the reduction of Aβ. Since both APP ectodomain and Aβ antibodies promoted surface APP reduction, although the latter were more effective, only Aβ antibodies reduced levels of cellular Aβ, and this suggests that the dissociation of the APP ectodomain antibody from βCTFs after BACE cleavage might preclude the enhanced degradation that occurs when Aβ antibody remains bound to the Aβ domain of C99. Our results support the scenario of Aβ antibodies inducing the internalization of APP from the plasma membrane to early endosomes where increased BACE cleavage appears to occur, followed by induction of the late endosomal-lysosomal-dependent clearance of βCTFs, and potentially Aβ. Since Aβ antibody alone tends to decrease levels of C99 in C99-N2a cells, our results suggest increased degradation of C99 and Aβ rather than γ-secretase inhibition. The lack of a statistically significant decrease of βCTFs in C99-N2a cells treated with DAPT and Aβ antibody compared with DAPT alone might be due to altered trafficking of C99 upon γ-secretase inhibition that thereby inhibits C99 degradation. In fact, altered βCTF trafficking was reported in neurons of PS1 conditional knock-out mice, where βCTFs accumulated abnormally at synapses (56). Our data cannot fully exclude that Aβ antibody treatment promotes βCTF processing by γ-secretase followed by increased degradation of the resultant Aβ-Aβ antibody complex rather than, or in addition to, primarily promoting degradation of the βCTF-Aβ antibody complex.

Aβ antibodies have been reported to block alterations of synapses induced by extracellular Aβ oligomers (50, 57). That C-terminal specific Aβ antibodies can also be protective in Aβ immunotherapy supports that antibody effects on extracellular Aβ are also involved (58, 59). Increasing evidence supports an as yet poorly understood dynamic relationship between extracellular and intracellular Aβ, modulation of which might be especially important in Aβ antibody induced therapeutic effects (60). High levels of extracellular Aβ were shown to induce up-regulation of newly generated intracellular Aβ42 (61). The mechanism whereby extracellular Aβ causes cell death in cultured neurons appears to be related to a dynamic relationship also between extracellular Aβ and cell surface APP, since toxicity did not occur in APP knock-out neurons (62) or cells harboring mutations in the YENPTY motif within the C terminus of APP (63). Thus, neurotoxicity might additionally require effects on intracellular Aβ.

In summary, in addition to effects on inflammatory mechanisms of Aβ clearance and on extracellular Aβ oligomers, among others, our data underscores that another mechanism whereby Aβ antibodies may play a critical role in Aβ immunotherapy is via reduction in intracellular Aβ. A better understanding of the molecular mechanism(s) whereby Aβ immunotherapy leads to reduced Aβ accumulation and improved cognitive function may lead to novel therapeutic approaches for AD.
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