Antibody Uptake into Neurons Occurs Primarily via Clathrin-dependent Fcγ Receptor Endocytosis and Is a Prerequisite for Acute Tau Protein Clearance*

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Background: How Tau immunotherapy clears Tau is not well known.
Results: Tau antibody uptake correlates with Tau levels, is primarily via clathrin-dependent FcγII/III endocytosis, and is required for acute Tau clearance.
Conclusion: Following receptor-mediated uptake into neurons, antibodies co-localize with Tau aggregates and promote Tau clearance.
Significance: Results support intracellular clearance as a viable route for immunotherapy and have major implications for future development.

A host of neurodegenerative diseases involve deposition of abnormal protein aggregates in the brain, including Alzheimer, Parkinson, Huntington, and prion diseases. In Alzheimer disease (AD),2 the defining lesions are amyloid-β plaques and neurofibrillary tangles composed of the microtubule-associated protein Tau. Active and passive immunotherapies are among the more attractive strategies for clearing these pathological proteins. Immunotherapy was first utilized in the context of AD to clear Aβ plaques and improve cognitive function in animal models (1–6). However, early clinical trials were suspended over concerns regarding encephalitis in a small percentage of participants (7). Although immunization was effective at reducing amyloid burden, there was no beneficial effect on synapses or clinical progression (8). Thus, plaque clearance alone was insufficient to alter the disease course. Following further refinement, additional clinical trials targeting various forms of Aβ are ongoing. Recent findings from phase III trials of bapineuzumab and solanezumab, antibodies recognizing amyloid-β, have been disappointing. No overall cognitive benefits were seen with either antibody, although subgroups showed significant albeit minimal slowing of memory deterioration in mild AD with solanezumab (9).

Besides Aβ, Tau lesions present an attractive target for disease-modifying interventions. Our group initially demonstrated the feasibility of targeting these aggregates by effectively utilizing active and passive Tau immunization in transgenic (Tg) models of tauopathy. Both approaches targeted the phosphoserine 396 and 404 region and consistently reduced pathological Tau and ameliorated related functional impairments (10–12). These findings have been confirmed and extended by other groups as well (13–18).

Recent results from our group have shown that Tau antibodies not only co-localize with intraneuronal pathological Tau but also with endosomal/lysosomal pathway markers (10, 19). Sim-
ilar findings have been observed by others utilizing antibodies for α-synuclein and Aβ (20–22). However, the mechanism by which antibodies enter cells remains unknown. To clarify this issue, we studied the uptake of labeled Tau antibodies in slice cultures prepared from Tg and wild type mice, as well as in primary neurons prepared from Tg mice. The antibody used in these studies, 4E6G7, was developed by our group and has previously shown promising results in preliminary experiments in transgenic animals (23). Mechanism of uptake was assessed via temperature manipulation, blocking with excess IgG, anti-Tau, or anti-Fc antibodies, and pharmacological interventions. In addition, the effect of antibody treatment on Tau levels in primary neuronal cultures was assessed. Our findings indicate that uptake of Tau antibodies is an energetic process and to a large extent occurs through a clathrin-mediated endocytic pathway via FcγII/III receptors. Importantly, antibody internalization is necessary for acute clearance of Tau in neuronal cultures.

EXPERIMENTAL PROCEDURES

Materials—Radioactive iodine was purchased from PerkinElmer Life Sciences, IODO-BEADS were from Pierce, and iodination reagent and desalting columns were from Fisher. Chlorpromazine, cytochalasin D, filipin III, dansylcadaverine, Superblock, and SX1-4 scintillation liquid were also purchased from Fisher. Neurobasal A, minimum Eagle’s medium, GlutaMAX™, sodium pyruvate, glutamine, HBSS, HEPES, DNase, and B27 vitamins were purchased from Invitrogen. Anti-Fc γ II/III (CD16/CD32) and FcγI (both rat anti mouse) were obtained from BD Biosciences; mouse IgG1κ was from eBioscience (San Diego); PHF-1 (mouse monoclonal) antibody was a gift from Dr. Peter Davies. pan-Tau rabbit polyclonal antibody corresponding to residues 243–441 was purchased from Dako (Carpinteria, CA). Rabbit polyclonal anti-Tau Ser(P)199 antibody was purchased from Santa Cruz Biotechnology (Dallas). Complete protease inhibitor mixture tablets were purchased from Roche Applied Science.

Animals—Animals utilized for slice cultures in experiments with 125I-labeled antibodies were aged adults (15–18 months) from three different mouse lines. The hTau/PS1 (12) line was utilized for the majority of the experiments. These animals express all six isoforms of human Tau in the absence of murine Tau (24). In addition, they express human presenilin containing the M146L mutation (25). For experiments correlating pathology with antibody uptake, additional aged animals from the 3×Tg line (26) and their wild type littermates were used. For experiments with fluorescently labeled antibodies, adult animals (3–4 months) from the JNPL3 (27) line were used. These animals express human 0N4R Tau containing the P301L mutation. JNPL3 pups at postnatal day 0 were utilized for primary neuronal cultures. In all lines, animals of both sexes were enrolled. All animals were housed in accordance with IACUC regulations in AAALAC approved facilities, and given free access to food and water ad libitum.

4E6G7 Preparation—Monoclonal antibodies were generated by GenScript Inc. Wild type (WT) BALB/c mice were immunized with a peptide corresponding to the phospho-Ser396/404 region (cTDHGAEVYK(pS)PVVSGLT(pS)PRHL). The peptide was conjugated to keyhole limpet hemocyanin via the cysteine residue, and mice showing a satisfactory immune response were used in hybridoma production. Monoclonal antibody 4E6G7 was selected and purified from the culture supernatant with a low endotoxin unit of the protein A affinity column. Further characterization of this antibody can be found in Gu et al. (42).

125I Labeling—Uptake studies utilized 4E6G7, an IgG1κ isotype monoclonal antibody developed by this laboratory; 4E6G7 was selected from a panel of antibodies made by subcontractor Genscript Inc. (Piscataway, NJ) against a phospho-epitope encompassing serine 396 and 404 as detailed above. This antibody selectively recognizes this region, primarily the phosphoserine 404, with lesser reactivity toward nonphospho-Tau. See Gu et al. (42) for a further characterization of this antibody. 4E6G7 and control IgG1κ were labeled with carrier-free Na125I using Pierce iodination beads and reagents according to the manufacturer’s instructions. Specific activity was determined as 2.04 and 2.12 μCi/μg, respectively.

Fluorescent Labeling—4E6G7 was labeled using the Alexa Fluor 568 labeling kit from Invitrogen. Briefly, the antibody was incubated with reactive dye at room temperature for 1 h with stirring. The elution column was prepared as per the instructions, and the antibody dye mixture was added, followed by antibody collection and verification of labeling.

Slice Cultures—Slice cultures were prepared as described previously (28). Briefly, mice were killed via cervical dislocation, and their brains were removed. The brainstem and cerebellum were discarded, and the two hemispheres were separated. Each hemisphere was cut into 400-μm sections on a tissue chopper from Brinkmann Instruments. Slices were separated in ice-cold culture buffer (124 mM NaCl, 1.5 mM KCl, 0.62 mM KH2PO4, 4.01 mM MgSO4, 1.35 mM CaCl2, 1.74 mM NaHCO3, 5 mM glucose, 1 mM ascorbic acid, 0.02 mM ATP) and distributed among six wells. Slices were left for 30 min at room temperature to recover. Following recovery, slices were placed into a Beinon BS3 brain slice chamber with oxygenated culture buffer.

Each apparatus contains six wells allowing each animal to be utilized for multiple conditions as well as serve as its own internal control. Because pathology is regional, slices are distributed among the wells so that each well contains a similar selection of brain regions.

Primary Neuronal Cultures—Neuronal cultures were prepared from JNPL3 pups at postnatal day 0. Briefly, plates were coated for 3 h with poly-l-lysine. Brains were harvested, and meninges and brainstem were removed. The remaining brain tissue was washed five times in HBSS+++ (975 ml Hanks’ balanced salt solution, 10 ml of 1 mM HEPES, 5 ml of penicillin/streptomycin, 10 ml of 100 mM sodium pyruvate) and then incubated with 200 μl of 0.5% trypsin for 15 min. Trypsin was neutralized with an equal volume of plating media (423.5 ml of minimum Eagle’s medium, 15 ml of GlutaMAX™ (100×), 5 ml of 200 mM glutamine, 50 ml of FBS, 4 ml of B27, 2.5 ml of penicillin/streptomycin), and 100 μg of DNase was added to further dissociate the cells. Tissue was again washed five times with HBSS+++ and centrifuged for 1 min at 0.5 × g. Tissue was then resuspended in 1 ml of plating media and mechanically dissociated to form a cell suspension. Cells were centrifuged again, and the resuspended cells were evenly distributed among the wells.
**Fcγ Receptor-mediated Antibody Uptake**

Plating medium was replaced with culture media (499 ml Neurobasal A, 1 ml of B27 supplement, 17 μl of basal medium Eagle) the following day. Cells were maintained in culture and allowed to develop processes prior to use in experiments.

**Antibody Uptake in Primary Neurons and Brain Slices**—Cells were grown as described on glass coverslips. Fluorescently labeled 4E6G7 was added to the culture media, and cells were incubated at 37 °C for 1 h. Cells were then washed and fixed for 10 min. Coverslips were washed again and mounted on slides using Dako Cytomation fluorescent mounting media. Images were collected on a Nikon Eclipse Ti confocal microscope. Additional cells were incubated with fluorescently labeled dextrans as well as 4E6G7 for 1 h at 37 °C.

Brain slices were incubated at 37 °C in the presence of fluorescently labeled 4E6G7 for 2 h. Slices were then fixed, sectioned, and stained with anti-Tau antibodies. Sections were mounted using Dako Cytomation fluorescent mounting media. Images were collected using a Zeiss 700 confocal microscope.

**Staining of Primary Neurons and Brain Slices**—Cells were cultured as described, washed, and fixed for 10 min. Cells were permeabilized with 0.3% Tween in PBS for an additional 10 min. Coverslips were incubated with primary antibody at room temperature for 2 h. Following primary antibody, coverslips were washed three times and then incubated with secondary antibodies for 1 h. Coverslips were washed an additional three times and then mounted on slides.

Sections from acute brain slice cultures were also utilized for staining. Sections were fixed and permeabilized with 0.3% Tween in PBS. Slices were incubated in primary antibodies overnight and secondary antibodies for 1 h at room temperature.

**Dose-response and Time Course Experiments**—Brain sections prepared as described above from hTau/PS1 mice (n = 3, age 15–18 months) were incubated with increasing concentrations of 125I-labeled 4E6G7 antibody. Concentrations of antibody in culture buffer ranged from 0.01 to 5 μg/ml. Each brain section was sectioned at 400 μm, and sections were divided evenly between the treatment groups.

Sections were maintained in oxygenated buffer at 37 °C. At 30, 60, and 120 min, sections were removed, weighed, and rinsed with acidified culture buffer, pH 5. Sections were washed a further three times in ice-cold culture buffer to remove surface-bound antibodies. Following rinsing, sections were placed in plastic vials with 5 ml of scintillation liquid, and radioactivity was measured on a Beckman LS 6500 liquid scintillation counter. Counts/min values were converted to nCi and corrected for differing tissue mass. Average and S.E. were determined from the results, plotted against the concentration of antibody in buffer, and fit to a linear curve.

To determine whether uptake was saturable, an additional group of brain slices from hTau/PS1 animals was incubated with increasing concentrations of 125I-labeled 4E6G7 with an additional 10 μg/ml dose added. For this experiment, all samples were collected after 60 min of incubation. Samples were prepared, and radioactivity was determined as described above. A saturation curve was also prepared using slices from wild type animals. All samples were collected after 60 min of incubation with up to 10 μg/ml 125I-labeled 4E6G7.

Further dose-response experiments were carried out utilizing WT animals and mice from the 3×Tg line (n = 3 per group, age 15–18 months). Brain sections were prepared as described and incubated for 1 h at 37 °C with increasing concentrations of 125I-labeled 4E6G7 (0.01–5 μg/ml). Sections were washed, and radioactivity was measured. As before, values were converted and plotted against the concentration of antibody in the incubation buffer.

With fluorescently labeled 4E6G7, antibody incubation and washing were carried out using sections from JNPL3 mice (n = 3 per group, age 3–4 months) exactly as with radiolabeled antibodies. Following washing, slices were homogenized and centrifuged for 15 min, and the values were corrected for total protein concentration in the samples. A standard curve was generated by adding antibodies to brain homogenate with a known protein concentration.

**Temperature Sensitivity**—Prior to experiments, the slice chamber was placed at 4 °C for 2 h. Sections from hTau/PS1 mice (n = 3, age 15–18 months) were prepared as described and incubated at 4 °C for 1 h in culture buffer containing 0.5 μg/ml of 4E6G7 antibody. Following treatment, slices were weighed and washed, and radioactivity was measured. Results were compared with those obtained from 37 °C control sections. As before, fluorescence experiments were carried out using the same procedure. For each group, averages and S.E. were determined. Two-tailed t test was used to determine significance.

**Effects of Excess IgG and 4E6G7**—In addition to temperature, the effects of an excess of mouse IgG on uptake were examined. For each animal (n = 3 hTau/PS1 mice, age 15–18 months), the brain sections were separated into three groups, control without antibody, 0.5 μg/ml 4E6G7, and 0.5 μg/ml 4E6G7 plus an excess of unlabeled mouse IgG1k (either 5 or 50 μg/ml). Samples were collected and processed as described above. The effect of excess 4E6G7 was tested using the same procedure. Samples were incubated with 0.5 μg/ml 125I-labeled 4E6G7 alone or 125I-labeled 4E6G7 in the presence of 10 or 100× excess unlabeled 4E6G7. Averages and S.E. were determined for each group. Fluorescence experiments were done with an additional group of JNPL3 mice (n = 3, age 3–4 months). As with temperature experiments, a two-tailed t test was used to determine significant differences between groups.

**Endocytosis Inhibitors**—Slice cultures were prepared from hTau/PS1 mice as described above. To understand the contribution of different types of endocytosis, three different inhibitors were utilized, filipin III (caveolin), chlorpromazine (clathrin), and cytochalasin D (phagocytosis). As with other experiments, slices were incubated with 0.5 μg/ml 4E6G7 for 1 h at 37 °C. Increasing concentrations of inhibitors were added to each sample, and radioactivity was determined. The same protocol was utilized to assess the effect of the drugs on primary neuronal cultures; with the exception that chlorpromazine was replaced with dansylcadaverine due to solubility issues in the cell culture media. Cells were washed in acidified HBSS+++ +, pH 5, and neutral HBSS+++ + and lysed with RIPA. As with slices, final radioactivity was determined for protein concentration. For both slices and neurons, a one-way ANOVA followed by Tukey’s HSD was utilized to determine significance.
**Fc Block**—To determine the contribution of Fc receptors to antibody uptake, slices were prepared as described above (hTau/PS1 mice, n = 3, age 15–18 months) and incubated at 37°C for 1 h with 0.5 µg/ml 4E6G7. Increasing concentrations of anti-CD16/CD32 Fc receptor blocking antibody (Fc block anti-CD16/CD32 (BD Biosciences) 0.05–50 µg/ml) were added to the culture buffer. Following incubation, slices were treated as described, and average radioactivity per treatment group was determined. Additional slices were incubated with increasing concentrations of an antibody against Fcγ receptor (anti-FcγRI, 0.05–50 µg/ml).

Fc block was also added to primary neuronal cultures prepared from JNPL3 pups (postnatal day 0, n = 6 wells per group). Cells were incubated with 0.5 µg/ml 4E6G7 and increasing concentrations (0.5–10 µg/ml) of Fc block for 1 h at 37°C. Cells were washed following treatment and processed as described. A second group of neurons was treated with anti-FcγRI (0.5–10 µg/ml) under the same conditions. Final radioactivity for all samples was obtained as described and normalized for total protein concentration. Significance in both slices and neurons was determined using a one-way ANOVA followed by Tukey’s honestly significant difference.

**Effects of 4E6G7 on Tau**—Primary neurons were prepared as described above to determine the effect of 4E6G7 on intraneuronal Tau levels. Cells were incubated for 24 or 72 h in the presence of 4E6G7 alone (1, 10, or 20 µg/ml, n = 6 wells per group) or 4E6G7 and 1 µg/ml dansylcadaverine. After treatment, cells were collected and processed for immunoblotting. Immunoblots for total Tau levels, Tau phosphorylated at Ser199, and actin were carried out. Data were analyzed using a two-way ANOVA and Bonferroni post hoc test.

**Immunoblotting**—Brain sections not used in uptake experiments and primary neurons were retained for immunoblotting. Each sample was homogenized in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, 1 µM complete protease inhibitor mixture (Roche Applied Science)) and subjected to a low speed spin (22 °C × g) to remove the membrane fraction. The supernatant was retained, and volumes were adjusted for total protein concentration. Samples were diluted in O+ buffer (62.5 mM Tris-HCl, pH 6.8, 5% glycerol, 2-mercaptoethanol, 2.3% SDS, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 µg/ml Roche Applied Science complete protease inhibitor mixture), boiled for 5 min, and loaded onto a 12% polyacrylamide gel. Gels were transferred at 100 V for 1 h and blots incubated overnight in appropriate dilutions of primary antibody. Blots were washed and incubated with a Peroxidase-labeled mouse secondary antibody for 1 h followed by further washing.

Blots were developed using a Fuji LAS-4000 and signal quantified with Multigauge. Chemiluminescent signal was measured and, when appropriate, plotted against radioactivity and fit to a linear curve.

**RESULTS**

**4E6G7 Recognizes Pathological Tau in Tissue**—In sections from 3Xtg and WT animals aged 22 months, 4E6G7 shows strong neuronal staining in the hippocampus of 3Xtg mice (Fig. 1A). PHF-1 also produced robust staining (Fig. 1B) in transgenic animals, although the pattern is less punctate. However, 4E6G7 did not stain tissue from WT mice of the same strain’s background (Fig. 1C). This region has prominent Tau and Aβ lesions in this model. See Gu et al. (42) for additional characterization of this antibody.

**Uptake Is Rapid and Saturable**—Slice cultures from hTau/PS1 transgenic mice (n = 3, 15–18 months) were incubated at 37°C with increasing concentrations (0–10 µg/ml) of 125I-labeled Tau antibody 4E6G7 raised against the Ser(P)396/404 region of the Tau protein. Individual sections were removed at 30, 60, and 120 min. Final intracellular radioactivity at all end points was linear up to antibody concentrations of 5 µg/ml at 1 h of incubation (Fig. 2A), with saturation occurring between 5 and 50 µg/ml (Fig. 2B). Similar values were obtained when utilizing fluorescent antibodies with JNPL3 slices, n = 3, age 3–4 months, with uptake reaching saturation between 5 and 10 µg/ml (Fig. 2C). This pattern appears fairly robust across mouse lines, as similar results were obtained in wild type animal using 125I-labeled 4E6G7 at 1 h of incubation (Fig. 2D). In addition, uptake was rapid and remained steady at each time point. On the basis of these results, other experiments were carried out using a 0.5 µg/ml antibody concentration and an incubation time of 1 h. In control experiments, we have found that slice cultures prepared from wild type animals show reactivity to PHF-1 on Western blots. It appears that the degree of PHF-1 reactivity is increased due to the process by which the slice cultures are made. When compared with sections allowed to recover at room temperature, PHF-1 chemiluminescent signal in flash-frozen hemispheres was reduced by 28% (Fig. 3).
**Fcγ Receptor-mediated Antibody Uptake**

![Graph](image)

**FIGURE 2. 4E6G7 uptake is rapid and saturable.** Brain sections prepared from hTau/PS1 mice (n = 3, 15–18 months) were incubated with increasing concentrations of 125I-4E6G7 antibody (Ab). Concentrations of antibody in buffer ranged from 0.01 to 5 μg/ml of buffer. A, sections were removed and assayed for radioactivity at 30, 60, and 120 min. Similar values were obtained for each time point indicating that uptake is rapid, occurring within 30 min of incubation. All points represent an average ± S.E. B, as above, brain slices from hTau/PS1 animals (15–18 months) were incubated with increasing concentrations of 125I-4E6G7. In this experiment, an additional 10 μg/ml dose was added, and all samples were collected after 60 min of incubation. Uptake of the antibody appears linear to 5 μg/ml, but it saturates at higher concentrations. C, similar results are obtained utilizing fluorescently labeled 4E6G7 with slice cultures prepared from JNPL3 mice (n = 3, 3–4 months). D, slices from wild type animals (n = 4, 12–17 months) were incubated with 125I-4E6G7 at increasing concentrations for 60 min. Similar to both transgenic lines, uptake saturated between 5 and 10 μg/ml.

To validate the system, we performed several control experiments. Slices that had been incubated with labeled 4E6G7 were further sectioned at 30 μm. Each section was added to 5 ml of scintillation fluid, and radioactivity was measured. There was no significant difference in radioactivity between sections taken at different levels (data not shown) indicating homogeneous distribution of antibody in tissue. Additionally, we compared sections with unsectioned hippocampus to determine the contribution of broken cell membranes to final radioactivity. There was a 30% decrease in radioactivity in unsectioned tissue, indicating that at least a portion of antibody uptake is via damaged membranes.

**Uptake Is Reduced at Low Temperatures**—To ascertain the effect of temperature on antibody uptake, the slice chamber was chilled for 2 h at 4 °C prior to the addition of brain slices. Slices were prepared as described above and incubated at 4 °C for 1 h with 0.5 μg/ml 125I-labeled 4E6G7. Samples were collected, weighed, and rinsed, and levels of radioactivity were determined. Lowered temperatures significantly reduced the uptake of antibody from 0.171 ± 0.06 to 0.031 ± 0.006 nCi/mg (p = 0.002; 63% decrease relative to 37 °C control), suggesting that antibodies are internalized mainly via an energy-dependent process. The same pattern of uptake was observed in JNPL3 slices incubated with fluorescently labeled 4E6G7 with 4 °C sections showing a 35% reduction in signal 0.048 ± 0.009 to 0.031 ± 0.004 μg of antibody/mg tissue (p = 0.05).

**Excess IgG and Unlabeled 4E6G7 Partially Block Antibody Uptake**—In addition to temperature, co-incubation with an excess of mouse IgG1κ also reduced uptake of 4E6G7. Slices from each animal were separated into three groups as follows: control 0.5 μg/ml 4E6G7 and 0.5 μg/ml 4E6G7 plus IgG1κ. The presence of a 10× excess of mouse IgG1κ resulted in a 41% decrease in radioactivity, a significant reduction relative to antibody alone (p = 0.04, Fig. 4A). A second group of slices was prepared the same way and incubated with 100× excess IgG1κ. Again, radioactivity was significantly reduced relative to 0.5 μg/ml 4E6G7 alone (40% decrease, p = 0.02, Fig. 4A), but the additional IgG1κ did not lead to further inhibition of uptake (Fig. 4A). Excess IgG also resulted in a 29% decrease in uptake when using fluorescently labeled antibodies (p = 0.05; Fig. 4B). Additional slices were incubated with 0.5 μg/ml 125I-labeled 4E6G7 and either 5 or 50 μg/ml nonradiolabeled 4E6G7. Addition of a 10- and 100-fold excess of 4E6G7 reduced labeled antibody uptake by 35 and 62%, respectively (p = 0.01 and 0.005; Fig. 4C). Both the mouse IgG and unlabeled 4E6G7 compete with 125I-4E6G7 for binding to the receptors on the cell surface. However, the apparently greater effect of 4E6G7 at the 100× dose (62% versus 40% reduction in radioactivity) may be related to its effect on retention. Unlike the mouse IgG, the unlabeled 4E6G7 can compete with 125I-4E6G7 for binding with intracellular Tau. Thus, the excess of 4E6G7 may affect both uptake and retention of the labeled antibody. In control experiments, we found that as much as 30% of the final radioactivity was the result of antibody uptake through damaged cells. Thus, this may mask the true extent of blockage and prevent complete blockage of uptake.

**Pathology Correlates with Antibody Uptake**—Slice cultures were created from animals of three different mouse lines (hTau/PS1, 3×Tg, and wild type (WT), n = 3 animals per...
group, age 15–18 months) with differing levels of Tau. Brain slices from each animal were incubated at 37 °C for 1 h with increasing concentrations of 125I-labeled 4E6G7 antibody. Average radioactivity was measured and nCi/mg of tissue determined. In addition, brain sections from each animal were washed three times and retained for Tau immunoblotting. Animals from the hTau/PS1 line showed significantly higher levels of total Tau and stronger PHF-1 immunoreactive bands in the Sarkosyl-insoluble fraction relative to both 3×/H11003 Tg and WT animals (Fig. 5, A–D). Both transgenic lines (hTau/PS1 and 3×/Tg) expressing Tau had significantly higher levels of radioactivity relative to WT mice at 1 μg/ml (49 and 34%, respectively; p = 0.002 and 0.04) and 5 μg/ml 4E6G7 (86 and 30% respectively; p = 0.008 and 0.02). Furthermore, at the 5 μg/ml
dose, animals from the hTau/PS1 line had significantly higher levels of uptake than the 3xTg line (43% increase, \( p < 0.02 \); Fig. 5E).

Within the identified mouse groups, levels of both total Tau and Sarkosyl-insoluble Tau correlated highly with final radioactivity (\( r^2 = 0.77 \) and 0.87, \( p = 0.002 \) and 0.0002, respectively; Fig. 5, F and G) for pooled samples. These results indicate that higher levels of intracellular Tau and the degree of phosphorylation at Ser\(^{396/404}\) may promote greater uptake or retention of the 4E6G7 antibody in the tissue. As stated above, the method used to make the slices produces an increase (28%) in Tau phosphorylated at Ser\(^{396/404}\) in WT animals (Fig. 3). This may account for the higher than expected degree of 4E6G7 uptake/retention in the WT mice.

IgG1\(\kappa\), the same isotype as 4E6G7, was also labeled with \(^{125}\)I and incubated with sections from hTau/PS1 and WT animals as described. As with 4E6G7, hTau/PS1 animals had higher levels of final radioactivity at 0.5, 1, and 5 \(\mu g/ml\) IgG concentrations (71, 73, and 48% increase, \( p = 0.003, 0.025, \) and 0.020, respectively, relative to WT mice). In both cases, however, the uptake was greatly reduced relative to 4E6G7-treated hTau/PS1 animals at 1 and 5 \(\mu g/ml\) IgG concentrations (84 and 91% decrease, \( p = 0.008 \) and 0.00007) (Fig. 5H). The difference in the overall levels of IgG1\(\kappa\) uptake relative to 4E6G7 may be explained by

FIGURE 5. 4E6G7 uptake correlates with pathological Tau levels. A and B, immunoblots using a pan-Tau antibody (Dako) and PHF-1 were carried out on the total and Sarkosyl-insoluble (aggregated) fractions, respectively, from three different mouse lines (\( n = 3 \) mice per group, age 15–18 months). C, total Tau levels from the low speed supernatant were quantified for each of the different mouse lines used. Bars represent average \( \pm \) S.E. D, Sarkosyl-insoluble fraction was isolated, and chemiluminescence was determined. Values were corrected for background and the average values obtained. Bars represent average \( \pm \) S.E. E, slices prepared from the same hTau/PS1, 3xTg, and WT mice were incubated for 1 h in varying concentrations of \(^{125}\)I-4E6G7 (\( n = 3 \) mice per group). Average radioactivity per mg of tissue was determined as described above, and values were fit to a linear curve (squares, hTau/PS1; circles, 3xTg; triangles, WT). All points represent averages \( \pm \) S.E. F, chemiluminescent signal was plotted against radioactivity for each sample (total \( n = 9 \)). A linear regression was fit to the data yielding \( r^2 \) of 0.77 (\( p = 0.02 \)). G, chemiluminescent signal from the Sarkosyl-insoluble fraction was plotted against final radioactivity for each animal (total \( n = 9 \)) and fit to a linear regression. An \( r^2 \) of 0.87 (\( p = 0.002 \)) was obtained indicating that levels of pathological Tau and antibody uptake are highly correlated. H, additional sections from hTau/PS1 and WT mice were incubated with \(^{125}\)I-IgG1\(\kappa\). Samples were processed as described. IgG uptake was greater in slice cultures made from hTau/PS1 relative to WT, but both were substantially less than 4E6G7 uptake.
 retention of the antibody in the tissue. 4E6G7 will be bound to Tau and thus less subject to recycling out of the cell than the unbound IgG. Additionally, excess IgG may be degraded more rapidly by the lysosomal system.

Antibody Uptake Occurs Mainly via Clathrin-mediated Endocytosis—Endocytosis inhibitors were utilized to further clarify the mechanism of antibody uptake. Brain slices from h Tau/PS1 mice were incubated with varying concentrations of either chlorpromazine, filipin III, or cytochalasin D. Phagocytosis inhibitor cytochalasin D did not alter uptake. Primary neurons from postnatal day 0 JNPL3 mice were incubated with 125I-4E6G7 and increasing concentrations (0.5–50 μg/ml) of drug (Fig. 6B). Addition of Dansylcadaverine produced significant reductions in final radioactivity at every dose used (p = 0.00006–0.000005) and up to 78% reduction in signal relative to control. In contrast, Filipin III produced a significant decrease in radioactivity at only the highest dose (p = 0.04, 30% reduction). Cytochalasin D had no significant effect. These data indicate that the majority of antibody uptake in neurons occurs via clathrin-mediated endocytosis, but that a smaller fraction is internalized by caveolin-dependent pathways. Other mechanisms may contribute fractionally to antibody uptake. Data are less clear in the slice cultures, where multiple cell types are present and drugs may not have fully penetrated the tissue. Longer incubation times with the inhibitors, or higher concentrations, may be necessary to observe the full effect in that model.

To ensure that observed uptake is receptor-mediated, as hypothesized, rather than fluid phase endocytosis, JNPL3 primary neurons were incubated with both 4E6G7 and fluorescently labeled dextran for 1 h at 37 °C. Images were collected and merged (Fig. 7, A–C). The numbers of red, green, and co-localized puncta were determined (~1700 puncta counted in total). Only 17 ± 1.2% of all the puncta were positive for both. Of those positive for 4E6G7 (red or yellow) 27 ± 1.4% were co-localized.
for both dextran and 4E6G7. Of those puncta positive for 4E6G7, 27 ± 1.4% were yellow, indicating that about one-fourth of antibody uptake occurs via fluid phase endocytosis (Fig. 8D).

Incubation with FcyRII/III Antibody, but Not FcyRI, Reduces 4E6G7 Uptake—Primary neurons cultured from JNPL3 mice were stained with Dako pan-Tau polyclonal antibody and FcγRII/III antibody (Fig. 8A–C). Images show that the receptors are distributed throughout the cells. Additionally, sections from JNPL3 mice were also stained with total Tau and FcγRII/III antibodies showing a similar pattern to that seen in the primary neurons (Fig. 8D–F). To visualize antibody uptake, additional cultured neurons were incubated with fluorescently labeled 4E6G7 for 1 h. Images show that the majority of the 4E6G7 antibody is visible as bright puncta bound to Tau within the neurons (Fig. 8G–I). Similar results were seen in sections from JNPL3 mice incubated with fluorescently labeled 4E6G7 (Fig. 8J–L).

To ascertain the contribution of Fc receptors, slices from adult (15–18 months) hTau/PS1 animals (n = 3) were incubated with 0.5 μg/ml 125I-4E6G7 and increasing concentrations of Fc block (anti-CD16/CD32, FcγRII/III). Fc block at 0.05 and 0.5 μg/ml produced a trend toward reduced radioactivity. Both 5 and 50 μg/ml concentrations of Fc block resulted in significantly reduced uptake (41 and 43% reduction, p = 0.05 and 0.04, respectively). The same protocol was used with an Fcy I antibody (anti-CD64) with no significant reduction in final radioactivity. FcγRII/I antibodies did not significantly affect uptake at any dose. Blocking FcγRII/III receptors reduces uptake.
whereas anti-FcγRI (0.05–10 μg/ml) did not affect uptake at any dose (Fig. 9B).

Incubation with 4E6G7 Reduces Intracellular Tau, and This Effect Can Be Blocked with Dansylcadaverine—To determine whether antibody entry into neurons is a prerequisite for Tau clearance, primary neurons cultured from P0 JNPL3 pups were incubated with 1, 10, or 20 μg/ml 4E6G7 with or without 1 μg/ml dansylcadaverine (DC) to block antibody uptake. Blots were incubated with mouse secondary alone, and chemiluminescence signal was determined for each. A, antibody uptake is dose-dependent with antibody accumulating in the cells with increased dose. B, samples incubated with 10 μg/ml 4E6G7 in the presence or absence of dansylcadaverine (DC) were compared to assess the effects of blocking clathrin-mediated endocytosis on antibody uptake. Uptake was reduced by 24% with dansylcadaverine present (p = 0.008; all samples run on the same gel). Bars represent average ± S.E. **, p < 0.01.

**FIGURE 10.** 4E6G7 uptake into cells can be visualized by immunoblot and is inhibited by dansylcadaverine. Primary JNPL3 neurons (n = 6 wells per group) were incubated with 4E6G7 at three different concentrations of 1, 10, or 20 μg/ml. Samples were collected and processed for immunoblotting as described. Blots were incubated with mouse secondary alone, and chemiluminescence signal was determined for each. A, antibody uptake is dose-dependent with antibody accumulating in the cells with increased dose. B, samples incubated with 10 μg/ml 4E6G7 in the presence or absence of dansylcadaverine (DC) were compared to assess the effects of blocking clathrin-mediated endocytosis on antibody uptake. Uptake was reduced by 24% with dansylcadaverine present (p = 0.008; all samples run on the same gel). Bars represent average ± S.E. **, p < 0.01.

Findings with radiolabeled antibody, incubation with 1 μg/ml dansylcadaverine significantly reduced uptake by 24% (p = 0.008; Fig. 10B). To assess Tau clearance, immunoblots for total Tau and Ser199-phosphorylated Tau were performed. None of the treatments significantly reduced actin levels. No change in Tau signal was observed in any of the treatment groups at 24 h (data not shown). However, at 72 h, 4E6G7 decreased the total Tau/actin ratio, and this effect was blocked by dansylcadaverine (two-way ANOVA dose, p < 0.0001; treatment, p < 0.0001; interaction, p < 0.0001). Post hoc analysis revealed that all doses of 4E6G7 (1, 10, and 20 μg/ml) reduced total Tau/actin (49, 61, and 58%, respectively, p < 0.001) (Fig. 11, A and C). In contrast, samples
incubated with both antibody and dansylcadaverine showed no change in total Tau/actin ratio (Fig. 11, B and C). These data show that neuronal uptake of 4E6G7 is a prerequisite for Tau clearance within these primary neurons.

To assess if phosphorylated Tau was preferentially targeted for clearance, additional immunoblotting was performed with a polyclonal antibody recognizing Tau phosphorylated at Ser<sup>199</sup>. Previously, we have shown that treatment with antibodies can reduce levels of phospho-Tau, including epitopes other than the one targeted (11). A similar pattern emerged as for total Tau, albeit less pronounced (two-way ANOVA treatment, \( p = 0.03 \); interaction, \( p = 0.03 \)). Post hoc analysis showed significant 42% decrease in Ser<sup>(P)199</sup>/actin at the highest dose of erine (Fig. 11s), consistent with previous findings indicating that Fc receptors on sensory neurons but not II or III (43). However, this is likely due to an intact blood brain barrier in WT mice preventing antibody-mediated Tau clearance within primary tauopathy neurons.

**DISCUSSION**

Our results show that neuronal uptake of an anti-Tau IgG antibody, 4E6G7, is required for Tau clearance. It is dose-dependent, saturable, and significantly reduced by temperature reduction and excess unlabeled IgG or 4E6G7. Furthermore, internalization strongly correlates with levels of pathological Tau species. Because effects of temperature on endocytosis are well established (29–31), our results indicated that uptake was likely an energy-dependent process. Treatment with clathrin-mediated endocytosis inhibitors, chlorpromazine and dansylcadaverine, significantly reduced uptake in slice cultures and primary neurons. Inhibition of phagocytosis had no effect, indicating limited microglial uptake. We further clarified the receptor type involved by determining that uptake was partially blocked by co-incubation with an Fcγ receptor antibody (CD16/CD32), corresponding to FcγII/III, in brain slices and primary neurons but not with anti-Fcγ. These results are consistent with previous findings indicating that FcγII/III receptor-mediated endocytosis occurs via the clathrin-dependent pathway (32–34). No treatment completely blocked uptake, however. This may be due to the nature of the slice cultures; sectioning brains causes disruption of some cell membranes. Other mechanisms may also be involved such as fluid phase endocytosis, although our data indicate that this represents a minority of uptake. Similar results were seen in endothelial cells transfected with neonatal Fc receptors (FcRn), in which fluorescently labeled Fc was taken up via FcRn and found in compartments containing receptors and not co-localized with dextran (35).

Interestingly, there was a higher degree of uptake in WT tissue than expected. In our prior study, antibodies were only detected in brains of Tg tauopathy mice and not WT animals when injected into the carotid artery (10). However, this is likely due to an intact blood brain barrier in WT mice preventing antibodies from crossing into the brain, which is removed in slice cultures. Additionally, slices are left to recover at room temperature, which has been shown to increase Tau phosphorylation in animals (36), and in our experiments this results in a 28% increase in PHF-1 signal by immunoblot. Thus, phosphorylated Tau levels in slice cultures are higher than in the brains of WT animals. Furthermore, 4E6G7 binds to nonphosphorylated Tau as well, at least on ELISA plates.

Involvement of FcRs in antibody uptake and protein clearance has been examined utilizing Aβ antibodies in AD models with conflicting reports. Early reports demonstrated that Aβ plaques and activated microglia in AD patients were immunoreactive for antibodies against Fcγ I, II, and III (37) with similar results also seen in Parkinson disease (38, 39). Deane et al. (41) indicated that FcR knock-out animals showed minimal clearance following administration of Aβ antibodies relative to FcR-positive animals, and additional data suggest that FcR binding is important for amyloid clearance (40). In contrast, other researchers found that in amyloid precursor protein transgenic animals, knocking out FcγR did not affect antibody clearance of plaques relative to animals expressing FcγR (1). It could be argued that microglial uptake may be important in the experiments described in this work. For example, antibody uptake highly correlates with the degree of Tau lesions. Such pathology may result in greater activation of microglia, which may phagocytose the antibody. Microglial activation may then be a factor in increased 4E6G7 uptake. However, blocking phagocytosis did not significantly affect uptake in either model system utilized, indicating minimal involvement of microglia, which should be abundant in slices and present to some extent in primary cultures. Indeed, we observe much less microglial activation in various tauopathy mouse models than in Aβ plaque models because most Tau lesions are intracellular. Additionally, in brain slice cultures, the majority of fluorescently labeled 4E6G7 was found in neurons, with some uptake in microglia but none in other glial cell types (42). Furthermore, data from primary neuronal cultures showed that uptake is primarily occurring via receptor-mediated clathrin-dependent endocytosis and to a large extent by FcγII/III receptors.

In addition to expression on glial cells, neurons also express Fcγ receptors. Earlier findings indicated the presence of Fcγ receptors on sensory neurons but not II or III (43). However, more recent studies have found expression of FcγIlb on Purkinje cells and parvalbumin neurons and expression of I, II, III, and IV in primary neuronal cultures (44–46). Additionally, neurons are responsive to extracellular IgG by up-regulating FcγR (44). These results, and data showing that antibodies against pathological proteins are taken up by neurons, suggest a mechanism by which the FcR contributes to protein clearance in immunotherapy (10, 19–22). Our results show that anti-Tau antibody uptake can be blocked to a large extent using FcγII/III antibodies suggesting that low affinity Fc receptors are the major route into neurons.

Of particular note is the finding that, in cultured neurons, partial blockade of receptor-mediated endocytosis with dansylcadaverine is sufficient to prevent Tau clearance. Importantly, this indicates that antibody-mediated Tau clearance takes place primarily within neurons. Previous data from our laboratory showed that antibodies were present in neurons of treated ani-
mals and that the treatment resulted in clearance of phospho-
Tau but not total Tau (10), which we subsequently confirmed in
other studies (11, 12, 19). Together, these prior studies suggest
involvement of intracellular Tau antibodies in Tau clearance,
and we now show in the primary Tg JNPL3 neuronal model that
this pathway is necessary for removal of Tau. The main differ-
ence between those prior animal studies and this experiment is
an observed Tau antibody-mediated decrease in total Tau in
culture and not in animals. This discrepancy may be explained
by numerous differences in these models and study design,
including antibodies and their concentration per tissue weight,
which is likely much greater in the culture. Our findings that
blocking Tau antibody uptake prevented Tau clearance indi-
cates that the intracellular clearance pathway is the primary one,
at least in this model and under relatively acute conditions.
Additionally, this treatment does not appear toxic at the con-
centrations used. Lactate dehydrogenase assays revealed no
change in neurons treated with 4E6G7 alone or 4E6G7 and
dansylcadaverine relative to control cells at up to 7 days of incub-
Actin levels were also not significantly altered at any of the
conditions used.

The involvement of FcR, coupled with our data showing
uptake-dependent intracellular Tau clearance, also suggests a
pathway for improving efficacy of immunotherapy. In Aβ-target-
ning experiments, antibodies with isotypes having higher
affinity for phagocytic FcRs led to better clearance (40). Indeed,
affinity for Fc receptors was more important for clearance
than affinity for Aβ. This suggests selection of antibodies could
be improved by focusing on specific isotypes or modifications
that would improve receptor binding. For example, mutations in
antibody Fc region are capable of increasing affinity for neo-
natal FcRs (47), whereas other mutations can shift the ratio
of FcγRIIA/FcγRIIB affinity leading activation of macrophages
(48). Thus, it is possible that antibodies containing mutations
that enhance binding to the desired receptor may provide
greater efficacy in clearing protein aggregates. It is uncertain
how or if such mutations would affect neuronal uptake, but
further studies are clearly warranted.

Besides intracellular aggregates, extracellular Tau may play
an important role in disease progression. Recent findings have
demonstrated that Tau pathology can spread across synapses
and that pathological Tau can be taken up by cells (17, 49–58).
Cultured cells, even healthy ones, have also been found to
secrete phosphorylated Tau (48), and extracellular Tau
increases intracellular calcium in cultured neurons (59). This
presents an additional mechanism by which treatment with
antibodies might alter disease progression. Antibodies may
prevent secretion by targeting intracellular Tau into protein
degradation pathways. Extracellularly, antibodies may bind to
Tau and promote its uptake into microglia or neurons for clear-
ance and thereby prevent spread of Tau pathology to neighbor-
ing neurons.

Previously our lab has shown that Tau antibodies clear Tau
aggregates and improve cognition and other Tau-related defi-
cits in Tauopathy mouse models. Also, these antibodies do not
only cross the blood brain barrier but enter neurons. Herein, we
have identified the mechanism of this neuronal uptake showing
it to be primarily receptor-mediated via FcγII/III receptors, to
correlate very well with the degree of Tau pathology, and to be
a prerequisite for Tau clearance. These important findings
identify a neuronal pathway that may be activated in response
to intraneuronal Tau aggregation and that can be manipulated
to improve the efficacy of immunotherapy.

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Lundbeck A/S.

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