IgG-Assisted Age-Dependent Clearance of Alzheimer's Amyloid β Peptide by the Blood–Brain Barrier Neonatal Fc Receptor

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The role of blood–brain barrier (BBB) transport in clearance of amyloid β -peptide ($A\beta$) by $A\beta$ immunotherapy is not fully understood. To address this issue, we studied the effects of peripherally and centrally administered $A\beta$ -specific IgG on BBB influx of circulating $A\beta$ and efflux of brain-derived $A\beta$ in $APP^{sw+/-}$ mice, a model that develops Alzheimer's disease-like amyloid pathology, and wild-type mice. Our data show that anti- $A\beta$ IgG blocks the BBB influx of circulating $A\beta$ in $APP^{sw+/-}$ mice and penetrates into the brain to sequester brain $A\beta$. In young mice, $A\beta$ -anti- $A\beta$ complexes were cleared from brain to blood by transcytosis across the BBB via the neonatal Fc receptor (FcRn) and the low-density lipoprotein receptor-related protein (LRP), whereas in older mice, there was an age-dependent increase in FcRn-mediated IgG-assisted $A\beta$ BBB efflux and a decrease in LRP-mediated clearance of $A\beta$ -anti- $A\beta$ complexes. Inhibition of the FcRn pathway in older $APP^{sw+/-}$ mice blocked clearance of endogenous $A\beta_{40/42}$ by centrally administered $A\beta$ immunotherapy. Moreover, deletion of the FcRn gene in wild-type mice inhibited clearance of endogenous mouse $A\beta_{40/42}$ by systemically administered anti- $A\beta$. Our data suggest that the FcRn pathway at the BBB plays a crucial role in IgG-assisted $A\beta$ removal from the aging brain.

Key words: antibody; amyloid β ; Alzheimer's disease; blood–brain barrier; amyloid; transport

Introduction

Although the amyloid hypothesis of Alzheimer's disease (AD) remains controversial, many investigators consider accumulation of neurotoxic amyloid β peptide ($A\beta$) in the brain as a key pathogenic event contributing to neurodegeneration (Hardy and Selkoe, 2002). The levels of $A\beta$ in the brain are controlled by its rates of production from the larger $A\beta$ -precursor protein (APP) and the rates of clearance (Tanzi et al., 2004; Zlokovic, 2005). According to recent studies, the blood–brain barrier (BBB) transport of $A\beta$ critically regulates the levels of brain $A\beta$ and particularly the receptor for advanced glycation end products (RAGE)-mediated influx of circulating $A\beta$ (Deane et al., 2003) and the low-density lipoprotein receptor-related protein 1 (LRP)-mediated efflux of brain-derived $A\beta$ (Shibata et al., 2000; Deane et al., 2004). Under physiological conditions, the LRP-mediated brain efflux of $A\beta$ prevails, whereas in animal models of AD and in AD brains, downregulation of LRP in concert with

upregulation of RAGE may create an unfavorable $A\beta$ gradient across the BBB, resulting in $A\beta$ retention in the brain (Tanzi et al., 2004; Zlokovic, 2004).

Vaccination and passive immunization to $A\beta$ lower brain $A\beta$ (Schenk et al., 1999; Sigurdsson et al., 2001; Das et al., 2003) and improve behavior in animal models of AD (Janus et al., 2000; Morgan et al., 2000; Dodart et al., 2002). Although clinical $A\beta$ immunization trials in AD patients were terminated because of adverse neuroinflammatory effect (Orgogozo et al., 2003), reduced $A\beta$ brain deposition (Nicoll et al., 2003) and slower cognitive decline (Hock et al., 2003) have been reported. How anti- $A\beta$ IgG clears brain $A\beta$ remains debatable. The “sink” theory suggests that the interaction of $A\beta$ with an $A\beta$ -specific IgG in plasma creates a concentration gradient across the BBB that promotes efflux of brain $A\beta$ into blood, as shown by passive and active immunization studies (DeMattos et al., 2001, 2002; Deane et al., 2003; Lemere et al., 2003). Conversely, some circulating anti- $A\beta$ antibodies cross the BBB and activate microglia-mediated Fc γ receptor-dependent (FcR γ) clearance of amyloid (Bard et al., 2000) and/or the FcR γ -independent clearance (Bacskai et al., 2002; Das et al., 2003). Microglia clear amyloid slowly (Frautschy et al., 1992; Paresse et al., 1997; Bacskai et al., 2002), whereas microglia-independent clearance of $A\beta$ seems to be more rapid (DeMattos et al., 2001; Wilcock et al., 2003; Deane et al., 2004) and may require efflux of $A\beta$ across the BBB (Shibata et al., 2000; Banks et al., 2003; Tanzi et al., 2004; Zlokovic, 2004).

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Here, we show in *APP^{Sw+/−}* mice (Hsiao et al., 1996), a model that develops AD-like amyloid pathology, that an A β -specific IgG prevents RAGE-mediated transport of circulating A β across the BBB and enters into the brain to sequester brain A β and promote its vigorous clearance to blood. We demonstrated that the major histocompatibility complex class I-related neonatal Fc receptor (FcRn), which is functionally and structurally distinct from the Fc γ receptors (Borvak et al., 1998; Ravetch and Bolland, 2001; Roopenian et al., 2003; Ober et al., 2004; Yoshida et al., 2004), is critical for the IgG clearance from brain and for the elimination of A β -anti-A β complexes through the BBB. We also show that FcRn-mediated IgG-assisted A β efflux across the BBB remains active with aging, in contrast to LRP-mediated clearance of A β -anti-A β complexes, which decreases with aging. Finally, we demonstrate that inhibition of the FcRn pathway in old *APP^{Sw+/−}* mice (Hsiao et al., 1996) completely blocks rapid clearance of endogenous A β 40/42 by centrally administered A β immunotherapy, whereas deletion of the FcRn gene in mice inhibits clearance of endogenous mouse brain A β by systemically administered anti-A β .

Materials and Methods

Animals. C57BL/6 and *APP^{Sw+/−}* (transgenic Tg2576) mice were from Taconic Farms (Germantown, NY). *β_2 M^{−/−}* mice (neonatal Fc receptor light chain β_2 -microglobulin null mice), *FcR γ ^{−/−}* mice (Fc γ receptor null mice), *RAP^{−/−}* (receptor-associated protein null mice), and *FcRn^{−/−}* mice (neonatal Fc receptor null mice) were from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized by ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). All procedures were according to National Institutes of Health guidelines approved by the University Committee on Animal Resources (University of Rochester).

Reagents. Human A β 1–40 was synthesized at the W. M. Keck Facility (Yale University, New Haven, CT), using solid-phase *N*-t-butyloxycarbonyl chemistry and purified by HPLC. We used human recombinant RAP (EMD Biosciences, San Diego, CA), monoclonal mouse antibody (ab) against C-terminal domain of human LRP β -chain, which cross reacts with mouse LRP (5A6, 1:350, 5 μ g/ml; EMD Biosciences), rat anti-mouse CD31 antibody (1:200; BD PharMingen, Lexington, KY), anti-RAGE IgG F(ab')₂ (20 μ g/ml; Dr. Shi Du Yan, Columbia University, New York, NY), rat anti-mouse F4/80, a microglial cell marker (1:100; Serotec, Indianapolis, IN), mouse anti-human GFAP (1:100; DakoCytomation, Glostrup, Denmark), mouse monoclonal anti-phosphotyrosine (1:2500; Sigma, St. Louis, MO), and monoclonal mouse anti-human A β (4G8; Signet Laboratories, Dedham, MA) against amino acids 17–24 of A β . Monoclonal anti-FcRn (1G3) was obtained as the condition medium of cultured mouse hybridoma cell line CRL-2434 (American Type Culture Collection, Manassas, VA) as described previously (Schlachetzi et al., 2002). The FcRn blocking antibody does not interact with 4G8 *in vitro*, as indicated by 4G8 ELISA (see below), which showed no change in 4G8 signal in the presence of the FcRn antibody. 4G8 F(ab')₂ was prepared as described previously (Pierce, Rockford, IL) and dialyzed overnight to remove elution buffer and digestion medium. The monoclonal antibody 1560, which recognizes A β epitope 1–17, was from Chemicon (Temecula, CA). All chemicals were obtained from Sigma, except protease inhibitor (Roche, Indianapolis, IN) and Triton X-100 (Electron Microscopy Sciences, Gibbstown, NJ). ¹²⁵I-Na and ¹⁴C-inulin were from Amersham Biosciences (Little Chalfont, UK). ^{99m}Tc was obtained from Cardinal Health 414 (Rochester, NY).

Radio-iodinated ligands. Radio-iodination of the A β peptide (10 μ g) was performed by mild "lactoperoxidase" method (Thorell and Johansson, 1971) using 2 mCi ¹²⁵I-Na. The mono-iodinated nonoxidized form of A β was purified by reverse-phase HPLC separation (Shibata et al., 2000). Typically, the specific activities were in the range of 45–65 μ Ci/ μ g peptide. 4G8 (50 μ g) and 4G8 F(ab')₂ (10 μ g) were radio-iodinated using IODO-BEADS or IODO-GEN (Pierce) and 0.5 and 0.7 mCi ¹²⁵I-Na, respectively. Typical specific activity was ~3–5 μ Ci/ μ g 4G8 or 4G8 F(ab')₂. ¹²⁵I-A β 40–4G8 complex was prepared by incubating

A β 40 with 4G8 at 5:1 molar ratio for 3 h at 37°C, and excess ¹²⁵I-A β was removed by ultrafiltration. Complex stability was confirmed by Tris-tricine native PAGE and autoradiography. ¹²⁵I-A β –4G8 complex was prepared in a similar manner.

Brain perfusion technique. This method is used to determine influx of radiolabeled ligands across the BBB and has been described in detail previously (LaRue et al., 2004). Briefly, the right common carotid artery was cannulated with a polyethylene tubing (PE10), and the brains were perfused at 1.0 ml/min (Ranin peristaltic pump), with an artificial plasma solution as described previously (LaRue et al., 2004). Radiolabeled test ligands (e.g., ¹²⁵I-A β 40, ¹²⁵I-4G8-A β 40, and ¹²⁵I-A β 40–4G8) and the reference molecules (e.g., ¹⁴C-inulin and ⁹⁹Tc-albumin) were infused simultaneously via a slow-drive syringe pump (Harvard Apparatus, Holliston, MA) at a rate of 0.1 ml/min. Influx of ¹²⁵I-A β 40 in *APP^{Sw+/−}* mice was determined at carrier concentrations corresponding to A β 40 plasma levels in *APP^{Sw+/−}* mice at different ages (Kawarabayashi et al., 2001). The effect of RAGE-specific IgG F(ab')₂ was tested at 20 μ g/ml. After a timed perfusion, typically 5–10 min, the brain was rapidly removed, and the ipsilateral hemisphere was homogenized for radioactivity quantification. The perfusion fluid was centrifuged, and supernatant (plasma) was counted. ¹²⁵I samples were subjected to TCA, SDS-PAGE, native PAGE, and/or HPLC analysis using the procedures we described previously (Shibata et al., 2000; Deane et al., 2003, 2004).

Brain clearance studies. Clearance of ¹²⁵I-A β 40, ¹²⁵I-4G8, or ¹²⁵I-A β –4G8 from brain interstitial fluid (ISF) was determined simultaneously with ¹⁴C-inulin (reference marker), using a procedure described previously (Shibata et al., 2000). Briefly, a stainless steel guide cannula was implanted stereotactically into the right caudate–putamen with the cannula tip coordinates of 0.9 mm anterior and 1.9 mm lateral to bregma and 2.9 mm below the surface of the brain. Animals were recovered after surgery before tracer studies. The experiments were performed before substantial chronic processes occurred, as assessed by histological analysis of tissue, i.e., negative staining for astrocytes (glial fibrillar acidic protein) and activated microglia (anti-phosphotyrosine), but allowing time for the BBB repair for large molecules, as reported previously (Cirrito et al., 2003; Deane et al., 2004). Isotope mixture (0.5 μ l) containing ¹²⁵I-labeled test molecule at 40 nM and ¹⁴C-inulin was injected over 5 min via an ultra micropump with a micro4-controller (World Precision Instruments, Sarasota, FL) into brain ISF. The recovery of both radiolabeled inulin and A β at zero time was 100%, indicating that 100% of injected material remains present for transport with no loss of tracers via tracking up the cannula. 4G8 was administered by two intraperitoneal injections at 200 μ g at 0 and 48 h, and ¹²⁵I-A β 40 clearance was measured at 1 and 120 h of 4G8 administration in nontransgenic mice and at 120 h in 18- to 20-month-old *APP^{Sw+/−}* mice. The levels of 4G8 in plasma and brain ISF within 120 h were determined by ELISA (see below). Unlabeled molecular reagents [4G8 (0.5 and 2 μ M), anti-FcRn (α FcRn, 60 μ g/ml), RAP (0.5 and 5 μ M), and fucoidan (1.5 mM)] were infused into brain ISF in control mice 30 min before radiolabeled ligands and then simultaneously with radioligands until the end of experiment. Clearance of ¹²⁵I-4G8 or ¹²⁵I-A β –4G8 was also studied in *FcRn^{−/−}*, *β_2 M^{−/−}*, *RAP^{−/−}*, or *FcR γ ^{−/−}* mice. In all studies, brains were sampled within 30 min after tracer injection and prepared for radioactivity analysis. TCA and/or SDS-PAGE/immunoprecipitation analyses were determined to confirm the molecular forms of test tracers in brain and plasma (Deane et al., 2003, 2004).

Brain capillary uptake. Brain capillaries from wild-type and *APP^{Sw+/−}* mice were isolated as described previously (Wu et al., 2003) and incubated with ¹²⁵I-4G8, ¹²⁵I-A β 40–4G8, or ¹²⁵I-4G8–A β 40 at 1 nM and ¹⁴C-inulin in mock CSF at 37°C for 1 min. The following potential inhibitors were used: 4G8 (0.5 and 2 μ M), RAP (0.5 and 5 μ M), Fc fragment (1 and 10 μ M), and LRP-specific IgG (20 μ g/ml). The capillary pellet was separated by centrifugation at 4°C, washed in ice-cold mock CSF, and prepared for radioactivity analysis along with samples of the incubating medium.

Radioactivity measurements. ¹²⁵I samples and ⁹⁹Tc-albumin radioactivity analysis were determined by gamma counter analysis (Wallac Wizard Gamma Counter; PerkinElmer, Meriden, CT). ¹⁴C samples were solubilized in 0.5 ml of tissue solubilizer (PerkinElmer) overnight, fol-

lowed by addition of 5 ml of scintillation cocktail (Packard Ultima Gold; PerkinElmer) and analysis on a liquid scintillation counter (Packard Tri-Carb 2100TR Liquid Scintillation Counter; PerkinElmer).

Calculations. The BBB influx was determined as cerebrovascular permeability surface area product (PS) $\times$ Cpl, where Cpl was the concentration of the test molecule in plasma. The PS product of [125 I]-labeled test molecule was calculated using 14 C-inulin correction: $PS \times T = [(^{125}\text{I cpm/g of brain tissue}) \times \text{TCA-precipitable radioactivity}/(^{125}\text{I cpm/ml of arterial plasma inflow}) \times \text{TCA-precipitable radioactivity}] - (^{14}\text{C dpm/g of brain tissue})/(^{14}\text{C dpm/ml of arterial plasma inflow})]$ (LaRue et al., 2004), where T is the infusion time and 14 C-inulin was infused simultaneously with the test molecule. Influx was expressed per gram brain ISF, assuming the ISF space of 0.1 ml/g of brain (LaRue et al., 2004).

For brain clearance studies, calculations of clearance parameters were as reported previously (Shibata et al., 2000). The percentage of radioactivity of the test ligand and inulin was determined as follows: % recovery in brain = $100 \times (N_b/N_i)$, where N_b is the radioactivity remaining in the brain at the end of the experiment, and N_i is the radioactivity injected into the brain ISF, i.e., the disintegrations per minute for 14 C-inulin and the counts per minute for TCA-precipitable 125 I-radioactivity. The percentage of 125 I-labeled test ligands (i.e., 4G8, A β 40–4G8, and A β 40) cleared through the BBB was calculated as $[(1 - N_{b(\text{TEST})}/N_{i(\text{TEST})}) - (1 - N_{b(\text{inulin})}/N_{i(\text{inulin})})] \times 100$, using a standard time of 30 min. The loss of tracers via the ISF bulk flow was used to calculate the percentage clearance of 125 I-labeled test ligands via the flow of brain ISF and was calculated as $(1 - N_{b(\text{inulin})}/N_{i(\text{inulin})}) \times 100$ (Shibata et al., 2000).

Brain capillary uptake of 125 I-labeled test molecules [4G8, 4G8–A β , and A β –4G8–F(ab') $_2$] were corrected for the distribution of 14 C-inulin and determined as the tissue to medium ratio as follows: cpm for TCA-precipitable 125 I-radioactivity (g capillary protein)/cpm for TCA-precipitable 125 I-radioactivity (ml medium), as reported previously (Deane et al., 2004).

4G8 ELISA. 4G8 titers in plasma and brain were determined within 120 h after two intraperitoneal injections of 4G8 (200 μ g/each) at 0 and 48 h by ELISA technique similar to that described previously (Das et al., 2001). Briefly, A β 42 was coated at 5 μ g/well overnight at 4°C in 50 mM carbonate buffer, pH 9.6, and 0.05% sodium azide on 96-well high-binding Stripwell immunoassay plates (Corning Life Sciences, Corning, NY) and blocked with 0.25% bovine serum albumin in PBS overnight at 4°C. After three washes with PBS/0.1% Tween 20, 100 μ l of diluted plasma or brain homogenates were added and incubated overnight at 4°C. After washes with PBS/0.1% Tween 20, plasma and brain 4G8 was detected using a goat anti-mouse conjugated with HRP (Sigma) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). 4G8 levels were obtained from the standard curve. The levels of 4G8 in plasma and brain ISF were expressed in nanomolar assuming the ISF volume of distribution of 0.1 ml/g brain.

Immunohistological analysis. Freshly frozen acetone-fixed brain tissues from APP $^{sw+/+}$ and control mice cut in the sagittal plane at 14 μ m, and paraffin-embedded human brain tissue from AD patients [Braak stage V–VI, CERAD (for Consortium to Establish a Registry for Alzheimer's Disease), frequent] and age-matched controls (Braak negative; CERAD negative) cut in 6 μ m sections were used for double immunostaining for FcRn and CD31 or von Willebrand Factor (vWF) (endothelial cell marker). Anti-FcRn antibodies were prepared from the condition medium from 1G3 cells. Biotinylated anti-mouse IgG was used as a secondary antibody and was detected with fluorescein streptavidin (1:1000; Vector Laboratories, Burlingame, CA). M.O.M kit (Vector Laboratories) was used to block endogenous IgG in studies on mouse brain tissue. For CD31 staining, mouse CD31-specific IgG was used as a primary antibody, and Alexa Fluor 594 donkey anti-rat IgG (1:500; Invitrogen, Carlsbad, CA) was used as a secondary antibody. The anti-vWF primary antibody was detected with fluorescein goat anti-rabbit IgG (1:150; Invitrogen). Image analysis was performed using Olympus Optical (Tokyo, Japan) AX70 microscope equipped with the SPOT digital camera. Ten randomly selected fields in each region from 10 sections spanning the entire hemisphere from four mice per group or from Brodmann areas

9/10 in humans were analyzed as we reported previously (Deane et al., 2004).

Western blotting. The cerebrovascular mouse system was washed with 100 ml of ice-cold PBS by cardiac perfusion, and cerebral microvessels were isolated as described previously (Zlokovic et al., 1993). Protein homogenates (5–15 μ g) were separated under reducing conditions (FcRn) or nonreducing conditions (GFAP and F4/80), electroblotted in Tris-glycine buffer on nitrocellulose membrane, and probed with anti-FcRn (1:1), anti-GFAP (1:100), or F4/80 (1:100). The signal was detected by enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

125 I-A β 40–4G8 complex in brain. After 120 h of the intraperitoneal 4G8 (200 μ g) administration at 0 and 48 h, 120 nM 125 I-A β 40 was microinfused into brain ISF in the caudate nucleus. After 30 min, the brain was removed, homogenized in cold PBS containing complete proteinase inhibitor cocktail (Roche), and centrifuged at $18,000 \times g$ for 30 min at 4°C. The supernatant was concentrated by lyophilization and analyzed by 4–20% Tris-tricine native gel electrophoresis, followed by autoradiography.

A β immunotherapy and A β quantification. Twenty 4-month-old APP $^{sw+/+}$ mice were anesthetized as above (see above, Animals) and placed in a stereotactic apparatus. 4G8 was injected into the right hippocampus (ipsilateral) in mock CSF in a volume of 0.5 μ l over 5 min through a 33 gauge injector attached to a 10 μ l Hamilton syringe. As a negative control for 4G8, a rabbit anti-mouse IgG against divalent metal transporter-1, which does not interact with A β but has an intact Fc region to interact with the FcRn receptor at the BBB, was injected into the contralateral hippocampus in the same animal [nonimmune IgG (NI IgG)]. The coordinates with respect to bregma were -2.7 mm posterior, $+2.5$ mm lateral, and -3.0 mm ventral to the skull. The following test antibodies were injected: 4G8 (2 μ g/0.5 μ l); α FcRn (anti-FcRn antibody, 10 μ g/0.5 μ l) followed by 4G8 (2 μ g/0.5 μ l) 30 min after α FcRn; NI-IgG (2 μ g/0.5 μ l) followed by 4G8 (2 μ g/0.5 μ l) 30 min after NI-IgG; and 4G8 F(ab') $_2$ (2 μ g/0.5 μ l). Brains were analyzed after 24 h.

Brain sections were incubated with rabbit polyclonal anti-pan-A β (1:100; BioSource International, Camarillo, CA) overnight at 4°C, washed, and incubated in Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen) for 1 h at a room temperature. Photomicrographs of hippocampi were taken using an AX70 Olympus Optical microscope (Spectra Services, Ontario, NY). The percentage difference between ipsilateral and contralateral hippocampus was determined as $[(\text{number of pixels ipsilateral} - \text{number of pixels contralateral})/\text{number of pixels contralateral}] \times 100$ (Oddo et al., 2004).

A β 40 and A β 42 were quantified by a sandwich ELISA kit (Signet Laboratories) according to the instructions of the manufacturer. A β levels were expressed as the percentage change between the ipsilateral compared with the contralateral hippocampus using the following formula: $[\text{A}\beta \text{ (ng/section) in the ipsilateral hemisphere} - \text{A}\beta \text{ (ng/section) in the contralateral hemisphere}]/[\text{A}\beta \text{ (ng/section) in the contralateral hemisphere}] \times 100$.

For soluble brain A β oligomer analysis, A β was extracted from 10 brain sections from the site of 4G8 or vehicle injection in 200 μ l of TBS buffer containing complete protease inhibitor cocktail (Roche), and separated from the insoluble pellet by centrifugation at $20,000 \times g$ for 30 min. Antibody 6E10 (Signet Laboratories) was used as the capturing and detection antibody to determine relative levels of TBS-extracted soluble A β oligomers, by using a sandwich ELISA similar to those described previously with different capturing/detecting antibodies (LeVine, 2004; Yang et al., 2005). In this assay, soluble A β oligomers captured by 6E10 can only be detected by biotinylated 6E10 (detecting antibody), if there is at least one more accessible epitope for the detecting biotinylated 6E10 antibody on A β oligomers. For the detection of biotinylated 6E10 antibody, streptavidin–HRP (BioSource International) was incubated for 2 h at room temperature and visualized using TMB substrate (Kirkegaard & Perry Laboratories). This method did not detect monomeric A β but detected soluble A β 40 or A β 42 oligomers of different sizes prepared *in vitro*, as described previously (Kayed et al., 2003) (data not shown). 4G8, at the levels used in intracerebral clearance studies, did not interfere with the 6E10 sandwich ELISA (data not shown). The optical density readings reflecting the number of accessible epitopes on A β oligomers were nor-

malized per total TBS-soluble brain protein to estimate the relative levels of oligomers.

The thioflavin S-positive amyloid load (Wilcock et al., 2003) was determined using the Image-Pro-Plus program (Media Cybernetics, Silver Spring, MD).

Mouse endogenous brain A β . Mouse endogenous A β 40 and A β 42 levels were determined by sandwich ELISA (Best et al., 2005). Cerebral cortex was homogenized in 2% SDS containing complete protease inhibitor cocktail (Roche) (Kawarabayashi et al., 2001). For mouse A β 40-specific sandwich ELISA, the capturing and biotinylated detecting antibodies were mouse monoclonal mouse A β raised against amino acid residues 1–20 (AMB0062; BioSource International) and rabbit polyclonal anti-A β 40 biotin conjugate (44-3489; BioSource International), respectively. For mouse A β 42-specific sandwich ELISA, the capturing and detecting antibody were AMB0062 and rabbit polyclonal anti-A β 42 biotin conjugate (44-3449; BioSource International), respectively. Murine synthetic A β 40 and A β 42 standards (American Peptide, Sunnyvale, CA) were prepared in the ELISA buffer (PBS, 0.05% Tween 20, 0.25% BSA, 0.05% sodium azide, and complete protease inhibitor cocktail). The capturing antibody was coated at 5 μ g/ml overnight at 4°C in 50 mM carbonate buffer, pH 9.6, and 0.05% sodium azide on 96-well high-binding Stripwell immunoassay plates (Corning Life Sciences), blocked with 0.25% bovine serum albumin in PBS overnight at 4°C, and 100 μ l of sample or standard was added and incubated overnight at 4°C. A β levels were obtained from the standard curve.

Statistical analysis. The results were compared by multifactorial analysis of variance and Student's *t* test. The differences were considered to be significant at *p* < 0.05. All values are mean $\pm$ SEM.

Results

Figure 1A shows that 4G8, an A β -specific monoclonal IgG2b raised to 17–24 residues of A β , reduces by fivefold to sixfold influx of circulating 125 I-A β 40 across the BBB in APP^{sw+/−} (Tg2576) mice at 2–3 and 4–6 months of age. At a later stage, 15- to 20-month-old APP^{sw+/−} mice, the influx of circulating A β into brain ISF, although significantly higher than in controls, declines spontaneously. Still, 4G8 produced a significant inhibition of A β influx in 15- to 20-month-old and 24- to 36-month-old APP^{sw+/−} mice (Fig. 1A).

In the cerebral arterial inflow, 125 I-A β 40 alone remains >98.5% in its free monomeric form, as shown by the SDS-PAGE and HPLC analysis (Fig. 1B), as reported previously (Deane et al., 2003). However, in the presence of 4G8, A β forms stable complexes in plasma, as demonstrated by the SDS-PAGE and HPLC analysis within 30 min of the cerebral arterial infusion (Fig. 1C), which is longer than typical infusion times of 5–10 min used for influx calculations in Figure 1A. In the present study, the concentrations of 4G8 that block A β 40 BBB influx (Fig. 1A) were by two orders of magnitude lower than previously reported anti-A β IgG plasma levels in actively (Das et al., 2003) or passively (DeMattos et al., 2002) immunized mice. Lowering the 4G8/A β 40 plasma ratio from 80 to 1 resulted in comparable >95% inhibition of A β 40 brain uptake (Fig. 1D). A β 40–4G8 complexes labeled on either A β or 4G8 residues did not penetrate across the BBB, and their behavior was not influenced by an RAGE blocking antibody. In contrast, a significant transport of free, circulating A β was by >80% inhibited by the blockade of RAGE at the BBB (Fig. 1E), as reported previously (Deane et al., 2003; LaRue et al., 2004). These findings suggest that 4G8 effectively reduces RAGE-mediated increase in circulating A β influx across the BBB in APP^{sw+/−} mice by sequestering circulating A β in plasma.

Figure 2, A and B, shows time-dependent 4G8 increases in plasma and brain ISF, respectively, within 120 h of two subsequent intraperitoneal injections of 4G8 (200 μ g). By using a brain clearance method (Shibata et al., 2000), we showed that efflux of

Figure 1. A β -specific IgG (4G8) blocks BBB influx of circulating A β 40. **A**, A β 40 BBB influx in APP^{sw+/−} (Tg2576) mice in the presence (circles) and absence (triangles) of a fourfold excess of 4G8 determined with the brain perfusion method (LaRue et al., 2004) with 125 I-A β 40 at carrier concentrations corresponding to A β 40 plasma levels in Tg2576 mice at different ages (Kawarabayashi et al., 2001), i.e., 4 nM (2–3 and 4–6 months), 3 nM (15–20 months), and 1.5 nM (24–36 months), and in age-matched littermate controls (non-Tg) at physiological A β 40 plasma levels (50 pM) without 4G8. **B**, Tris-tricine SDS-PAGE (top) and HPLC (bottom) analysis of 125 I-A β 40 radioactivity in the arterial inflow without 4G8. **C**, Tris-tricine native PAGE (top) and HPLC (bottom) analysis of 125 I-A β 40 radioactivity in the arterial inflow with a fourfold excess of 4G8 or NI IgG. **D**, 125 I-A β 40 brain uptake at varying 4G8/A β 40 plasma ratio in 4- to 6-month-old Tg2576 mice expressed as the percentage of control uptake. **E**, Transport across the BBB of 125 I-A β 40 and A β 40–4G8 complexes labeled on either A β 40 or 4G8 in the absence and presence of RAGE-specific IgG F(ab')₂ in 4- to 6-month-old Tg2576 mice. Mean $\pm$ SEM; *n* = 3–6. **p* < 0.001; and ***p* < 0.05; NS, not significant.

centrally administered 125 I-A β 40 from mouse brain ISF determined over 30 min was moderately but significantly increased by 15% at 1 h of peripheral 4G8 intraperitoneal administration (Fig. 2C). Because the levels of 4G8 in brain ISF were barely detectable after 1 h of peripheral 4G8 injections (Fig. 2B) and intracerebrally administered 125 I-A β 40 does not form complexes with plasma-derived 4G8 after 1 h of peripheral 4G8 administration, as indicated by the autoradiographic analysis of brain aqueous extracts (Fig. 2E), an increase in brain to blood clearance of centrally administered A β at this early time point is likely to be mediated by peripheral action of 4G8. Thus, an increased 125 I-A β 40 efflux at 1 h (Fig. 2C) probably reflects the sink effect of 4G8, similar to that reported for other peripheral A β sequestering agents by different groups (DeMattos et al., 2002; Deane et al., 2003; Lemere et al., 2003). Namely, after 1 h, plasma levels of 4G8 are already high and $\sim$ 5 nM (Fig. 2A), which likely mops up completely the endogenous free plasma A β (50–100 pM), alters the A β equilibrium at the BBB in favor of A β efflux from brain. However, after 120 h of two subsequent 4G8 systemic injections, the BBB efflux of 125 I-A β 40 was substantially increased by 50% (Fig. 2D). Because at 120 h there was a significant increase in 4G8 levels in brain ISF (Fig. 2B), we hypothesized that enhanced clearance of centrally administered 125 I-A β 40 by peripherally administered 4G8 at 120 h (Fig. 2D) is not attributable to peripheral sink action (Fig. 2A). Indeed, the autoradiographic analysis of brain extracts indicated that intracerebrally administered 125 I-A β 40 forms within 30 min complexes with systemically administered 4G8 at 120 h of 4G8 administration (Fig. 2E). This result suggests that peripheral

Figure 2. Plasma and brain ISF distribution of systemically administered 4G8 and its effect on ^{125}I -A β 40 clearance from brain ISF. **A**, Plasma 4G8 levels determined with ELISA within 120 h in mice that received 4G8 (200 μg) intraperitoneally at 0 and 48 h. **B**, Brain ISF 4G8 levels in mice at 1 and 120 h of 4G8 treatment. **C**, **D**, Clearance of ^{125}I -A β 40 (40 nM) via BBB transport and ISF bulk flow within 30 min of ^{125}I -A β 40/ ^{14}C -inulin microinjections into brain ISF in mice at 1 h (**C**) and 120 h (**D**) of 4G8 treatment as in **A**. **E**, Formation of ^{125}I -A β 40–4G8 complexes in mouse brain ISF determined by Tris-tricine native PAGE of the aqueous brain extracts 30 min after ^{125}I -A β 40 (120 nM) microinfusion into brain ISF and after either 1 h (left) or 120 h (right) of 4G8 treatment as in **A**. Mean $\pm$ SEM; $n = 3$ –5. * $p < 0.001$ and ** $p < 0.05$.

Figure 3. FcRn-mediated BBB transcytosis of ^{125}I -4G8, ^{125}I -A β 40–4G8, and ^{125}I -A β 42–4G8 from brain to blood. **A**, The BBB clearance of 40 nM ^{125}I -4G8, ^{125}I -A β 40–4G8, or ^{125}I -A β 42–4G8 within 30 min of brain ISF microinjections with and without unlabeled 4G8 (2 μM), RAP (5 μM), fucoidan (1.5 μM), αFcRn (anti-FcRn, 60 $\mu\text{g}/\text{ml}$), and A β 40 (1 μM) and in $\text{FcRn}^{-/-}$, $\beta_2\text{M}^{-/-}$, $\text{RAP}^{-/-}$, and $\text{FcR}\gamma^{-/-}$ mice. **B**, **C**, Serum levels of ^{125}I -4G8 (**B**) and of ^{125}I -A β 40–4G8 (**C**) from experiments in **A** expressed as the percentage of injected dose (%ID). Western blot analysis of FcRn in brain microvessels in control and $\beta_2\text{M}^{-/-}$ mice (inset). **D**, FcRn/CD31 double immunostaining in brains of nontransgenic mice (left) Scale bar, 50 μm . Western blot analysis (right) of FcRn, F4/80 (phagocytic cells), and GFAP (astrocytes) in isolated brain microvessels (lane 1) and capillary-depleted brain (lane 2). **E**, Uptake of ^{125}I -4G8, ^{125}I -4G8–A β 40, ^{125}I -A β 40–4G8, or ^{125}I -A β 40–4G8–F(ab')₂ (1 nM) at the abluminal side of isolated mouse brain microvessels within 1 min at 37°C. 4G8 (5 μM), RAP (5 μM), Fc (10 μM), and αLRP (20 $\mu\text{g}/\text{ml}$) were applied as potential inhibitors. Mean $\pm$ SEM; $n = 3$ –5. * $p < 0.05$, ** $p < 0.001$, inhibitors or gene deletion versus the corresponding controls; * $p < 0.01$, 125I-A β 40–4G8 versus 125I-4G8; NS, not significant.

4G8 may enhance clearance of brain-borne A β after its crossing into brain ISF. ^{125}I -A β 40 clearance via the ISF bulk flow was not altered by 4G8.

To test whether A β complexed to an A β -specific IgG is cleared from brain, we measured the BBB efflux of centrally administered 4G8 alone and of A β 40–4G8 and A β 42–4G8 complexes in wild-type 2- to 3-month-old mice, using our clearance method (Shibata et al., 2000). The BBB clearance of ^{125}I -4G8 was inhibited by the increasing concentrations of centrally administered unlabeled 4G8 (data not shown) and abolished by 2 μM 4G8 (Fig. 3A), suggesting a concentration-dependent IgG transcytosis at the BBB. RAP, which inhibits A β BBB clearance via LRP (Shibata et

al., 2000; Deane et al., 2004), or fucoidan, an inhibitor of the scavenger receptor on microglia, did not affect 4G8 clearance (Fig. 3A). Immunostaining for activated microglia was negative in the present model (data not shown), as reported previously (Cirrito et al., 2003; Deane et al., 2004).

Because IgG is carried across biological membranes by the FcRn (Roopenian et al., 2003; Ober et al., 2004; Yoshida et al., 2004) and FcRn is expressed in vascular endothelium and at the BBB (Borvak et al., 1998; Schlachetzki et al., 2002), we explored whether deletions of the *FcRn* gene and the FcRn light chain $\beta_2\text{M}$ gene affect ^{125}I -4G8 BBB efflux. Figure 3A shows significantly reduced 4G8 clearance in *FcRn* $^{-/-}$ mice and $\beta_2\text{M}$ $^{-/-}$ mice by 68 and 63%, respectively, whereas deletions of the *RAP* gene (functional LRP knock-out) (Deane et al., 2004) or the *FcR* γ gene were without effect. Excess of 4G8 in brain ISF in *FcRn* $^{-/-}$ mice resulted in complete inhibition of ^{125}I -4G8 BBB efflux (data not shown), consistent with the data demonstrating that unlabeled 4G8 inhibits ^{125}I -4G8 clearance at the BBB in wild-type mice (Fig. 3A). After central administration of ^{125}I -4G8, the radioactivity appearing in plasma was readily detectable and 100% TCA precipitable, suggesting transcytosis of intact ^{125}I -4G8 from brain into blood (Fig. 3B), as confirmed by the autoradiography (data not shown). ^{125}I -4G8 serum levels were significantly reduced (>90%) by central administration of unlabeled excess 4G8 and in *FcRn* $^{-/-}$ mice (70%) (Fig. 3B), indicating that the FcRn pathway is required for clearance of an anti-A β IgG across the BBB.

Next, we showed that ^{125}I -A β 40–4G8 was cleared across the BBB of 2- to 3-month-old wild-type mice at a rate 2.5-fold faster than ^{125}I -4G8 (Fig. 3A). Unlike 4G8, clearance of A β 40–4G8 complexes was inhibited by central administration of either unlabeled 4G8 or RAP and was abolished by the combination of both, suggesting that in young mice ^{125}I -A β 40–4G8 complexes can be cleared by an IgG-dependent mechanism and/or via a RAP-sensitive LRP mechanism (Shibata et al., 2000; Deane et al., 2004). That both the IgG-assisted and LRP-dependent transport are important for clearance of A β 40–4G8 complexes in young mice has been confirmed by demonstrating ~60% reductions in ^{125}I -A β 40–4G8 BBB efflux in $\beta_2\text{M}$ $^{-/-}$ mice and $\text{RAP}^{-/-}$ mice (Fig. 3A). After central administration of ^{125}I -A β –4G8 complexes, the radioactivity appearing in plasma was 100% TCA precipitable (Fig. 3C), suggesting no degradation of ^{125}I -A β 40 from the complex, as well as transcytosis of intact complexes, which has been confirmed by the autoradiography (data not shown). Serum levels of centrally infused ^{125}I -A β 40–4G8 complexes were substantially

Figure 4. FcRn-dependent ^{125}I -A β 40–4G8 BBB clearance in old $\text{APP}_{\text{sw}}^{+/-}$ mice and FcRn BBB expression in AD. **A**, ^{125}I -A β 40 (40 nM) BBB and ISF clearance in 18- to 20-month-old $\text{APP}_{\text{sw}}^{+/-}$ (Tg2576) mice 30 min after brain ISF microinjections and 120 h after vehicle or 4G8 intraperitoneal treatment, as in Figure 2A. **B**, The BBB clearance of ^{125}I -A β 40 or ^{125}I -A β 40–4G8 complexes (40 nM) in 20- to 23-month-old Tg2576 mice within 30 min of brain ISF microinjections. **C**, ^{125}I -A β 40–4G8 (1 nM) uptake by isolated cerebral microvessels of Tg2576 mice at 3–5 and 20–23 months of age determined within 1 min at 37°C without and with Fc fragment (10 μM) and RAP (5 μM). **D**, FcRn colocalization with vWF (endothelial intracellular marker) in the brain of 10-month-old Tg2576 mice studied by confocal microscopy. **E**, Western blot analysis of FcRn in brain microvessels of control and Tg2576 mice at 5–7 and 20–23 months of age (top) and its relative abundance by scanning densitometry corrected for β -actin (bottom). **F**, FcRn and vWF double immunostaining in brains of AD and age-matched individuals (left) and FcRn-positive vascular profiles (right). Scale bar, 50 μm . Mean $\pm$ SEM; $n = 3$ –6.

reduced by centrally administered unlabeled 4G8 and/or RAP, as well as by deletion of the $\beta_2\text{M}$ gene and RAP gene (Fig. 3C). We also demonstrated that A β 42–4G8 complexes are cleared by the FcRn pathway (Fig. 3A), as well as A β 42 bound to anti-A β 1560 (data not shown).

By using isolated cerebral microvessels positive for FcRn and negative for microglia and astrocytic markers (Fig. 3D), we corroborated our *in vivo* findings by showing that ^{125}I -4G8 rapid brain capillary uptake at 37°C, which reflects its clearance by brain capillaries, in 2- to 3-month-old mice was dose dependently (data not shown) and completely blocked by excess unlabeled 4G8 (Fig. 3E) but was not affected by RAP. Under the present experimental conditions, uptake of ^{125}I -radiolabeled 4G8 and its complexes with A β occurred at the abluminal side of brain capillaries because short, 1 min exposure times precluded a significant diffusion of the studied tracers into the capillary lumen, which is required for the uptake at the luminal side (Deane et al., 2004). This has been confirmed by a negligible uptake of ^{14}C -inulin, which does not interact with the cells and is taken up by the isolated capillaries only via passive diffusion into the lumen. Regarding ^{125}I -labeled 4G8–A β 40 complexes (with the label on either 4G8 or A β 40 residues), both the unlabeled Fc fragment and RAP or an LRP-specific IgG blocked clearance of complexes on brain capillaries from 2- to 3-month-old young mice (Fig. 3E). As expected, A β 40–4G8 F(ab') $_2$ complexes lacking the Fc domain were cleared by an Fc-independent RAP-sensitive mechanism, confirming the involvement of LRP, as shown for A β -mediated clearance (Deane et al., 2004). That A β molecule in anti-A β –A β complexes is critical for interaction with LRP was shown by inhibition of brain capillary clearance of ^{125}I -labeled 4G8–A β 40 complexes (the label was on 4G8) by excess of unlabeled A β 40 (data not shown).

Figure 4A shows that 4G8 systemic administration over 120 h increases by approximately threefold efflux of centrally administered ^{125}I -A β 40 in 18- to 20-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice (Fig. 4A). To test whether old $\text{APP}_{\text{sw}}^{+/-}$ mice clear A β from brain

through an IgG-assisted efflux, we compared clearance of A β 40 alone and A β 40–4G8 complexes. Consistent with a significant downregulation of LRP at the BBB with aging, and particularly in transgenic $\text{APP}_{\text{sw}}^{+/-}$ mice (Deane et al., 2004), the BBB efflux of A β 40 alone was significantly reduced in old $\text{APP}_{\text{sw}}^{+/-}$ mice (Fig. 4B) compared with control young mice (Fig. 2C,D) or young $\text{APP}_{\text{sw}}^{+/-}$ mice (data not shown). However, central infusion of ^{125}I -A β 40–4G8 complexes in 20- to 23-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice resulted in an approximately fivefold greater clearance than of ^{125}I -A β 40 alone (Fig. 4B), suggesting that IgG-assisted efflux of A β remains active in old $\text{APP}_{\text{sw}}^{+/-}$ mice and is even somewhat higher than in young control wild-type mice (Fig. 3A).

In a next set of studies, we showed that both RAP and Fc fragment effectively block clearance of 4G8–A β complexes on isolated cerebral microvessels derived from younger, 3- to 5-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice (Fig. 4C). In contrast, RAP did not block clearance of 4G8–A β complexes on brain capillaries derived from 20- to 23-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice (Fig. 4C). The loss of inhibitory effect of RAP on capillary clearance in aged $\text{APP}_{\text{sw}}^{+/-}$ mice is consistent with a significant loss of LRP expression in these animals at an older age (Deane et al., 2004). Conversely, the Fc fragment inhibited significantly by 67% clearance of complexes on aged $\text{APP}_{\text{sw}}^{+/-}$ mouse brain capillaries (Fig. 4C), confirming that an IgG-dependent clearance of IgG–A β complexes remains functional with aging in $\text{APP}_{\text{sw}}^{+/-}$ mice. These results are consistent with a significant FcRn expression in brain endothelium in older $\text{APP}_{\text{sw}}^{+/-}$ mice and an age-dependent increase in the FcRn levels in brain capillaries of $\text{APP}_{\text{sw}}^{+/-}$ mice (Fig. 4D,E). We also observed an age-dependent increase in the FcRn levels in control mice, although the levels of FcRn in $\text{APP}_{\text{sw}}^{+/-}$ mice were substantially higher than in their corresponding age-matched littermate controls, at both 5–7 and 20–23 months of age, respectively (Fig. 4E). Studies on human tissue indicated an increase in FcRn-positive brain vascular profiles in AD compared with age-matched controls (Fig. 4F).

Next, we determined whether inhibition of the FcRn pathway affects intracerebral 4G8 immunotherapy in 24-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice. We limited studies to 24 h to minimize possible influence of the antibody on microglia-dependent clearance, because microglia are typically activated in older $\text{APP}_{\text{sw}}^{+/-}$ mice after 24 h of an anti-A β antibody intracerebral injection (Wilcock et al., 2003), as confirmed in this study (data not shown). Our data corroborate findings by the previous studies (Wilcock et al., 2003; Oddo et al., 2004) by showing that centrally administered 2 μg of 4G8 significantly reduces A β immunostaining and the levels of human endogenous A β 40 and A β 42 in the hippocampus of 24-month-old $\text{APP}_{\text{sw}}^{+/-}$ mice by 45, 47, and 25%, respectively (Fig. 5A,D–F). These results correlated well with the observed >35% reductions in the levels of TBS-insoluble/SDS-soluble human endogenous A β 40 and A β 42 by 4G8. It is noteworthy that the levels of TBS-soluble human A β oligomers were also reduced by 25% by 4G8, as demonstrated by an ELISA developed previously for determining the relative levels of A β oligomers (Levine, 2004; Yang et al., 2005) (Fig. 5G). However, 4G8 did not affect thioflavin S-positive amyloid load (Fig. 5H), consistent with our finding that 4G8 did not affect the levels of SDS-resistant/formic acid-soluble A β 40 and A β 42, using methods described previously (Kawarabayashi et al., 2001). This result corroborates findings by a previous study demonstrating that, within 24 h of intracerebral delivery, the A β -specific antibody does not reduce thioflavin S staining (Wilcock et al., 2003). Although 4G8 binds amyloid plaques on tissue sections *ex vivo*, its binding to plaques

Figure 5. FcRn-dependent clearance of endogenous A β in *APPsw*^{+/−} mice by centrally administered 4G8. **A**, Intrahippocampal 4G8 (2 μg/0.5 μl) reduces A β immunostaining 24 h after injection in 24-month-old Tg2576 mouse (top; ipsi) compared with nonimmune IgG-treated contralateral hippocampus (bottom; contra). **B**, 4G8 (2 μg/0.5 μl) was injected into the ipsilateral hippocampus in 24-month-old Tg2576 mouse 30 min after αFcRn (anti-FcRn antibody; 10 μg/0.5 μl) (top) or vehicle (bottom; contralateral hippocampus). **C**, 4G8 F(ab')₂ (2 μg/0.5 μl) injected into the hippocampus in 24-month-old Tg2576 mouse (top) versus vehicle-treated contralateral hippocampus (bottom). Scale bar, 350 μm. **D–F**, A β load (**D**), A β 40 (**E**), and A β 42 (**F**) levels: the percentage change (%) in the ipsilateral versus contralateral hippocampus in Tg2576 mice. **p* < 0.05. **G**, TBS-soluble A β oligomers (**G**) and thioflavin S-positive amyloid load (**H**) in 4G8-treated (ipsilateral) versus vehicle-treated (contralateral) hippocampus in 24-month-old Tg2576 mice at 24 h. Mean ± SEM; *n* = 5 mice per group and 6–8 sections close to the injection site per mouse. **I**, ¹²⁵I-4G8 F(ab')₂ (40 nm) and ¹⁴C-inulin brain retention 30 min after brain ISF microinjection of the tracer mixture. Mean ± SEM; *n* = 3. NS, Not significant.

after intracerebral *in vivo* administration was minimal under the present experimental conditions (data not shown).

In contrast to vehicle-treated *APPsw*^{+/−} mice, pretreatment of *APPsw*^{+/−} mice with intracerebral infusions of FcRn-specific blocking antibodies 30 min before administration of 4G8 completely abolished A β lowering effects of centrally administered 4G8 immunotherapy (Fig. 5*B,D–G*). A nonimmune IgG did not have any effect on A β clearance (Fig. 5*A–C*, bottom). Similarly, within 24 h, we failed to demonstrate significant reductions in A β load and A β 40 and A β 42 levels by 4G8 F(ab')₂ in 24-month-old *APPsw*^{+/−} mice (Fig. 5*C–F*). Our clearance study with radiolabeled ¹²⁵I-4G8 F(ab')₂ failed to show clearance of Fab fragments across the BBB (Fig. 5*I*), in contrast to a rapid removal of the full length 4G8 (Fig. 3*A*).

To determine whether the FcRn pathway is critical for removal of endogenous brain A β by systemically administered 4G8, we compared the effects of peripheral 4G8 immunotherapy (administered as in Fig. 2) on levels of endogenous mouse brain A β 40 and A β 42 in *FcRn*^{+/+} mice and *FcRn*^{−/−} mice. To minimize a possible confounding effect of LRP-mediated clearance, we performed these studies in 9-month-old mice, at an age when LRP expression at the BBB is already substantially reduced in mice (Shibata et al., 2000; Deane et al., 2004), as confirmed in this study (data not shown). By using an ELISA similar to that reported recently (Best et al., 2005), we showed that treatment of

Figure 6. FcRn-dependent clearance of mouse endogenous brain A β by 4G8 systemic administration: studies in *FcRn*^{+/+} and *FcRn*^{−/−} mice. Mice were treated for 5 d with 4G8 (open bar), 200 μg intraperitoneally at 0 and 48 h, or vehicle (filled bar), and the levels of endogenous mouse A β 40 (**A**) and A β 42 (**B**) determined in the cerebral cortex in *FcRn*^{+/+} mice and *FcRn*^{−/−} mice. Mean ± SEM; *n* = 5. NS, Not significant.

FcRn^{+/+} wild-type mice with systemically administered 4G8 results in significant 35 and 40% reductions in brain endogenous mouse A β 40 and A β 42 levels, respectively (Fig. 6*A,B*). In contrast, there was a negligible 5–7% reduction in endogenous A β 40 and A β 42 brain levels in *FcRn*^{−/−} mice (Fig. 6*A,B*). A reduction in endogenous mouse brain A β levels by systemic 4G8 in *FcRn*^{+/+} mice compared with *FcRn*^{−/−} mice was associated with a corresponding significant increase in plasma A β , which was >97% G-protein precipitable (G-protein binds the Fc fragment of 4G8) (data not shown), indicating that the majority of A β in plasma after systemic therapy with the antibody is bound to 4G8, similarly as shown by previously passive and active immunization studies (DeMattos et al., 2001, 2002; Lemere et al., 2003).

Discussion

Our study shows that an anti-A β antibody regulates brain A β by exerting important peripheral and central actions on A β transport across the BBB. The mechanism of peripheral A β sequestration observed in the present study with 4G8 in *APPsw*^{+/−} mice may be similar to that of other A β binding agents, i.e., apolipoproteins E2 and 3 (Martel et al., 1997), ganglioside M and gelsolin (Matsuoka et al., 2003), the soluble forms of RAGE (Deane et al., 2003) or LRP (Deane et al., 2004), and/or other antibodies to A β that reduce its influx across the BBB (Pan et al., 2002; Banks et al., 2005). This peripheral binding of A β may create a sink effect, providing that the activity of the LRP efflux pathway (Deane et al., 2004) is not lost or is sufficient to maintain elimination of brain A β and A β –IgG complexes. The central action of certain anti-A β IgGs may, however, critically depend on sequestering brain A β , followed by the FcRn-mediated rapid BBB clearance. Importantly, the FcRn IgG-dependent clearance for A β remains active at the BBB during normal aging and in *APPsw*^{+/−} mice, and increased FcRn expression at the BBB in AD may likely be of a therapeutic value for A β immunotherapy.

Circulating IgG may enter the brain and have widespread extracellular distribution within the CNS and CSF from sites deficient in a BBB such as subarachnoid space pial surface in the Virchow-Robin spaces and subpial cortical gray matter and circumventricular organs (Balin et al., 1986; Kozlowski et al., 1990; Broadwell and Sofroniew, 1993). It has been reported that an antibody directed at 1–17 region of A β can use the extracellular pathways for its passive entry into the CNS (Banks et al., 2002). In addition, some IgG species are also transported slowly across the BBB via adsorptive-mediated transcytosis (Zlokovic et al., 1990; Pardridge, 1991). Our results indicate that systemically administered 4G8 exerts a significant clearance effect on endogenous brain A β that is abolished in *FcRn*^{−/−} mice at an age when LRP expression is substantially downregulated (Deane et al., 2004). Although 4G8 recognizes APP in addition to A β , it is unlikely that the A β -lowering effect of systemically administered 4G8 is attrib-

utable to inhibition of APP/A β production because this effect is lost in *FcRn*^{-/-} compared with *FcRn*^{+/-} mice. Inhibition of the FcRn pathway influences also the outcome of centrally administered A β immunotherapy in old *APP*^{sw^{+/-} mice. Although intracerebral immunotherapy has limitations regarding direct insight into what happens with peripheral passive or active approaches, studies using the intracerebral approach have been useful in delineating clearance pathways of A β from brain (Tanzi et al., 2004; Zlokovic, 2005) and have been used extensively to demonstrate that antibody in the brain exerts a clearance action on brain A β (Bacskai et al., 2001, 2002; Wilcock et al., 2003; Oddo et al., 2004). For example, it has been shown that intracerebral delivery of 2 μ g of 4G8 or anti-A β 1560 (Oddo et al., 2004) and 2 μ g of 6E10 (Wilcock et al., 2003), substantially reduces A β load in 12-month-old triple-transgenic AD mice and in 20-month-old *APP*^{sw^{+/-} mice, respectively. Our data suggest that the FcRn pathway is required for clearance of TBS-insoluble/SDS-soluble endogenous A β 40 and A β 42, which possibly represents A β in diffuse plaques that is not yet converted into a hard-core thioflavin S-positive amyloid. Although we have not demonstrated FcRn-mediated clearance of thioflavin-S-positive amyloid, it is possible that, over longer periods of time (>24 h), centrally administered 4G8 will reduce thioflavin S-positive load from brain (Wilcock et al., 2003; Oddo et al., 2004), which, at least in part, could involve the FcRn pathway.}}

Topical administration of Fab fragments alone can reduce A β immunostaining in *APP*^{sw^{+/-} mice within 3 d (Bacskai et al., 2002). Our data show that this effect could be mediated via LRP efflux system, which can clear A β 40–4G8 F(ab')₂ complexes. In contrast, Fab fragments alone are eliminated slowly from brain ISF at a rate comparable with that of a reference marker, inulin. However, we also demonstrated that 4G8 F(ab')₂ cannot exert a vigorous effect on A β pathology within 24 h in 24-month-old *APP*^{sw^{+/-} mice, which could be explained by substantial down-regulation of LRP levels at the BBB in old *APP*^{sw^{+/-} mice (Deane et al., 2004).}}}

FcRn plays a central role in delivering IgGs within and across the cells (Ober et al., 2004). Although FcRn was discovered as a developmental IgG receptor, recent studies suggest its expression in different cell types in adult rodents and humans, including polarized epithelia and endothelia, which are able to translocate the FcRn-bound cargo bidirectionally, from either the lumen or tissue spaces into the opposite pole of the cell (Roopenian et al., 2003; Yoshida et al., 2004). FcRn is directly involved in IgG exocytosis (Ober et al., 2004) and binds IgG best at an intracellular pH 6.0–6.5 (Roopenian et al., 2003). It is expressed at low levels in the cell membrane because of its rapid recycling after incomplete fusion with the plasma membrane, known as “kiss and run” (Ober et al., 2004). It is likely that the FcRn-mediated transcytosis of IgG across the BBB may require an additional binding step to another IgG receptor at the abluminal cell membrane, which will facilitate IgG endocytosis before its FcRn-mediated migration to the exocytotic site at the luminal membrane, analogous to the IgG transport across other biological membranes (Roopenian et al., 2003). This hypothesis is supported by our work in progress showing a single 55 kDa band (data not shown) after biotinylation of the abluminal mouse brain capillary membranes and 4G8 affinity purification. This band corresponded to a recently described IgG cell surface receptor in the placental endothelium (Gafencu et al., 2003). The exact cellular mechanisms involved in possible interaction between a putative 55 kDa IgG receptor and FcRn during IgG and A β -anti-A β complex internalization at the

abluminal side of the BBB and exocytosis across the luminal side remain, however, to be investigated.

Based on the present work, we propose two possibilities for enhanced efficacy of immunization therapy directed at an improved clearance of A β across the BBB: (1) to increase a peripheral sink of an anti-A β antibody and facilitate brain efflux of A β and A β -IgG complexes by increasing the activity of the BBB LRP efflux pathway at an older age; and (2) to optimize the FcRn pathway for enhanced IgG-mediated A β clearance across the BBB in the aging brain.

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