BRI2 (*ITM2b*) Inhibits A β Deposition *In Vivo*

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Analyses of the biologic effects of mutations in the BRI2 (*ITM2b*) and the amyloid β precursor protein (*APP*) genes support the hypothesis that cerebral accumulation of amyloidogenic peptides in familial British and familial Danish dementias and Alzheimer's disease (AD) is associated with neurodegeneration. We have used somatic brain transgenic technology to express the BRI2 and BRI2-A β 1–40 transgenes in APP mouse models. Expression of BRI2-A β 1–40 mimics the suppressive effect previously observed using conventional transgenic methods, further validating the somatic brain transgenic methodology. Unexpectedly, we also find that expression of wild-type human BRI2 reduces cerebral A β deposition in an AD mouse model. Additional data indicate that the 23 aa peptide, Bri23, released from BRI2 by normal processing, is present in human CSF, inhibits A β aggregation *in vitro* and mediates its anti-amyloidogenic effect *in vivo*. These studies demonstrate that BRI2 is a novel mediator of A β deposition *in vivo*.

Key words: BRI2; ITM2b; amyloid β protein; Alzheimer's disease; somatic brain transgenesis; adeno-associated virus

Introduction

Familial British and Danish dementias (FBD and FDD, respectively) are neurodegenerative dementias pathologically characterized by parenchymal preamyloid and amyloid deposits, cerebral amyloid angiopathy (CAA), neuronal loss, and neurofibrillary tangles (Ghisso et al., 2006). Two mutations in the *ITM2b* gene encoding the BRI2 protein have been identified as the cause of FBD and FDD. BRI2 is a 266-aa-long type 2 transmembrane protein of unknown function. It is expressed at high levels in the brain and cleaved by furin or furin-like proteases at its C terminus to produce a 23 aa peptide (Bri2–23) (Kim et al., 1999; Choi et al., 2004) (Fig. 1*B*). Disease-causing mutations result in the production of C-terminally extended 277 aa mutant BRI2 proteins, which are cleaved at the normal furin processing site to generate distinct 34 aa peptides (ABri in FBD and ADan in FDD) that accumulate in the brains of affected patients (Vidal et al., 1999, 2000). Notably, synthetic ABri and ADan undergo rapid aggregation and fibrillization into amyloid, and they are neurotoxic (Gibson et al., 2005; Ghiso et al., 2006). Thus, there are clear

pathological and clinical similarities between FBD, FDD, and Alzheimer's disease (AD). Indeed, genetic analyses of FBD, FDD, and familial forms of AD support a unifying pathologic mechanism in which accumulation of amyloidogenic peptides triggers a complex pathological cascade leading to neurodegeneration (Golde, 2003).

Our interest in the BRI2 protein developed in the course of studies using BRI2-A β fusion proteins to express individual A β peptides (McGowan et al., 2005; Kim et al., 2007). By crossing BRI2-A β 1–40 or BRI-A β 1–42 transgenic mice with Tg2576 mice, we previously demonstrated that A β 1–40 and A β 42 have opposing effects on amyloid deposition (McGowan et al., 2005; Kim et al., 2007). To study the anti-amyloidogenic effect of A β 1–40 in another amyloid β precursor protein (APP) mouse model, we used recombinant adeno-associated virus 1 (rAAV1)-mediated gene transfer to deliver the BRI2-A β 1–40 and BRI2 transgenes to the brains of postnatal day 0 (P0) TgCRND8 hAPP_{KM670/671NL+V717F} APP mice (Levites et al., 2006b). This methodology of gene transfer, which we have termed somatic brain transgenesis, leads to consistent widespread and permanent expression of the transgene in forebrain and hippocampal neurons (Fig. 1*A*) and enables one to rapidly and cost-effectively evaluate the effects of transgene expression on the amyloid deposition phenotype (Levites et al., 2006b).

Materials and Methods

rAAV1 construction and preparation. rAAV1 expressing BRI2, BRI2-A β 1–40, BRI2del244–266, nonspecific single-chain variable fragment (scFv ns), or enhanced green fluorescent protein (eGFP), under the control of the cytomegalovirus enhancer/chicken β actin (CBA) promoter were generated by calcium-phosphate transfection of pAM/CBA-pI-WPRE-BGH, rAAV1 *cis*-plasmid pH21 (AAV1 helper plasmid), and p Δ 6 into a HEK293 cell line. rAAV1-scFv ns construct was reported previously (Levites et al., 2006b). At 48 h after transfection, cells were

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Figure 1. BRI2 expression inhibits A β deposition *in vivo*. **A**, Somatic brain transgenesis results in consistent transduction of forebrain and hippocampal neurons. rAAV1-EGFP was delivered into cerebral ventricles of newborn mice (4×10^9 genomes per mouse), and widespread EGFP expression was detected by immunohistochemistry with an anti-GFP antibody at ~ 1 month of age. The top panel is a saline-injected control, and the next six panels are representative of three separate somatic brain transgenic experiments performed on four different litters using two different rAAV1-EGFP preparations. Note that the forebrain and hippocampus are routinely transduced, whereas hindbrain structures (e.g., cerebellum) are more variably transduced. **B**, Schematic of BRI2-fusion constructs. BRI2 and BRI2-A β 1-40 are cleaved by furin and other kex2 proteases to release Bri2-23 and A β 1-40, respectively. **C**, P0 TgCRND8 mice were transduced by the intracerebroventricular injection of rAAV1-BRI2 ($n = 8$) or BRI2-A β 1-40 ($n = 11$). Total brain A β levels (pooled values of the SDS-soluble and SDS-insoluble FA extracts) from 3-month-old mice were analyzed by A β end-specific ELISA. Control refers to both noninjected and PBS-injected mice ($n = 8$). **D**, Cortical sections of 3-month-old mice were immunostained with anti-A β 1-16 antibody 33.1.1. Sections representing the mice with the median levels of biochemical A β deposition are shown. CAA was not increased by the BRI transgenes and was almost completely absent in the TgCRND8 mice at 3 months of age. Magnification, $200\times$. **E**, Amyloid plaque burdens and thioflavin (Thio S)-positive plaques were quantified from the stitched images of whole cerebral cortex. Cored plaques, identified by Thio S staining, were counted individually. $**p < 0.01$ versus no injection control (ANOVA).

lysed in the presence of 0.5% sodium deoxycholate and 50 U/ml benzodase (Sigma) by repeated rounds of freeze/thaws at -80°C and -20°C . The virus was isolated using a discontinuous Iodixanol gradient and then affinity purified on a HiTrap HQ column (GE Healthcare). Samples were eluted from the column and buffer exchanged to PBS using an Amicon Ultra 100 Centrifugation device (Millipore). The genomic titer of each virus was determined by quantitative PCR using the ABI 7900 (Applied Biosystems). The viral DNA samples were prepared by treating the virus with DNase I (Invitrogen), heat inactivating the enzyme, and then digesting the protein coat with Proteinase K (Invitrogen), followed by a second heat inactivation. Samples were compared against a standard curve of supercoiled plasmid.

rAAV1 injection to neonatal mice. TgCRND8 mice expressing mutant human APP (KM670/671NL and V717F) gene under the control of hamster prion promoter were reported previously (Chishti et al., 2001). Hemizygous male TgCRND8 mice were crossed with female B6C3F1 wild-type mice. Tg2576 mice expressing mutant human APP (KM670/671NL) gene under the control of hamster prion promoter were reported previously (Hsiao et al., 1996). Hemizygous female Tg2576 mice were mated with male B6SJL wild-type mice. The injection procedures were performed as described previously (Passini et al., 2003; Broekman et al.,

2006; Levites et al., 2006b). Briefly, P0 pups were cryoanesthetized on ice for 5 min. Two microliters of AAV1 construct (1×10^{12} genome particles/ml) were bilaterally injected into the cerebral ventricle of newborn mice using a 10 ml Hamilton syringe with a 30 gauge needle. The pups were placed on a heating pad until they recovered from cryoanesthesia and then returned to their mother for further recovery. Negative control groups (total $n = 20$) were noninjection ($n = 4$), PBS injection ($n = 4$), eGFP ($n = 5$), and nonspecific scFv ($n = 7$) groups. Experimental groups were BRI2-A β 1-40 ($n = 11$), BRI2 ($n = 8$), and BRI2del244-266 ($n = 13$). Biochemical and histochemical A β loads in the control groups were equivalent. All animal procedures were approved by Mayo Clinic Institutional Animal Care and Use Committee in accordance with National Institutes of Health (NIH) guidelines.

Quantification of amyloid deposition. Hemibrains were immersion fixed in 10% formalin then processed for paraffin embedding. Brain tissue sections ($5 \mu\text{m}$) were immunostained with the anti-total A β antibody [33.1.1; 1:1000 (Levites et al., 2006a)] on a DAKO autostainer. The cortical A β plaque burden and the number of Thio S-positive plaques were quantified as previously reported (Kim et al., 2007). Three to six sagittal sections per brain, $50 \mu\text{m}$ apart, were analyzed.

A β sandwich ELISA. For brain A β ELISAs from TgCRND8 mice,

hemi-forebrains were homogenized in 2% SDS with 1 $\times$ protease inhibitor mixture (Roche) dissolved in H₂O and then ultracentrifuged at 100,000 $\times$ g for 1 h. The SDS-insoluble A β peptides were extracted using 70% formic acid (FA). For brain A β ELISAs from 2-month-old Tg2576 mice, hemi-forebrains were homogenized in radioimmunoprecipitation assay buffer (0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl) and then ultracentrifuged at 100,000 $\times$ g for 1 h. To measure the endogenous mouse A β levels, hemi-forebrains of nontransgenic littermates of the TgCRND8 mice expressing BRI2 were homogenized in 0.2% diethylamine buffer containing 50 mM NaCl and 1 $\times$ protease inhibitor mixture (Roche). Endogenous mouse A β levels were measured using the previously validated rodent-specific A β ELISA system as previously reported (Eckman et al., 2006). For plasma A β analysis, blood was collected in EDTA-coated tubes after cardiac puncture. Blood samples were centrifuged at 3000 rpm for 10 min at 4°C, and then the plasma was aliquoted and stored at –80°C until used. A β levels were determined by human A β end-specific sandwich ELISAs as previously described (Kim et al., 2007).

Mouse anti-A β IgG ELISA. To test whether mice generate anti-A β antibody responses, anti-A β IgG antibody titers were determined by standard ELISA techniques, as described previously (Das et al., 2001). Briefly, microtiter plates (Maxi Sorp; Dynatech) were coated with aggregated A β 42 at 2 μ g/well. After washings, serial dilutions of plasma (1:500 dilution) were added and incubated overnight at 4°C. After washes with PBS/0.1% Tween 20, plasma IgG was detected using an anti-mouse IgG antibody conjugated with HRP (1:2000; Sigma) and TMB substrate (KPL).

Western blotting. Snap-frozen forebrain samples were homogenized in 2% SDS buffer with 1 $\times$ protease inhibitor mixture (Roche). The homogenate was centrifuged at 100,000 $\times$ g for 1 h at 4°C. Protein concentration in supernatants was determined using the BCA Protein Assay kit (Pierce). Protein samples (20 μ g) were run on Bis-Tris 12% XT gels (Bio-Rad) with XT-MES buffer or Bis-Tris 4–12% XT gels (Bio-Rad) with XT-MOPS buffer and transferred to 0.2 μ m nitrocellulose membranes. Blots were microwaved for 2 min in 0.1 M PBS twice and probed with the antibody 82E1 (anti-A β 1–16; 1:1000; IBL), CT20 (anti-APP C-terminal 20 aa; 1:1000; T. E. Golde) and ITM2b (GenWay). Blots were stripped and reprobed with anti β -actin (1:1000; Sigma) as a loading control. Relative band intensity was quantified using ImageJ software (NIH).

In vitro A β aggregation assay using native gel electrophoresis. Synthetic A β 1–42 and A β 1–40, treated with hexafluoroisopropanol and dried (Bachem), and Bri2–23 peptides (Bachem) were dissolved in DMSO and then diluted in TBS at molar ratios as indicated. A β 1–42 and Bri2–23 peptide mixtures were incubated for 3 h at either 0°C or 37°C without shaking. Mixtures were run on 4–20% Tris-HCl gels under nondenaturing conditions and transferred to 0.4 μ m polyvinylidene fluoride membrane as previously described (Klug et al., 2003; Kim et al., 2007). The blot was probed with Ab9 (anti-A β 1–16; 1:1000; T. E. Golde). Relative band intensity was quantified using ImageJ software (NIH).

In vitro A β 1–42 aggregation assay using thioflavin T and atomic force microscopy studies. Bri2–23 peptides (Bachem) were reconstituted in 1 mg/ml Tris-HCl, pH 8.0. The lyophilized synthetic A β 1–42 (Mayo Clinic Peptide Synthesis Facility) was dissolved at 0.5–2.0 mM in 20 mM NaOH 15 min before size exclusion chromatography on Superdex 75 HR 10/30 column (GE Healthcare) to remove any preformed A β aggregates. The concentration of monomeric A β was determined by UV absorbance with a calculated extinction coefficient of 1450 cm^{–1} $\times$ M^{–1} at 276 nm (Rangachari et al., 2006). A β 1–42 aggregation reactions were initiated in siliconized Eppendorf tubes by incubating 25–50 μ M freshly purified A β 1–42 monomer in 10 mM Tris-HCl and 150 mM NaCl, pH 8.0, buffer without agitation at 37°C. Monomeric A β 1–42 aggregation process in the presence or absence of Bri2–23 peptide were monitored using a thioflavin T (ThT) assay as previously reported (Rangachari et al., 2006). Atomic force microscopy images were obtained with a NanoScope III controller with a Multimode AFM (Veeco Instruments) as described previously (Nichols et al., 2005). Images are shown in amplitude mode, where increasing brightness indicates greater damping of cantilever oscillation.

HPLC/mass spectrometry analysis of Bri2–23 peptides. Conditioned media or CSF was filtered through a 0.45 μ m syringe filter to remove large particulate matter. A 50 μ l aliquot of the sample was injected into an Agilent 1100 Series HPLC with a Zobax Eclipse XDB-C8 column and running buffer of acetonitrile/H₂O (ACN:H₂O) with 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Initial solvent composition was 20:80 ACN:H₂O; this composition was held for 3 min and then linearly ramped up to 37:63 ACN:H₂O over the next 7 min. A fraction was collected between 9.4 and 10.4 min (as the BRI-23 standard was seen to elute at 9.8 min) for a total of 1 ml.

The collected fraction was then blown down in nitrogen at 37°C to ~100 μ l in volume. A 1 μ l aliquot of this concentrated sample was applied to a Bio-Rad gold array chip and allowed to air dry. After the sample dried, 1 μ l of saturated α -cyano-4-hydroxycinnamic acid (MALDI matrix) in 70:20:10 ACN:H₂O:MeOH with 0.1% TFA was applied on top of dried sample and allowed to air dry. This was then analyzed on a Bio-Rad Ciphergen ProteinChip SELDI time-of-flight system. A laser intensity of 750 μ J was used to collect spectra from 3975 laser shots, which were averaged into the final spectra. The finished spectra were baseline corrected.

Statistical analysis. One-way ANOVA with *post hoc* Holm-Sidak multiple-comparison test or two-tailed Student's *t* test was used for statistical comparison (SigmaStat 3.0 version). If the data did not meet the parametric test assumptions, nonparametric statistics was performed, either Kruskal–Wallis test (one-way ANOVA on ranks) followed by *post hoc* Dunn's multiple-comparison procedures or Mann–Whitney rank sum test (SigmaStat 3.0 version). Variability of the estimates was reported as SEM.

Results

BRI2 and BRI2-A β 1–40 suppress amyloid deposition in APP transgenic mice

The effects of the virally delivered BRI2-A β 1–40 transgene were compared with effects of the rAAV1-delivered human BRI2 transgene and a noninjection control (Fig. 1*B*). Expression of BRI2 was intended to serve as a second control, because we had established that rAAV1-hGFP delivery and mock virus delivery did not alter A β deposition in the CRND8 APP mouse model (Levites et al., 2006b). Three months after rAAV1-mediated transgene delivery, mice were killed and brain A β deposition was analyzed using both biochemical and histochemical methods. These analyses revealed a dramatic suppressive effect of both the BRI2-A β 1–40 and BRI2 transgenes on parenchymal A β 1–40 and A β 1–42 accumulation as measured by biochemical and histochemical assessments of A β levels (Fig. 1*C–E*).

The reduction in A β deposition observed in the mice expressing the rAAV1 BRI2-A β 1–40 transgene was consistent with our previous transgenic mice studies (Kim et al., 2007), whereas the reduction of A β deposition observed in the mice expressing BRI2 was unexpected. Previous studies had demonstrated a potential interaction between BRI2 and APP and noted that BRI2 overexpression increased APP C-terminal fragment β (CTF β) and reduced A β secretion in cultured cells (Fotinopoulou et al., 2005; Matsuda et al., 2005). As in the studies of BRI2-A β transgenic mice crossed into Tg2576 mice (McGowan et al., 2005; Kim et al., 2007), we found no evidence for alterations in the steady-state levels of APP or APP CTF β in TgCRND8 mice expressing the virally delivered BRI2-A β 1–40 or BRI2 transgenes (Fig. 2*A,B*). Moreover, levels of endogenous rodent A β levels in the brains of the nontransgenic littermates of the TgCRND8 mice expressing the BRI2 transgene were not altered (Fig. 2*C*). BRI2-A β 1–40 expression slightly increased plasma A β 40 levels, attributable to brain to plasma efflux of A β 1–40; plasma A β 1–42 levels were not significantly changed by BRI2 expression (Fig. 2*D*). Because of the rapid onset of A β deposition in TgCRND8 mice, it is not

possible to measure steady-state A β levels; therefore, we conducted additional experiments in Tg2576 mice to determine whether BRI2 altered steady-state A β before plaque deposition. rAAV1-mediated delivery of BRI2 to P0 Tg2576 did not lower steady-state A β levels in brains of 2-month-old mice (Fig. 2*E*). Because anti-A β antibodies reduce A β deposition in mice and expression of virally encoded A β peptides in the periphery has been shown to generate an anti-A β response, we examined whether CNS delivery of the transgene induced a humoral immune response to A β . There was no evidence for an anti-A β titer in any of the rAAV1-injected mice (Fig. 2*F*). Collectively, these results in TgCRND8, wild-type TgCRND8 littermates, and Tg2576 mice demonstrate that the reduction of A β accumulation by BRI2 and BRI2-A β 1–40 transgenes was not likely to be attributable to alterations in APP processing resulting from altered A β production or induction of an anti-A β immune response.

Bri2–23 inhibits A β 1–42 aggregation *in vitro*

To understand the mechanism by which BRI2 reduced A β accumulation, we tested whether the Bri2–23 peptide could directly inhibit A β 1–42 fibrillogenesis *in vitro*. Several methods were used, including a native gel assay previously used to demonstrate that A β 1–40 inhibits A β 1–42 aggregation (Kim et al., 2007). When A β 1–42 aggregation was assessed using the native gel assay, we observed loss of the monomeric A β 1–42 signal and the appearance of high-molecular weight (HMW) aggregates (Fig. 3*A*, lane 10). Addition of Bri2–23 or A β 1–40 to the reaction resulted in retention of the monomeric A β 1–42 signal, suggesting direct inhibition of aggregation (Fig. 3*A*, compare lane 10 with lanes 4, 6, and 8). Quantification by an ELISA capable of only recognizing monomeric A β confirmed that the Bri2–23 peptide retained A β 1–42 in its monomeric state, and this effect was more robust with increasing concentrations of Bri2–23 (Fig. 3*B*). To further analyze the effect of Bri2–23 on A β aggregates, we examined the effect of equimolar concentrations (25 or 50 μ M) of the Bri2–23 peptide on monomeric A β 1–42 aggregation into A β 1–42 fibrils or protofibrils using ThT fluorescence assay. Prolonged incubations of Bri2–23, by itself, did not result in aggregation or β -sheet formation as assessed by ThT fluorescence assay, change in circular dichroism spectra, or insolubility (data not shown) (Gibson et al., 2005). Coaggregation of A β 42 and Bri2–23 demonstrates that Bri2–23 appears to initially increase the rate of aggregate formation during the first 12 h of incubation, but inhibits fibril formation at later time points (Fig. 3*C*). After 120–200 h of incubation, Bri2–23 inhibited A β 1–42 aggregation by $46 \pm 9\%$ ($n = 6$; $p = 0.0004$). Atomic force microscopy (AFM) imaging confirmed the inhibitory effects of Bri2–23 on A β 1–42 aggregation in these assays (Fig. 3*D*). These data show that Bri2–23 has a complex effect on aggregation of monomeric A β 1–42; however,

Figure 2. Effects of BRI2 and BRI2-A β 1–40 on steady-state levels of APP, APP CTF, and A β . **A**, To analyze whether APP processing was altered by expression of BRI2 and BRI2-A β 1–40 in TgCRND8 mice, SDS-soluble forebrain extracts were analyzed by Western blotting probed with 6E10. The virally delivered construct (BRI2 or BRI2-A β 1–40) or control (PBS) is shown below the panel, and the CRND8 hAPP transgene status is listed in parentheses. **B**, Quantification of APP and CTF β protein level after normalization to β -actin level showed no change in the relative levels of both proteins in all groups. **C**, The steady-state endogenous mouse A β levels in nontransgenic littermates of TgCRND8 mice, measured by rodent A β -specific ELISA, were comparable between PBS injection control and rAAV1-BRI2 group. **D**, A β levels in plasma of the somatic brain transgenic CRND8 mice were analyzed by A β end-specific ELISA. There was no change in A β level in BRI2 group, compared with no injection control group. BRI2-A β 1–40-injected TgCRND8 mice had significantly increased plasma A β 40 levels, compared with no injection and BRI2 injection groups (* $p < 0.05$). A β 42 levels were comparable between all groups ($p > 0.05$). **E**, P0 Tg2576 mice were injected with PBS or rAAV1-BRI2 construct, and then the steady-state A β levels in 2-month-old Tg2576 mice were measured by A β end-specific ELISA. The expression of BRI2 did not lower the steady-state A β levels compared with control group. **F**, BRI2-A β 1–40 and BRI2 expression does not result in a humoral immune response to A β . The levels of anti-A β IgG antibody in plasma were measured by anti-A β antibody ELISA. Cerebral expression of BRI2 and BRI2-A β 1–40 in TgCRND8 mice did not trigger anti-A β immune response in all groups, except in the positive control group immunized with fibrillar A β 42.

both assays are consistent with a net inhibitory effect of Bri2–23 peptide on amyloid formation, presumably through inhibition of a later stage in fibril assembly.

The Bri2–23 sequence is required to suppress A β deposition *in vivo*

These observations suggested that the anti-amyloidogenic effect of the BRI2 protein is mediated by an interaction between Bri2–23 and A β . To further test this idea *in vivo*, we generated a cDNA that expresses a truncated BRI2 protein lacking the Bri2–23 peptide (BRI2del244–266) (Fig. 4*A*) and used rAAV gene transfer to deliver this construct to newborn TgCRND8 mice. Transgene positive mice were killed at 3 months of age, and biochemical and histochemical A β loads were examined. Analyses of A β loads showed no significant difference between BRI2del244–266 and the control groups (Fig. 4*B–D*). Western blot analyses of brain lysates demonstrated that the somatic brain transgenic methodology produced approximately equivalent expression levels from the BRI2 and BRI2del244–266 constructs and somewhat higher levels from BRI2-A β 1–40 (Fig. 4*E*). These later data and the lack of anti-amyloidogenic effect from BRI2del244–266 demonstrate that the Bri2–23 peptide sequence is critical for the inhibitory effect of BRI2 *in vivo*. Together with the data demonstrating that Bri2–23 directly inhibits A β aggre-

Figure 3. Bri2–23 peptide inhibits A β aggregation *in vitro*. **A**, Synthetic A β 1–42, A β 1–40, and Bri2–23 peptides were mixed at the concentrations indicated and incubated at 0 or 37°C for 3 h. After incubation, the extent of A β aggregation into HMW aggregates was assessed by native gel electrophoresis and Western blotting with Ab9 (anti-A β 1–16) antibody that recognizes A β fibrils, oligomers, and monomers. **B**, Quantitative analysis of a second dose–response study showed the difference in monomeric A β 1–42 levels between the 37 and 0°C incubations. $n = 3$ for each condition. * $p < 0.05$ and ** $p < 0.01$ versus 1.5 μ M A β 42 aggregation (ANOVA). **C**, Monomeric A β 1–42 isolated by size-exclusion chromatography was incubated in the presence or absence of Bri2–23 peptide. At the indicated times, aliquots of aggregation reaction mixtures were analyzed for the extent of aggregation by ThT assay. **D**, AFM analysis of aggregates at 72 h of incubation. Representative images, shown in height mode, are $10 \times 10 \mu$ m. Scale bars, 1 μ m.

gation *in vitro*, these data support an anti-amyloidogenic function for BRI2 mediated by the Bri2–23 peptide.

The Bri2–23 peptide is present in human CSF

Our mouse data suggested that endogenous BRI2 could function, at least in part, by secretion of the Bri2–23 peptide as an anti-amyloidogenic binding partner of A β . To date, studies of normal BRI2 processing and secretion relied mainly on epitope-tagged versions of the Bri2–23 peptide (Kim et al., 1999; Choi et al., 2004). First-generation antibodies were not sensitive enough to detect the Bri2–23 peptide in biological samples. Thus, we developed an HPLC/mass spectrometry (HPLC/MS)-based assay to detect secreted Bri2–23. We validated this methodology by detecting untagged Bri2–23 peptide secretion from H4 cells transfected with BRI2 but not BRI2del244–266 (Fig. 4F). We then tested normal human CSF and were able to detect the endogenously secreted Bri2–23 peptide in all samples tested (Fig. 4G). This finding strengthens the notion that the anti-aggregation effects of Bri2–23 peptide in our experiments may be physiologically relevant to human AD, FDD, and FBD.

Discussion

We have used somatic brain transgenic technology to deliver the BRI2 and BRI2-A β 1–40 transgenes to the brains of APP mouse models. The studies with BRI2-A β 1–40 confirmed previous studies obtained using conventional transgenic mice expressing BRI2-A β 1–40 (McGowan et al., 2005; Kim et al., 2007). Thus, the somatic brain transgenic BRI2-A β 1–40 studies provide additional validation for this rapid cost-effective method of manipulating gene expression in the brain (Levites et al., 2006b).

The novel result from these studies was the finding that BRI2 suppresses A β deposition in APP CRND8 transgenic mice to an extent equivalent to A β 1–40. Although it is not possible to completely rule out subtle effects on A β generation that could influence deposition, we found no evidence that the suppressive effect was mediated by alterations in APP processing or A β production. Instead, we find that the suppressive effect of BRI2 is likely to be mediated by inhibition of A β aggregation by the secreted peptide. We demonstrate that expression of the BRI2del244–266 construct that lacks a secreted peptide sequence has no effect on A β deposition after expression *in vivo*. BRI2del244–266 encodes a protein containing the region of BRI2 previously shown to interact with APP and interfere with APP processing in cell culture. Coupled with their inhibition of aggregation *in vitro*, we conclude that the A β 1–40 and Bri2–23 peptides are directly responsible for reduced A β deposition in our experiments rather than any other part of the BRI2 protein scaffold on which they were delivered. Notably, in FDD brains, A β and the ADan peptide are codeposited and bind to each other *in vitro* (Tomidokoro et al., 2005). These later findings suggest that the FDD-linked BRI2 mutation may corrupt a normally protective anti-amyloidogenic mechanism resulting in coaggregation of the mutant

peptide with a normal binding partner. In support of our observations, Bri2–23 contains the sequence FENKF that is homologous to peptide-based A β aggregation inhibitors incorporating a Fxxx motif (Sato et al., 2006). Moreover, solid-state nuclear magnetic resonance analysis demonstrated direct binding of an 8 aa peptide containing the sequence FEGKF with the glycine zipper (G33xxxG37) segment of A β 1–40, a sequence proposed to be critical for formation and stability of β -sheet structure (Liu et al., 2005; Sato et al., 2006).

Beyond the genetic link to FDD and FBD, little is known about the function of BRI2 and its homologues. BRI2 is encoded by the *ITM2b* gene located on chromosome 13q14.3, and is a member of a gene family consisting of BRI1 (*ITM2A*) and BRI3 (*ITM2C*) (Vidal et al., 2001; Akiyama et al., 2004; Choi et al., 2004). Orthologs are only found in higher eukaryotes. The BRI proteins share ~50% identity at the amino acid level, and are all expressed at modest (BRI1) to extremely high levels in the brain (BRI2, BRI3). They are relatively small (~260 aa) type 2 membrane proteins with single transmembrane domains, extracellular BRI-CHOS domains, and furin cleavage sites near their C termini. At their C termini, they encode small peptides that, for BRI2 and BRI3, have been shown to be released and secreted after the furin cleavage (Kim et al., 1999; Wickham et al., 2005). Based on limited data, others have proposed that the BRICHOS domain targets the protein to the secretory pathway, performs an intramolecular chaperone-like function, and assists the specialized intracellular protease-processing system (Sanchez-Pulido et al., 2002). Very recently, BRI2 has been shown to undergo sequential cleavage by ADAM10 to release its ectodomain and intramem-

brane proteolysis by SPPL2a and b (Martin et al., 2007). BRI2 has also been shown to undergo axonal transport (Choi et al., 2004). Nevertheless, other than the genetic link between BRI2 and FBD and FDD, almost nothing is known about the function of the BRI proteins (Ghisso et al., 2006).

Further study of BRI2 and the Bri2–23 peptide as well as analogous peptides released from the BRI2 homologues (which contain the conserved FxxxF motif) will be required to fully understand their anti-amyloidogenic action and other functions. The robust inhibitory effect of BRI2 on A β deposition *in vivo* and aggregation *in vitro* BRI2 indicates that BRI2 is a novel factor that modulates A β aggregation and deposition. These data support a novel approach to AD therapy or prevention based on increasing levels of BRI2 and more specifically the Bri2–23 peptide in the brain.

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Figure 4. The Bri2–23 peptide is required for the anti-amyloidogenic effect of the BRI2 protein *in vivo* and is present in human CSF. **A**, Schematic of BRI2 and BRI2del244–266 constructs. BRI2del244–266 construct does not encode the Bri2–23 peptide. P0 TgCRND8 mice were transduced by intracerebroventricular injection of rAAV1-BRI2del244–266. **B**, **C**, Cortical sections (magnification, 200 $\times$) of 3-month-old TgCRND8 mice were immunostained with anti-A β 1–16 antibody (33.11; **B**), and amyloid plaque burdens were quantified (**C**). **D**, Total-brain A β levels were analyzed by A β end-specific ELISA 3 months after transduction. **E**, Western blot analysis of steady-state levels of the rAAV1-delivered transgenes. Three lysates representing the typical range of expression are shown for the BRI2, BRI2-A β 1–40, and BRI2del244–266 transgenes. Anti- β -actin is used as a loading control, and a lysate from transgenic BRI2-A β 40 mice is used as a positive control (TgBRI2-A β 40). **F**, HPLC/MS detection of Bri2–23 in cells transfected with BRI2. Synthetic Bri2–23 yielded a peak that was in excellent agreement with its predicted mass-to-charge ratio (m/z) of 2630. Spectra from the conditioned media of cells transiently transfected with pcDNA3-BRI2 yielded a peak absent from both the blank and conditioned media from cells transfected with pcDNA3 BRI2del244–266. The peak has an assessed mass-to-charge ratio within 1 unit of the Bri2–23 peptide standard. **G**, HPLC/MS detection of Bri2–23 in human CSF. Spectra from the CSF of three normal subjects each yielded a peak absent from the blank with an assessed mass-to-charge ratio nearly identical to the Bri2–23 peptide standard. yo, Years old; F, female; M, male.

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