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Expression of BRI2 mRNA and protein in normal human brain and familial British dementia: its relevance to the pathogenesis of disease

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Abstract

Introduction—Two different disease-specific mutations in the *BRI2* gene, situated on chromosome 13, have been identified as giving rise to familial British dementia (FBD) and familial Danish dementia (FDD). Each mutation results in extension of the open reading frame generating the disease-specific precursor proteins which are cleaved by furin-like proteolysis releasing the amyloidogenic C-terminal peptides ABri and ADan in FBD and FDD, respectively.

Material and methods—To understand the mechanism of the formation of amyloid lesions in FBD, we studied the origin of the precursor proteins and furin in the human brain. We used control brains, cases of sporadic Alzheimer's disease (AD), variant AD with cotton wool plaques and FBD to study BRI2 mRNA expression using *in situ* hybridization. Furin and BRI2 protein expression was investigated using Western blotting and immunohistochemistry.

Results—BRI2 mRNA and BRI2 protein are widely expressed primarily by neurones and glia and are deposited in the amyloid lesions in FBD. They were, however, not expressed by cerebrovascular components. Furin expression showed a similar pattern except that it was also present in cerebrovascular smooth muscle cells.

Conclusions—These findings suggest that neurones and glia are a major source of BRI2 protein and that in FBD, the mutated precursor protein may undergo furin cleavage within neurones to produce the amyloid peptide ABri. The failure to demonstrate BRI2 in blood vessels under the conditions tested suggests that vascular amyloid peptide production does not contribute significantly to cerebral amyloid angiopathy (CAA) in FBD and FDD, lending indirect support to the drainage hypothesis of CAA.

Keywords

Alzheimer's disease; familial British dementia; furin; human brain; immunohistochemistry; *in situ* hybridization

Introduction

Two familial neurodegenerative diseases, familial British dementia (FBD) and familial Danish dementia (FDD), are associated with different mutations in the *BRI2* gene located on chromosome 13 [1–12]. Extensive cerebral amyloid angiopathy (CAA) and neurofibrillary degeneration are features of both diseases, and these are associated with parenchymal amyloid plaques, cerebellar degeneration and white matter changes similar to those seen in Binswanger's disease in FBD and with parenchymal pre-amyloid lesions in FDD [2,3,7,10, 13–15]. Thus, as in Alzheimer's disease (AD), cerebral amyloid deposition is accompanied by neurofibrillary degeneration in both FBD and FDD.

The *BRI2* gene, belonging to a family of genes with three members [16–19], encodes a 266-amino-acid-long type II transmembrane protein, BRI2, of unknown function. In FBD, a stop codon mutation in the *BRI2* gene leads to the production of an elongated 277-amino-acid-long precursor protein, while in FDD a 10-nucleotide duplication insertion abolishes the normal stop codon, extending the open reading frame and also producing a 277-amino-acid-long precursor protein. Proteolytic cleavage of each of these mutated precursor proteins generates the 34-amino-acid amyloidogenic peptides, ABri in FBD and ADan in FDD. ABri and ADan have unique C-termini recognized by specific antibodies [11,12]. In FBD, ABri forms fibrillar (amyloid) deposits in blood vessels, and both fibrillar and non-fibrillar (pre-amyloid) parenchymal lesions in the brain and systemic organs [7,20]. ADan amyloid lesions in FDD are predominantly found in the cerebral vasculature where they may be accompanied by amyloid- β (A β) deposition, while cerebral parenchymal deposits are more frequently in a non-fibrillar, pre-amyloid conformation [10]. Co-deposition of A β and ADan in FDD may reflect the proposed interaction between BRI2 and amyloid precursor protein (APP) [21,22].

Proteolytic cleavage by the ubiquitous proprotein convertase (PC) furin generates a C-terminal 23-amino-acid fragment from the wild-type protein, while the mutated BRI2 precursor proteins are cleaved to produce the amyloidogenic peptides ABri and ADan. Cleavage of the mutated precursor proteins is more efficient than that of the wild type, and furin is more effective in producing this cleavage than other PCs expressed in brain [23,24].

The origin and mechanism of deposition of ABri and ADan in plaques and blood vessel walls are not known. ABri and ADan may be produced by cellular components of the central nervous system and a neuronal origin, similar to that proposed for A β in AD, is supported by our observation of perineuronal ABri in FBD [7]. This hypothesis is further supported by our previous Northern blot studies, demonstrating that *BRI2* mRNA is expressed throughout the human brain with high levels of expression in a number of areas, including the hippocampus, amygdala and cerebellum [11]. Previous protein studies have shown that both ABri and furin are present in neurones, and furin was also localized in both reactive astrocytes and in ABri parenchymal amyloid lesions in FBD, indicating possible sites for cleavage of the precursor protein [25,26]. In this study, we wished to correlate *BRI2* mRNA expression with that of *BRI2* protein and furin to obtain further information regarding the cellular elements involved in *BRI2* production and processing in the central nervous system, as this will further elucidate the disease pathogenesis. We explored these in control brain tissue and in both FBD and AD to determine whether there is any relationship between parenchymal amyloid and pre-amyloid lesions and either *BRI2* or furin expression.

Materials and methods

Tissue

Samples of brain tissue which had been flash-frozen and stored at -80°C following brain removal at autopsy were obtained from four normal controls, one case of FBD, four cases of AD and one case of variant AD with cotton wool plaques associated with the presenilin-1 $\Delta\text{I83}/\Delta\text{M84}$ mutation [27] (Table 1). AD cases were selected to provide a range of plaque types (diffuse, mature and cotton wool), and CAA severity (mild–severe) was judged by published criteria [28,29]. Appropriate consent was obtained in all cases and approval for the study was granted by the Local Research Ethics Committee.

For Western blot analysis, tissue from the cerebellum of four of the control cases and frontal lobe from the FBD case was used. To study the distribution of furin and BRI2 in the normal brain using immunohistochemistry and *in situ* hybridization, sections were cut from the frontal cortex, basal ganglia, hippocampus and cerebellum in all control cases. In addition, parietal cortex and midbrain were available in three of the cases, temporal and occipital cortices in two of the cases, and thalamus and pons in one of the cases. Hippocampus and frontal cortex were available from the FBD case, while hippocampus and temporal cortex were used from the AD cases. Frontal cortex and hippocampus were also used for the double immunohistochemical study in a normal control case and the FBD case.

Western blot analysis

Frozen tissue was homogenized in $10\times$ its volume in phosphate buffered saline (PBS)-containing protease inhibitor (PBS/Complete, Roche, Sussex, UK), and 1 ml of homogenate was centrifuged (12 000 r.p.m., 5 min) to remove any tissue debris. The supernatant was added to $4\times$ NuPage sample buffer with NuPage-reducing agent (Invitrogen, Paisley, UK), heated to 70°C for 10 min, and electrophoresis was performed at 200 V for 35 min on a NuPage 10% Bis-Tris gel with 2-(N-morpholino) ethane sulphonic acid (MES) running buffer (Invitrogen). Protein transfer to polyvinylidene fluoride membrane was carried out at 30 V for 2 h. After blocking in 5% non-fat milk, the membranes were incubated overnight at 4°C with either Ab340 antibody (1:500; which had been raised against residues 223–233 of BRI2 – CHDKETYKLQR – situated 21 residues N-terminal of the furin cleavage site, and is therefore expected to recognize wild-type BRI2 and also the mutated precursor proteins, but not the amyloidogenic peptide ABri), immuno-absorbed Ab340, Ab340 pre-immune serum or antibody to furin convertase. Following washes, the membrane was transferred to a rabbit secondary antibody conjugated to HRP (1:1000, Vector, Peterborough, UK), incubated for 1 h at room temperature, washed and visualized using the ECL system (Amersham, Amersham, UK).

Northern blot analysis

RNA was extracted following a published protocol [30], and the RNA integrity was confirmed by the presence of 16S and 28S bands representing ribosomal RNA in denaturing agarose gels. RNA samples were separated by electrophoresis (60 V) on a 1% agarose and formaldehyde gel before transfer to Hybond-N plus nylon membrane using a standard procedure.

Complementary DNA (cDNA) probes for *BRI2* and *GAPDH* were produced by polymerase chain reaction (PCR) using the respective primers listed in Table 2, and labelled using ^{35}S with a High Prime kit (Roche). Twenty-five ng/ μl cDNA was denatured by heating in boiling water and chilling on ice. High Prime was mixed according to the manufacturer's instructions, and $4\text{ }\mu\text{l}$ was added to the denatured DNA, followed by $5\text{ }\mu\text{l}$ of $50\mu\text{Ci }^{35}\text{S}$. The mixture was incubated for 20 min at 37°C and the reaction halted with $2\text{ }\mu\text{l}$ of 0.2 M EDTA (pH 8.0).

Hybond-N membranes were placed in a sterile hybridization cylinder, wet with 2× SSC and then incubated in pre-hybridization solution (5× SSPE, 5× Denhardt's solution, 50% formamide, 0.1% SDS, 200 µg/ml salmon sperm in DEPC water) for 30 min at 42°C. Pre-hybridization buffer was then replaced by hybridization buffer (5 ml: pre-hybridization buffer containing 800 µl of the labelled probe), and hybridized overnight at 42°C. The membranes were washed (3 × 10 min) in pre-heated 2× SSC solution containing 0.1% SDS followed by 0.5× SSC containing 0.1% SDS at 55°C (2 × 15 min). Labelled bands were visualized by autoradiography.

BRI2 mRNA *in situ* hybridization

Sections of frozen brain tissue were cut (15 µm), mounted on glass slides and fixed in 4% paraformaldehyde in PBS at room temperature for 5 min. After washing twice in PBS followed by sterile saline, the sections were dehydrated in graded ethanol in DEPC-treated water (Sigma, Dorset, UK) and air-dried. Three oligonucleotide probes complementary to residues 28–68, 609–648 and 802–836 of BRI2 cDNA (Table 2) were labelled using the digoxigenin (DIG) oligonucleotide 3'-End Labelling Kit according to the manufacturer's instructions (Roche). The labelled probes were diluted in hybridization buffer (Sigma) to a concentration of 200 fmol/ml, with 0.1 mg/ml salmon sperm DNA and hybridized overnight at 37°C. After washes in SSC at 55°C and at room temperature followed by distilled water, sections were blocked with 10% non-fat milk for 30 min and incubated with anti-DIG antibody (1:250; Roche) at 4°C overnight. Biotinylated rabbit anti-mouse antibody (1:200; Dako, Ely, UK) was applied for 30 min at room temperature followed by ABC reagent for 30 min (Dako). After washing, the sites of probe hybridization were visualized using the glucose oxidase nickel di-aminobenzidine method [31] and counterstained with haematoxylin. Negative control experiments were performed by omitting the labelled probe and by competitive inhibition with 100-fold excess of unlabelled probe. Neither of these controls showed any labelling (data not shown).

Immunohistochemical staining for furin

Frozen tissue sections were cut at 10 µm thick, air-dried and post-fixed in acetone. Endogenous peroxidase activity was blocked with methanol/0.3% H₂O₂ and non-specific protein binding by incubation in 10% non-fat milk for 30 min. Primary antibody to furin convertase (Table 3) was incubated overnight at 4°C, followed by incubation with the secondary swine anti-rabbit antibody (Dako) and then a further 30-min incubation in ABC reagent. Bound antibody was visualized using di-aminobenzidine enhanced with nickel and activated with glucose oxidase [31].

Immunohistochemical staining for BRI2 and ABri

Frozen tissue sections were cut as described above and air-dried followed by fixation in 4% paraformaldehyde for 30 min. Endogenous peroxidase activity and non-specific protein binding were blocked as described above. Sections were incubated for 1 h at room temperature with the primary antibody Ab340 (1:500) (Table 3). Subsequent steps in the staining procedure were as described above. For negative controls, Ab340 was replaced with the pre-immune serum or immuno-absorbed Ab340 (see below), the primary antibodies were also omitted. Immunohistochemical staining for ABri was carried out as described above using the antibody Ab338 (Table 3) [7]. Immuno-absorption of Ab340 was carried out using full-length immunizing synthetic peptide (100 µg of peptide per 50 µl of antibody) incubated for 1 h at 37°C and 16 h at 4°C, followed by centrifugation at 14 000g for 5 min.

Double-immunofluorescent staining for furin/GFAP and furin/NeuN

For the double staining, the single immunohistochemical method for furin was followed as above, replacing the chromogen with tetramethylrhodamine TSA kit (Perkin Elmer, Beaconsfield, UK). After washing, the primary antibodies to GFAP or NeuN were applied for 1 h at room temperature (Table 3) followed by incubation with swine anti-rabbit (Dako) for 30 min and subsequently ABC reagent. Both GFAP and NeuN were visualized using the fluorescein TSA kit.

Double-immunofluorescent staining for BRI2/GFAP, BRI2/CD68 and BRI2/NeuN

The single-immunohistochemical method for BRI2 was followed as described above, replacing the chromogen with tetramethylrhodamine TSA kit. Staining for GFAP, CD68 and NeuN was performed as previously described for double staining with furin.

Double staining for CR3/43 and Thioflavine-S

Paraffin sections were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase and non-specific protein binding were blocked as described above. Sections were pre-treated in the pressure cooker for 10 min in citrate buffer (pH 6.0), followed by incubation with the primary antibody CR3/43 (Table 3) (Dako). Subsequently, biotinylated anti-mouse secondary antibody (Dako) and ABC complex (Vector) were applied and visualization was completed using tetramethylrhodamine TSA kit. Sections were counterstained in 1% Thioflavine-S solution and differentiated in 70% alcohol.

Results

The demographic details of the cases used are given in Table 1 and the major findings of the study in Table 4.

Northern blotting

Northern blot analysis of mRNA derived from the cerebellum showed good preservation of mRNA with hybridization of the probe for the control housekeeping gene *GAPDH*, producing a strong 1.4-kb band. A 2.0-kb species representing BRI2 mRNA was identified using the BRI2 probe (Figure 1A).

Western blot analysis

The monoclonal antibody Ab340 was characterized by immunoblotting of cerebellar tissue from four controls and one FBD case, and was found to identify a strong band at 39–41 kDa, the expected molecular weight of BRI2 (Figure 1B), which was absent when the pre-immune serum or the Ab340 absorbed with immunizing peptide, were used (data not shown). The presence of furin was confirmed by immunoblotting using antibody to furin convertase in four control cases and one FBD case. This demonstrated two bands at 90 and 96 kDa, corresponding with the zymogen form and the cleaved, lower-molecular-weight, active form of furin (Figure 1D).

Distribution of BRI2 mRNA assessed by *in situ* hybridization in normal human brain

The three different probes showed a very similar staining pattern, the figures have been illustrated using the sequence complementary to residues 28–66 of BRI2 cDNA, as this gave the strongest staining pattern. The findings were similar in each of the control brains. In all areas examined, neurones of all types showed strong labelling of the cytoplasm with extension into the apical dendrites of pyramidal cells. Such labelling was easily identified in all regions, and was particularly marked in the granule cells of the dentate fascia. Both Purkinje cells and cerebellar granule cells expressed BRI2 mRNA in proportion to the volume of their cytoplasm.

In the white matter, glial cells showed a small amount of cytoplasmic staining. There was no identifiable labelling of vascular smooth muscle or endothelial cells (Figure 2). Control sections in which the labelled probe was diluted 100 fold by unlabelled probe showed no activity (result not shown).

Distribution and staining pattern of BRI2 in normal human brain

The distribution of the full-length precursor protein BRI2 was demonstrated using the antibody Ab340, which was raised against a region of BRI2 proximal to the furin cleavage site (see above). This showed strong diffuse cytoplasmic labelling in neurones of all sizes in all regions of the brain examined, with the exception of the cerebellar granule cell layer where only a proportion of neurones was clearly stained, reflecting the paucity of cytoplasm in these cells (Figure 3A and B). Blood vessels were consistently unstained by Ab340. Cells with astrocytic morphology were also found to be immunoreactive for BRI2, and these were confirmed as astrocytes by double labelling with GFAP (Figure 5, row E). Neuronal staining was also confirmed by double-immunofluorescent labelling using the neuronal marker NeuN together with Ab340 (Figure 5, row D).

Distribution and staining pattern of furin in normal human brain

Neurones of all sizes and in all cortical, subcortical and cerebellar regions examined showed strongly positive staining for furin with a punctate cytoplasmic distribution extending into the apical dendrite of pyramidal cells (Figure 3C). Similar to the pattern seen with BRI2 mRNA *in situ* hybridization and BRI2 immunohistochemistry, a narrow rim of staining was evident around the nucleus of the cerebellar granule cells in keeping with the volume of cytoplasm in these cells. In all areas of the brain, vascular smooth muscle cells were found to contain furin (Figure 3D). Double-immunofluorescent staining with antibodies to furin and NeuN confirmed the finding of neuronal cytoplasmic furin expression (Figure 5, row A). Astrocytes labelled by GFAP immunofluorescence showed no furin expression (Figure 5, row B).

Distribution and staining pattern of ABri in FBD

Immunohistochemical staining for ABri using the antibody Ab338 showed a similar pattern of staining to that described previously [7]. In the hippocampus, amyloid plaques of all types were stained in addition to the diffuse deposits in the entorhinal cortex. In the frontal lobe, parenchymal deposits were limited to perivascular white matter lesions. Amyloid-laden blood vessels were stained in the hippocampus, frontal cortex and underlying white matter (Figure 4A and B).

Distribution of BRI2 mRNA, BRI2 and furin in FBD and AD

In a case of FBD, *in situ* hybridization for BRI2 mRNA and immunohistochemical labelling for BRI2 in the hippocampus and frontal lobe showed the same pattern of staining in neurones as that seen in the control brain tissue. In addition, cells with glial morphology were found to contain BRI2 mRNA (Figure 4D, insert). Parenchymal amyloid lesions contained both BRI2 mRNA (Figure 4C) and BRI2 protein (Figure 4E, insert), although pre-amyloid lesions were unstained. In blood vessels, both BRI2 mRNA (Figure 4C and D) and BRI2 (Figure 4E and F) were found in association with ABri amyloid deposits; however, smooth muscle cells contained neither BRI2 mRNA (Figure 4C, insert) nor BRI2, and vessels lacking amyloid deposits were unstained. Immunohistochemical staining for furin in FBD (Figure 4G and H) showed similar findings with staining of parenchymal lesions (Figure 4H) and amyloid-laden blood vessels (Figure 4H), as well as those structures which were stained in control cases. The majority of astrocytes did not contain furin (Figure 5, row B). The marked microglial response in FBD [7] allowed us to study furin and BRI2 expression by microglial cells. These studies showed that both proteins are diffusely expressed by CD68-positive microglia (Figure 5, rows

C and F). In contrast, microglia containing ABri amyloid following phagocytosis, which could also contain BRI2 as this is present in amyloid, showed a punctate staining pattern (Figure 5, row G).

In both sporadic AD and variant AD with cotton wool plaques, the distribution of BRI2 mRNA, BRI2 and furin was similar in neurones and glia to that found in controls and in FBD. In variant AD with cotton wool plaques, in which severe CAA is a prominent feature, BRI2 mRNA was found in the walls of amyloid-laden blood vessels, although the cotton wool plaques, which are largely composed of A β in pre-amyloid conformation, did not contain the mRNA. In sporadic AD, the amyloid plaques and amyloid-laden vessels contained BRI2 mRNA. No deposition of BRI2 or furin was found in either amyloid or pre-amyloid lesions in sporadic or variant AD.

Discussion

An important step in our understanding of the pathogenesis of FBD is to localize the sites of BRI2 production in the central nervous system (CNS) and to determine how this relates to expression of the enzyme furin which is thought to be responsible for proteolytic production of the ABri amyloidogenic peptide. Using *in situ* hybridization, we have shown that neurones throughout the brain express BRI2 mRNA and also contain levels of BRI2 protein and furin which are readily detected using immunohistochemistry. Furin and BRI2 mRNA were found in glia, and it was also possible to identify BRI2 in both astrocytes and microglia; in addition, furin was found in microglia. Vascular smooth muscle cells were found to have detectable levels of furin, but neither BRI2 mRNA nor BRI2 were identified in this site under the current experimental conditions. The cellular expression of BRI2 mRNA, BRI2 and furin was similar to controls in cases of FBD and AD. In FBD, vascular and parenchymal amyloid lesions, but not preamyloid deposits, contained BRI2 mRNA, BRI2 and furin. In AD, BRI2 mRNA was found in vascular and parenchymal amyloid deposits, but there was no lesional deposition of either BRI2 or furin.

This *in situ* hybridization investigation of the distribution of BRI2 mRNA confirmed widespread mRNA expression with especially strong labelling of the hippocampal dentate fascia neurones and cerebellar Purkinje cells, and was supported by the findings of BRI2 immunoblot and immunohistochemical studies. A study using *in situ* hybridization and immunoblotting to demonstrate mouse BRI2 mRNA and BRI2, respectively, has also suggested that both mRNA and protein are widely expressed in the mouse brain with greatest expression in the cerebellum, granular layer of the hippocampus, and olfactory bulb [32]. This latter study also emphasized that structures with a high level of mRNA expression are among those in which neurogenesis takes place in adult mammalian brain [33]. This is interesting in view of the role the BRI2 homologue BRI1 has been shown to play in differentiation [16,32]. Our observations confirmed that the hippocampus and cerebellum are among the structures with a high level of BRI2 mRNA expression, and corresponding protein expression correlates with the finding of a high degree of ABri deposition with subsequent neurodegeneration in these structures in FBD [3,7,10,13]. BRI2 mRNA and protein expression alone may not determine the development of pre-amyloid and amyloid lesions in different cerebral anatomical areas, as we observed strong positivity in both BRI2 mRNA *in situ* hybridization and BRI2 immunohistochemical studies in structures such as the striatum and the substantia nigra, which are unaffected or only relatively mildly affected in FBD [7,10].

Our data confirm and extend the previously published findings that BRI2 is expressed by virtually all neuronal types throughout the normal human brain [26] and in both FBD and AD. The amino acid sequence recognized by Ab340 used to detect BRI2 is 11 to 21 residues upstream of the furin cleavage site [11–21]. Therefore, this antibody is expected to recognize

not only the full-length, but also the 243-amino-acid-long N-terminal fragment generated by furin-like processing of both the wild-type and mutated precursor proteins, making it possible that these fragments contribute to the positive immunohistochemical results. The biological function of BRI2 remains unknown. Its presence in dystrophic neurites in AD, axonal swellings in neuroaxonal dystrophy (Revesz, unpub. obs.), axonal torpedoes, Lewy bodies and Lewy neurites [26], together with its axonal localization in transfected neurones [34], suggests a role in axonal transport. Recent studies have identified an interaction between BRI2 and APP which is dependent upon the transmembrane domain of each protein and is independent of the extracellular domains, indicating an interaction between proteins expressed in the same cell. BRI2 was found to modulate APP processing with a reduction in the production of A β [21, 22]. This raises the possibility that BRI2, by its interaction with APP, may have an important role in the pathogenesis of AD.

This study has shown that in FBD, both the vascular and parenchymal amyloid lesions contain furin as previously described [25], and also BRI2 mRNA and BRI2. Furin, which has been suggested to be involved in the processing of BRI2 [23,24], may have a role in other neurodegenerative diseases due to cerebral amyloid deposition, as it has also been implicated in the proteolytic processing of several molecules with a role in AD. These include BACE, which mediates cleavage of APP to produce the N-terminus of the A β peptide in AD; ADAM10 and ADAM17, which have been implicated in α -secretase activity, leading to cleavage within the A β sequence of APP and the protein CLAC, which colocalizes to amyloid plaques in AD. Furin also cleaves gelsolin, the precursor protein for the amyloidogenic peptide AGel, deposition of which results in a form of familial cerebral amyloid angiopathy [35–40].

The demonstration of both furin and BRI2 (wild type or mutated) in the amyloid lesions of FBD points to the possibility that proteolytic processing may occur in the lesions. Furin and BRI2 deposition in ABri amyloid lesions is unlikely to be due to non-specific binding to amyloid peptides, as neither was identified in A β amyloid lesions in AD. Our *in situ* hybridization studies showed that BRI2 mRNA is also present in vascular and parenchymal amyloid deposits in FBD and AD. Previous studies analysing the non-proteinaceous components of senile plaques have identified about 50 different mRNA species, including APP, tau and furin, and it has been suggested that both normal and degenerating neurones and their processes, which are in close contact with the amyloid plaques in AD, are a source of mRNA in amyloid lesions [41,42]. A neuronal origin of deposited BRI2 mRNA is supported by BRI2 mRNA deposition in ABri amyloid lesions which are surrounded by abnormal neurites while mRNA is not present in the non-neuritic pre-amyloid lesions of FBD or cotton wool plaques in variant AD. RNA, together with amyloid-associated proteins including complement components, may have a role in the formation of amyloid lesions [43,44] as it has been demonstrated to be a cofactor in the conversion of normal cellular prion protein to the protease-resistant form which may be deposited as amyloid in prion disease [45].

The current study shows that although there is local furin expression, there is no significant BRI2 mRNA or BRI2 protein expression *in vivo* by the cellular elements, including smooth muscle cells, of the walls of cerebral arteries and arterioles in normal human brains. This is an important observation, as vascular elements have previously been implicated in the pathogenesis of CAA related to A β deposition [46,47]. Our results suggest that CAA is unlikely to be due to local production of ABri or ADan by cerebrovascular components in FBD and FDD, lending indirect support to the drainage hypothesis of CAA [48]. According to this proposal, A β , produced primarily by CNS cellular elements, is drained along the periarterial spaces of the brain parenchyma and leptomeninges, and CAA occurs due to deposition of the amyloid proteins along these drainage pathways. This is also supported by transgenic models of AD in which neuronal transgene expression is associated with both parenchymal and vascular A β deposition [49]. In favour of the neuronal hypothesis are two lines of evidence

from our previous studies of FBD and FDD [7,10]. In these, we demonstrated that small arteries and arterioles are usually more affected than large arteries which contain a greater number of smooth muscle cells, and also that some arteries, as in AD, are completely unaffected. Furthermore, capillaries which lack smooth muscle cells may have abundant amyloid deposits. It remains possible that a small amount of BRI mRNA and BRI2, at levels below the threshold of the methods used in this study, could be expressed by vascular components as indicated by cell culture experiments, demonstrating the presence of BRI mRNA and BRI2 in endothelial and vascular smooth muscle cells [50]. Should this be the case, it seems likely that this would represent a minor contribution to the deposition of protein in CAA. It is of note that in both FBD and FDD, the amyloid peptides have been shown to be present in the plasma at levels 20 times higher than that of A β , and amyloid deposition in systemic organs has also been documented in FBD [20]. It remains to be investigated, perhaps with the help of future transgenic animal models and other experimental approaches, how efficiently the blood brain barrier separates the central and peripheral pools of ABri and ADan, and thus determines the role systemically produced ABri species may play in the pathogenesis of cerebral amyloid lesions in FBD.

In conclusion, this study has presented evidence that BRI2 mRNA is produced by neurones and glia in normal human brain. The presence of furin in neurones and glia suggests that cleavage of the wild-type and mutated precursor proteins can take place in these cells, resulting in the generation of carboxy-terminal fragments, including ABri and ADan. These findings confirm previous suggestions for a cerebral, neuronal and possibly glial origin of these amyloid peptides. The failure to demonstrate BRI2 mRNA with the current methodologies in cerebrovascular cells suggests that local production of ABri and ADan by vessels may not represent a significant source of these peptides, and gives indirect support to the drainage hypothesis of CAA.

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Figure 1.

Characterisation of RNA integrity and antibodies. **(A)** Northern blot analysis of RNA extracted from the cerebellum of a normal control showing a 2.0-kb band corresponding to BRI2 (1) and a 1.4-kb band corresponding to GAPDH (2). **(B)** Western blot analysis for BRI2 using the Ab340 antibody demonstrates a band of 39–41 kDa corresponding to BRI2 in the cerebellum of normal controls (1–4) and in the frontal cortex in a case of familial British dementia (FBD) (5). **(C)** Western blot analysis of furin using furin convertase antibody demonstrating the expected two bands of 90 and 96 kDa in the cerebellum of normal controls (1–4) and the frontal cortex of a case of FBD (5). The higher molecular weight represents the zymogen form of furin, while the lower molecular weight species corresponds to the active form of the enzyme.

Figure 2.

In situ hybridization for BRI2 mRNA demonstrated strong expression in neurones in all areas of the normal brain. (A) In the frontal cortex, neurones of all sizes express the mRNA with extension into the apical dendrite of pyramidal cells (insert), while vessels are negative (arrows). (B) The granule cells of the hippocampal dentate fascia strongly express BRI2 mRNA. (C) In the cerebellum, the Purkinje cells and outer stellate cells of the cerebellar molecular layer (arrows) are strongly stained, while only a proportion of granule cells show a narrow rim of staining around the nucleus. Glial mRNA expression was demonstrated in the cerebellar white matter (insert in C). (D) Positive staining of neurones in the dentate nucleus. Bar in A represents 60 μ m in A; 30 μ m in B, C, D and insert in A, and 20 μ m in insert in C.

Figure 3.

Immunohistochemical staining with the Ab340 antibody which recognizes the full-length wild-type BRI2 and N-terminal fragments of both wild-type and mutant precursor proteins produced by furin cleavage was used to demonstrate the distribution of BRI2 in normal brain. (A) In the parietal cortex, protein expression was demonstrated in all neuronal types. (B) Cerebellar Purkinje cells show abundant cytoplasmic staining, and there is a narrow band of staining around the nucleus in some of the granule cells. Vessels are unstained (arrows).

Immunohistochemical staining for furin was used to determine the distribution of protein expression in the normal brain. (C) Temporal cortex showing neuronal expression of furin. (D) A vessel in the frontal white matter showing furin expression in smooth muscle cells. Bar in A represents 60 μ m in A and C, and 30 μ m in B, D and inserts in A and C.

Figure 4.

In a case of FBD, the distribution of BRI2 mRNA, wild-type or mutant BRI2 and furin was examined and compared with that of ABri. **B, D, F** and **H** represent serial sections of a small white matter arteriole. (**A** and **B**) Immunohistochemical staining with antibody Ab338 confirms the presence of ABri in amyloid-laden vessels (arrow in **A**) and parenchymal plaques. (**C** and **D**) BRI2 mRNA is present in neurones and glia (insert in **D**) as well as in parenchymal (**C**) and vascular amyloid deposits (**D** and arrow in **C**). The insert in **C** shows a leptomenigeal artery from the medial temporal lobe with BRI2 mRNA deposited in amyloid (arrow) while the smooth muscle cells (double arrow) are unstained. In **D**, an amyloid-laden arteriole (see also **B**) with complete loss of smooth muscle cells is strongly labelled for BRI2 mRNA. (**E** and

F) Antibody Ab340 stains parenchymal amyloid plaques (insert in **E**) and amyloid-laden vessels (**F** and arrow in **E**) in addition to neurones. As in the normal brain, unaffected vessels contained neither BRI2 mRNA nor BRI2 (not shown). (**G** and **H**) Furin immunohistochemistry demonstrates enzyme in parenchymal and vascular amyloid deposits in addition to neurones. (**A**, **C**, **E** and **G**) Hippocampal CA4 sub-region, arrows indicate amyloid-laden small vessels. (**B**, **D**, **F** and **H**) Frontal white matter. Bar in **A** represents 60 μm in all panels and 30 μm in the inserts in **C** and **D**.

Figure 5.

Double-immunofluorescence labelling and confocal microscopy in control brain tissue shows colocalization of furin innunoreactivity with that of the neuronal marker Neu N (row **A**), and also between BRI2 and Neu N (row **D**). Furin could not be demonstrated in astrocytes, as labelling did not colocalize with GFAP (row **B**), although BRI2 expression in astrocytes could be visualized (row **E**). Expression of furin and BRI2 by microglia was investigated in a case of familial British dementia (FBD) by double labelling using CD68 as a microglial marker. This revealed a diffuse pattern of both furin (row **C**) and BRI2 (row **F**) expression in microglia. A granular pattern of ABri amyloid accumulation in microglia in FBD (arrows in row **G**) indicating that phagocytosis of amyloid is apparent when double labelling for thioflavine S as

a stain for amyloid and CR3/43 as a marker of activated microglia is employed (row **G**). This is different from the diffuse staining pattern for BRI2 shown in panel **F**. Bar in row **A** = 12 μm in row **A**, 30 μm in row **B**, 10 μm in row **C**, 20 μm in rows **D** and **E**, and 23 μm in rows **F** and **G**.

Table 1

Cases used for immunohistochemistry and Western blotting

Number	Age (years)	Sex	Diagnosis
1	43	M	Myocardial infarction
2	63	M	Congestive cardiac failure
3	75	M	Pulmonary embolism
4	81	M	Chronic obstructive airway disease
5	68	F	FBD
6	79	M	AD
7	73	F	AD
8	78	M	AD
9	43	F	AD
10	46	F	Variant AD with cotton wool plaques

M, male; F, female; FBD, familial British dementia; AD, Alzheimer's disease.

Table 2
Sequences of polymerase chain reaction (PCR) primers and oligonucleotide probe used in the study

A			
Transcript	Sense	Antisense	Product size (bp)
BR12	CGTTCAACTCCGGCTCTGGC	GCAAACTCTTGGGACAGGCACAC	378
GAPDH*	ACCACAGTCCATGCCATCAC	TCCACCACTCTGTGCTGTA	433
B			
Transcript	Oligonucleotide probe sequence		Probe length (nt)
BR12 28–66	CCGCTCTGGGCTCGTCCTCTTGGCTCCTTCTGGGCCAG		41
BR12609–648	ACCCAGGTGATCAATGTTTCAATGCGATCAGTAATAACC		39
BR12802–836	TATTAATTTTCCTCAATAATGTTTTTCTTGACTGT		34

A, PCR primers used in analysis of gene transcription. Primers marked with * are based on previously published data [51]. B, oligonucleotide sequences used for *in situ* hybridization, complementary to residues 28–66, 609–648 and 802–836 of BR12 cDNA.

Table 3

Antibodies used in the study

Antibody	Source	Concentration for immunoblotting	Concentration for immunohistochemistry
Ab338	[11]	1:2000	1:1000
Ab340	[50]	1:500	1:500
Ab340 pre-immune serum		1:500	1:500
Ab340 immuno-absorbed		1:500	1:500
Furin convertase	Affinity Bioreagents	1:1000	1:50
GFAP	Dako	-	1:1000
NeuN	Chemicon	-	1:1000
CR3/43	Dako	-	1:150
CD68	Dako	-	1:200

Table 4
Summary of the findings for BRI2 mRNA, BRI2 protein and furin in control brains and amyloid lesions in FBD and AD

	Neurones	Glia	Vessels	ABri amyloid lesions			A β amyloid lesions		
				ABri pre-amyloid lesions	V	P	A β pre-amyloid lesions	V	P
BRI2 mRNA	+	+	-	-	+	+	-	+	+
BRI2	+	+	-	-	+	+	-	-	-
Furin	+	+	+	-	+	+	-	-	-

V, vascular; P, plaque; FBD, familial British dementia; AD, Alzheimer's disease.