Danish and British dementia ITM2b/BRI2 mutations reduce BRI2 protein stability and impair glutamatergic synaptic transmission

Received for publication, August 17, 2020, and in revised form, November 9, 2020. Published, Papers in Press, November 10, 2020, https://doi.org/10.1074/jbc.RA120.015679

Tao Yin , Wen Yao , Alexander D. Lemenze , and Luciano D’Adamio

From the 1 Department of Pharmacology, Physiology & Neuroscience New Jersey Medical School, Brain Health Institute, Jacqueline Krieger Klein Center in Alzheimer’s Disease and Neurodegeneration Research, Rutgers, The State University of New Jersey, Newark, New Jersey, USA; 2 Department of Pathology, Immunology, and Laboratory Medicine, New Jersey Medical School, The State University of New Jersey, Newark, New Jersey, USA

Edited by Paul Fraser

Mutations in integral membrane protein 2B (ITM2b/BRI2) gene cause familial British and Danish dementia (FDD and FBD), autosomal dominant disorders characterized by progressive cognitive deterioration. Two pathogenic mechanisms, which may not be mutually exclusive, have been proposed for FDD and FBD: 1) loss of BRI2 function; 2) accumulation of amyloidogenic mutant BRI2-derived peptides, but the mechanistic details remain unclear. We have previously reported a physiological role of BRI2 in excitatory synaptic transmission at both presynaptic termini and postsynaptic termini. To test whether pathogenic ITM2b mutations affect these physiological BRI2 functions, we analyzed glutamatergic transmission in FDD and FBD knock-in mice, which carry pathogenic FDD and FBD mutations into the mouse endogenous Itm2b gene. We show that in both mutant lines, spontaneous glutamate release and AMPAR-mediated responses are decreased, while short-term synaptic facilitation is increased, effects similar to those observed in Itm2bKO mice. In vivo and in vitro studies show that both pathogenic mutations alter maturation of BRI2 resulting in reduced levels of functional mature BRI2 protein at synapses. Collectively, the data show that FDD and FBD mutations cause a reduction of BRI2 levels and function at synapses, which results in reduced glutamatergic transmission. Notably, other genes mutated in Familial dementia, such as APP, PSEN1/PSEN2, are implicated in glutamatergic synaptic transmission, a function that is altered by pathogenic mutations. Thus, defects in excitatory neurotransmitter release may represent a general and convergent mechanism leading to neurodegeneration. Targeting these dysfunction may offer a unique disease modifying method of therapeutic intervention in neurodegenerative disorders.

ITM2b/BRI2 gene is a gene that codes a type II membrane protein called BRI2. The immature BRI2 precursor (imBRI2) is cleaved at the C-terminus by proprotein convertase to produce the mature NH2-terminal BRI2 (mBRI2) and a COOH-terminal peptide of 23 amino acid called Bri23 (1). Two autosomal dominant mutations in ITM2b/BRI2 cause FDD and FBD. The FDD mutation consists of a 10-nucleotide duplication one codon before the normal stop codon (2). This produces a frameshift in the BRI2 sequence generating a precursor protein 11 amino acids larger than normal. In the British kindred, a point mutation at the stop codon of BRI2 results in a read-through of the 3'-untranslated region and the synthesis of a BRI2 molecule containing 11 extra amino acids at the COOH-terminus (3). Convertase-mediated cleavage of immature mutant Danish and British BRI2 generates two distinct 34 amino acid long peptides, called ADan and ABri, respectively. Both peptides are deposited as amyloid fibrils. For clarity, we will refer to the wild-type imBRI2 as BRI2-Adan, to the Danish mutant imBRI2 as BRI2-Adan, and to the British mutant imBRI2 as BRI2-Abri. Since all three imBRI2 proteins form the same mBRI2 polypeptide, all mBRI2 proteins will be referred to as mBRI2.

Using a genetic approach to inactivate Itm2b in either presynaptic (CA3), or postsynaptic (CA1), or both (CA3+CA1) neurons of the hippocampal Schaeffer-collateral pathway (SC–CA3>CA1 synapses) in mice, we found a physiological role of BRI2 in the excitatory synaptic transmission (4). Loss of Bri2 impairs glutamatergic neurotransmitter release and AMPAR-mediated responses in SC–CA3>CA1 synapses by a presynaptic and postsynaptic mechanism, respectively. This evidence suggests that Bri2 facilitates glutamate transmission via both presynaptic and postsynaptic mechanism. Given the relevance of excitatory synaptic transmission to cognition and that BRI2 mutations cause neurodegeneration and dementia in humans, we tested whether the pathogenic Danish and British dementia mutations will alter, one way or another, this physiological function of BRI2. To test this hypothesis, we studied glutamatergic neurotransmission in SC–CA3>CA1 synapses of mice carrying the pathogenic Danish and British dementia mutations (Itm2bD/D and Itm2bB/B mice) into the Itm2b mouse gene (5–7). We choose a knock-in (KI) approach because: a) KIs mimic familial dementia genetics and make no assumption about pathogenic mechanisms; b) expression of mutant genes is controlled by endogenous regulatory elements, thereby allowing us to test the hypothesis in a biologically relevant

* For correspondence: Luciano D’Adamio, luciano.dadamio@rutgers.edu.
Itnm2b and excitatory transmission

model organism. This strategy allows examination of the effects of pathogenic Itnm2b mutations on the function of Bri2 in excitatory synaptic transmission on both presynaptic and postsynaptic neurons of the Schaeffer-collateral pathway.

Results

Pathogenic Bri2 mutations impair glutamatergic transmission at excitatory hippocampal SC–CA3>CA1 synapses

Itnm2b^{WT/WT} and Itnm2b^{B/B} mice were derived as described previously (5–7). To test whether these pathogenic mutations alter the synaptic function of Bri2 in the excitatory synaptic transmission at SC–CA3>CA1 synapses (4), we compared the following genotypes: Itm2b^{WT/WT}, Itm2b^{KO/KO} (mice carrying two knockout Itm2b alleles), Itm2b^{DD} (mice carrying two Itm2b alleles with the Danish mutation), and Itm2b^{B/B} (mice carrying two Itm2b alleles with the British mutation). Synaptic transmission was studied in 4 to 6-month-old Itm2b^{WT/WT} (three males and three females), Itm2b^{KO/KO} (three males and three females), Itm2b^{DD} (three males and three females), Itm2b^{B/B} (four males and three females) mice. First, we analyzed miniature excitatory postsynaptic currents (mEPSC), the frequency of which is also determined, in part, by the probability of synaptic vesicles release (P). With our previous report is probably due to the lower number of recordings analyzed here. Yet, both Itm2b^{DD} and Itm2b^{B/B} mice showed increased PPF as compared with control Itm2b^{WT/WT} animals at 50 ms ISI (Fig. 1). Overall, our data indicate that Itm2b inactivation and both Danish and British pathogenic Itm2b mutations alter glutamatergic synaptic transmission at excitatory hippocampal SC–CA3>CA1 synapses analogously.

Reduced synaptic levels of mBri2 in Itm2b^{B/B} and Itm2b^{DD} mice

The evidence that genetic inactivation of Bri2 expression in pre- and postsynaptic neurons reduced excitatory synaptic transmission at hippocampal SC–CA3>CA1 synapse indicates that neuronal, and perhaps synaptic, mBri2 expression is important for physiological glutamate transmission. To determine whether the synaptic impairment in Itm2b^{B/B} and Itm2b^{DD} mice is associated with loss of mBri2 at synapses, we examined Bri2 expression in synaptoneurosomes fraction (Fig. 2A shows the quality of synaptosomal fractions) prepared from whole brain lysates of 4 to 6-month-old mice (n = 6 for each genotype). We observed the following: 1) Bri2 is present in synaptoneurosomes (Fig. 2, A–B); 2) only mBri2 is found in synaptoneurosomes (Fig. 2, A–B), which is consistent with the evidence that only mature Bri2 transported to surface membrane (9); 3) mBri2 expression is significantly and similarly reduced in Itm2b^{B/B} and Itm2b^{DD} mice compared with Itm2b^{WT/WT} mice (Fig. 2, B–C). These results may explain why glutamatergic synaptic transmission is similarly affected in Itm2b^{KO/KO}, Itm2b^{B/B}, and Itm2b^{DD} mice.

The synaptoneurosome preparation includes synaptic elements from both excitatory and inhibitory neurons. To ascertain Itm2b mRNA expression in various neuronal populations, we analyzed publicly available scRNA-seq to test Itm2b mRNA coexpression with GABA<sub>A</sub>-receptor (Gabra1, Gabrb3, Gabrg2), AMPA-receptor (Gria1, Gria2, Gria3, Gria4), and NMDA-receptor (Grin1, Grin2a, Grin2b) genes. This analysis shows similar levels of coexpression of Itm2b with these neuronal markers (Fig. 2D). In addition, we determine coexpression of Itm2b with Scl32a1, which encodes for the vesicular inhibitory amino acid transporter Viaat and is a marker of inhibitory neurons, Scl17a7 and Scl17a6, which encode for vesicular glutamate transporter 1 Vglut1 and vesicular glutamate transporter 2 Vglut2, respectively, and are markers of excitatory neurons. The results suggest that Itm2b mRNA is equally expressed in excitatory and inhibitory neurons (Fig. 2D). This data suggest that it will be worth, in future experiments, testing the effect of Bri2 and the effects of the Danish and British mutations on GABAergic transmission as well.

Trafficking and maturation of mutant human BRI2 proteins are altered in transfected cell line

To investigate how the British and Danish pathogenic mutations affect BRI2 expression, we transfected HeLa cell with constructs driving expression of either BRI2-Bri23, BRI2-Adan, or BRI2-ABri fused to a Flag-peptide epitope at the
Figure 1. The Danish and British mutations reduce glutamatergic synaptic transmission at hippocampal SC–CA3–CA1 synapses. A, representative recording traces of mEPSC at SC–CA3–CA1 pyramidal cell synapses. B, the deletion of Itm2b (Itm2bKO/KO), the British and Danish mutations (Itm2bB/B and Itm2bD/D) cause a significant decrease in mEPSC frequency [ANOVA summary, F(3, 40) = 35.44, p < 0.0001**]; post-hoc Tukey’s multiple comparisons test: Itm2bWT/WT versus Itm2bKO/KO, p < 0.0001****; Itm2bWT/WT versus Itm2bB/B, p < 0.0001****; Itm2bWT/WT versus Itm2bD/D, p = 0.9982 (ns); Itm2bKO/KO versus Itm2bB/B, p < 0.0001****; Itm2bKO/KO versus Itm2bD/D, p = 0.9969 (ns); Itm2b−/− versus Itm2bB/B, p = 0.9982 (ns); Itm2bKO/KO versus Itm2bD/D, p = 0.9825 (ns); Itm2bB/B versus Itm2bD/D, p = 0.9969 (ns). C, cumulative probability of AMPAR-mediated mEPSC frequency inter event intervals. D, Itm2bKO/KO, Itm2bB/B and Itm2bD/D mice show a significant decrease in mEPSC amplitude [ANOVA summary, F(3, 40) = 16.59, p < 0.0001****; post-hoc Tukey’s multiple comparisons test: Itm2bWT/WT versus Itm2bKO/KO, p = 0.0004***; Itm2bWT/WT versus Itm2bB/B, p < 0.0001****; Itm2bWT/WT versus Itm2bD/D, p < 0.0001****; Itm2bKO/KO versus Itm2bB/B, p = 0.5223 (ns); Itm2bKO/KO versus Itm2bD/D, p = 0.2569 (ns); Itm2bB/B versus Itm2bD/D, p = 0.9516 (ns). E, cumulative probability of AMPAR-mediated mEPSC amplitude. F, in contrast, decay time of mEPSC was modestly increased, but only in the Itm2bD/D mice [F(2, 30) = 3.284, p = 0.0304*; post-hoc Tukey’s multiple comparisons test: Itm2bWT/WT versus Itm2bKO/KO, p = 3.590 (ns); Itm2bWT/WT versus Itm2bB/B, p = 0.6339 (ns); Itm2bWT/WT versus Itm2bD/D, p = 0.0176*; Itm2bKO/KO versus Itm2bB/B, p = 0.9585 (ns); Itm2bKO/KO versus Itm2bD/D, p = 0.4707 (ns); Itm2bB/B versus Itm2bD/D, p = 0.2433 (ns)]. G, average mEPSC of the three groups depicts differences in amplitude. H, AMPA/NMDA ratio is significantly decreased in all three mutant mice [ANOVA summary, F(3, 65) = 22.73, p < 0.0001****; post-hoc Tukey’s multiple comparisons test: Itm2bWT/WT versus Itm2bKO/KO, p = 0.0004***; Itm2bWT/WT versus Itm2bB/B, p < 0.0001****; Itm2bWT/WT versus Itm2bD/D, p = 0.0176*; Itm2bKO/KO versus Itm2bB/B, p = 0.0679 (ns); Itm2bKO/KO versus Itm2bD/D, p = 0.9256 (ns); Itm2bB/B versus Itm2bD/D, p = 0.9506 (ns); PPF at 200 ms ISI: ANOVA summary F(3, 63) = 0.7463, p = 0.5285 (ns)]. Representative traces are shown on top of the panels. Data are represented as mean ± SD and were analyzed by ordinary one-way ANOVA followed by post-hoc Tukey’s multiple comparisons test when ANOVA showed significant differences. We used n = 6 animals for each genotype, three males and three females.
**Itm2b and excitatory transmission**

Figure 2. Expression of mBri2 is reduced in synaptoneurosome fractions derived from Itm2b<sup>WT/WT</sup> and Itm2b<sup>Δ/Δ</sup> mice. A, synaptoneurosome fractions (SP) were separated from total brain homogenates (S1) of Itm2b<sup>WT/WT</sup> and Itm2b<sup>Δ/Δ</sup> and were further separated into triton-X soluble (SPs) and insoluble fraction (SPI). Presynaptic proteins (such as Vamp2) are enriched in SP as compared with S1 and are mainly distributed in the SPs fraction. Postsynaptic proteins (such as NmdaR-2b and Psd95) are enriched in SP as compared with S1 and are mainly distributed in the SPi fraction. These data attest the good quality of the SP preparation. Consistent with pre- and postsynaptic activity of Bri2, mBri2 is found in both pre- and postsynaptic enriched fractions. Since

![Graph showing expression levels of mBri2 and Psd95 in different genotypes](image_url)
NH2-terminus (ITM2bF\textsuperscript{WT}, ITM2bF\textsuperscript{D}, and ITM2bF\textsuperscript{B}, driving expression of F-BRI2-Bri23, F-BRI2-Adan, and F-BRI2-Atri, respectively). The levels of mF-BRI2 were significantly higher in ITM2bF\textsuperscript{WT} lysate as compared with ITM2bF\textsuperscript{B} and ITM2bF\textsuperscript{D} (Fig. 3, A–B). Also, the levels of F-BRI2-Bri23 were significantly higher compared with F-BRI2-Adan and F-BRI2-Atri (Fig. 3, A–B). In addition, the ratio of mF-BRI2 to immature F-BRI2 precursor proteins was lower in ITM2bF\textsuperscript{B} and ITM2bF\textsuperscript{D} as compared with ITM2bF\textsuperscript{WT} transfected cells: this difference was highly significant for ITM2bF\textsuperscript{D} samples (Fig. 3, A–B). Although potential differences in transfection efficiencies may be confounding factors, together these data suggest that the British and Danish pathogenic mutations reduce stability and maturation of precursor BRI2-Adan and BRI2-Atri as compared with wild-type BRI2-Bri23.

To further analyze the effect of pathogenic mutation on BRI2 polypeptides trafficking to cell membranes, HeLa cells transfected with BRI2 constructs were surface biotinylated, and the lysates were precipitated with streptavidin (SA) beads to isolate cell membrane proteins (fraction B). Unbound fraction (supernatant fraction U) contains proteins that are not present on the cell membrane and/or exposed to extracellular space. Analysis of the total lysate (T) and fractions B and U confirmed that mBRI2, but not BRI2-Bri23, BRI2-Adan, and BRI2-Atri, was present on the cell surface (fraction B, Fig. 3, C–D). Given the reduction in mBRI2 levels, ITM2bF\textsuperscript{B} and ITM2bF\textsuperscript{D} transfected cells presented significantly reduced levels of mBRI2 localized to the plasma membrane compared with ITM2bF\textsuperscript{WT} transfected cells (fraction B, Fig. 3, C–D).

Plasma membrane BRI2 undergoes an additional cleavage by ADAM10 in its ectodomain (10). This cleavage releases a soluble variant of BRI2 and a membrane-bound BRI2 N-terminal fragment (BRI2-NTF). As shown in Figure 3C, BRI2-NTFs are visible (and are biotinylated indicating the presence on plasma membranes) in cells transfected with WT BRI2 but not FDD and FBD mutant BRI2, supporting the notion that more mBRI2 is generated from BRI2-Bri23 as compared with BRI2-Adan and BRI2-Atri.

While in synaptoneurosome (Fig. 2B) the two pathogenic mutations seem to reduce equally mBri2 levels, there is a more pronounced difference for BRI2-Adan than BRI2-Atri on mBri2 in HeLa cells, suggesting that the Danish and British mutations may impair maturation of Bri2 in a quantitatively distinct manner in different cell types.

To better describe trafficking of wild-type and mutant BRI2 proteins, we transfected the F-BRI2 constructs in N2A cells with differentiation and visualized the distribution of BRI2 polypeptides by immunocytochemistry (Fig. 4A). This experiment is not used as a proxy for synaptic localization of BRI2. We defined two types of BRI2 staining patterns: identifiable surface membrane staining (compatible with plasma membrane BRI2 localization) versus internal staining compatible with BRI2 localization in endoplasmic reticulum, Golgi apparatus, and/or other cytosolic organelles (Fig. 4B). Based on these parameters, we quantified a significant reduction of cells with identifiable surface membrane staining but a significant increase in cells with internal staining in ITM2bF\textsuperscript{B} and ITM2bF\textsuperscript{D} transfected cells compared with ITM2bF\textsuperscript{WT} transfected cells (Fig. 4C). Consistent with these changes, surface biotinylation of transfected N2A cells showed reduced levels of mBRI2 localized to the plasma membrane in ITM2bF\textsuperscript{B} and ITM2bF\textsuperscript{D} transfected cells compared with ITM2bF\textsuperscript{WT} transfected cells (Fig. 4C). Also, in this experiment, BRI2-NTFs are visible and detectable on the plasma membrane in cells transfected with WT BRI2 but not FDD and FBD mutant BRI2.

In summary, these observations indicate that pathogenic mutation reduces maturation and plasma membrane localization of mBRI2.

Increased lysosomal mediated degradation of BRI2-Adan and BRI2-Atri

Transmembrane proteins that enter the ER, but fail to attain the native structure, are rapidly degraded to prevent toxic accumulation of aberrant gene products. These proteins are delivered to endo-lysosome for clearance by autophagic and nonautophagic pathways collectively defined as ER-to-lysosomes-associated degradation (ERLAD) (11). To investigate whether such a mechanism could underlie the maturation deficit and the reduction of mBRI2 caused by the Danish/British mutations, we prepared primary hippocampus neurons from ITM2b\textsuperscript{WT/WT}, ITM2b\textsuperscript{B/B}, and ITM2b\textsuperscript{D/D} mice. Primary neurons were treated with the lysosomal inhibitor chloroquine.

The first noteworthy observation is that without treatment, the steady-state levels of mBri2, Bri2-NTF as well as the ratio of m/im forms of Bri2 were significantly reduced in ITM2b\textsuperscript{B/B} and ITM2b\textsuperscript{D/D} neurons as compared with ITM2b\textsuperscript{WT/WT} neurons (Fig. 5A). This observation parallels what seen in cell lines expressing human wild-type and mutant BRI2. The evidence that chloroquine significantly reduced the LC3A I/LC3A II and LC3B I/LC3B II ratios indicates inhibition of lysosome-mediated degradation. In contrast, GAPDH levels are not changed by chloroquine treatment indicating specificity and lack of toxicity (Fig. 5, A–B). In neurons derived from ITM2b\textsuperscript{WT/WT} hippocampi, chloroquine caused a significant and predominant accumulation of mBri2, suggesting that mBri2 may be degraded by lysosome after endocytosis from plasma...
membranes. In contrast, chloroquine treatment caused exclusively a considerable accumulation of BRI2-ABri and BRI2-ADan in Itm2bB/B and Itm2bD/D neurons, respectively (Fig. 5, A–B).

Discussion

Our studies suggest that BRI2-ADan and BRI2-ABri may be targeted for ERLAD. The evidence that ADan and ABri peptides tend to aggregate and form amyloid deposits (2, 3) is consistent with the hypothesis that the immature mutant BRI2 proteins, which contain these sequences at the COOH-terminus, may misfold in the ER (Fig. 6). However, a fraction of BRI2-ADan and BRI2-ABri must escape lysosomal degradation and traffic through the trans-Golgi where a convertase removes the ADan and ABri peptides, since: 1) ADan and ABri peptides are produced in humans carrying the Danish and British mutation, respectively (2, 3); 2) cells transfected with ITM2bF-D and ITM2bF-B, Itm2bB/B and Itm2bD/D brain lysates and primary neurons contain some mBri2 (Figs. 2–5). In the central nervous system, these maturation anomalies lead to partial loss of mBri2 in synaptoneurosomes (Fig. 2), reduced glutamatergic neurotransmitter release, and AMPAR-mediated responses in Itm2bB/B and Itm2bD/D animals (Fig. 1).

Although future studies are needed to assess whether these excitatory synapses dysfunctions described here are pathologically relevant, the evidence presented here is noteworthy because also other genes mutated in Familial forms of dementia, such as APP, PSEN1, and PSEN2, are implicated in glutamatergic synaptic transmission. APP modulates glutamate release (12–15), a function that is altered by a pathogenic APP mutation (the Swedish mutation) (15). In addition, long-term potentiation (LTP), a cellular surrogate for memory and memory impairment caused by extracellular Aβ and Tau oligomers are APP-dependent (16). PSEN1 and PSEN2 regulate glutamate release by a presynaptic mechanism, a function that is altered by pathogenic mutation (17–19). Finally, a genetic variant of TREM2 that increases risk of sporadic AD in humans changes glutamatergic transmission (20) in a rat KI model (21). Our findings that BRI2 also physiologically fine-tunes glutamate transmission with both pre- and postsynaptic mechanisms and that pathogenic mutations dysregulate this function are intriguing and support the hypothesis that defects in excitatory neurotransmitter release may
represent a general and convergent mechanism leading to neurodegeneration.

Years ago, we proposed that familial Danish, British, and Alzheimer’s dementias may share a pathogenic sameness. This hypothesis was based on the following evidence: 1) APP and BRI2 physically interact (9, 22–24); 2) BRI2 alters APP processing (9, 24, 25); 3) APP and APP processing mediate LTP deficits as well as learning and memory deficits of Danish and British KI mice (26–31). In this context, the evidence that both APP and BRI2 tune excitatory synaptic transmission and that these functions are altered by pathogenic mutations in both APP and BRI2 may functionally interact if in tuning glutamatergic transmission. Future studies will be needed to test this hypothesis.

**Experimental procedures**

**Mice and ethics statement**

Mice were handled according to the Ethical Guidelines for Treatment of Laboratory Animals of the NIH. The procedures were described and approved by the Institutional Animal Care and Use Committee (IACUC, protocol number PROTO201702513).

**Mice slice preparation**

Mice were deeply anesthetized with isoflurane and intracardially perfused with an ice-cold cutting solution containing (in mM) 120 choline chloride, 2.6 KCl, 26 NaHCO3, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgCl2, 1.3 ascorbic acid, 15 glucose,
Figure 5. Distinct degradation pathways of wild-type and mutant Bri2 forms in primary neuron isolated from \textit{ltm2\textsuperscript{WT/WT}}, \textit{ltm2\textsuperscript{B/B}}, and \textit{ltm2\textsuperscript{D/D}} mice. 

\(A\), western immunoblot analysis of primary hippocampal neurons isolated from \textit{ltm2\textsuperscript{WT/WT}}, \textit{ltm2\textsuperscript{KO/KO}}, \textit{ltm2\textsuperscript{B/B}}, and \textit{ltm2\textsuperscript{D/D}} P1 pups treated with 50 \(\mu\text{M}\) chloroquine for 18 h. 

\(B\), quantification of imBri2, mBri2, Bri2 NTF, GAPDH, and LC3A/B levels. Data are represented as mean ± SD and analyzed by ordinary two-way ANOVA followed by post-hoc Sidak’s multiple comparisons test when ANOVA showed significant differences [ANOVA summary, LC3A: \(F_{\text{interaction}}(3,16) = 2.99, p = 0.0621, F_{\text{treatment}}(1, 16) = 395, p < 0.0001, F_{\text{genotype}}(3, 16) = 4.645, p = 0.0161, \) post-hoc Sidak’s multiple comparisons test: veh versus Chlor, \(p < 0.0001^{****}, \) \textit{ltm2\textsuperscript{B/B}}: veh versus Chlor, \(p < 0.0001^{****}, \) \textit{ltm2\textsuperscript{D/D}}: veh versus Chlor, \(p < 0.0001^{****}, \) \textit{ltm2\textsuperscript{WT/WT}}: veh versus Chlor, \(p < 0.0001^{****}, \) \textit{ltm2\textsuperscript{B/B}}: veh versus Chlor, \(p < 0.0001^{****}, \) \textit{ltm2\textsuperscript{D/D}}: veh versus Chlor]
prebubbled with 95% O₂/5% CO₂ for 15 min. The brains were rapidly removed from the skull. Coronal brain slices containing the hippocampal formation (350 μm thick) were prepared in the ice-cold cutting solution bubbled with 95% O₂/5% CO₂ using Vibratome VT1200S (Leica Microsystems) and then incubated in an interface chamber in ACSF containing (in mM): 126 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.3 MgCl₂, 2.4 CaCl₂, 26 NaHCO₃, and 10 glucose (at pH 7.3), bubbled with 95% O₂ and 5% CO₂ at 30 °C for 1 h, and then kept at room temperature. The hemi-slices were transferred to a recording chamber perfused with ACSF at a flow rate of ~2 ml/min using a peristaltic pump. Experiments were performed at 28.0 °C ± 0.1 °C.

Whole-cell electrophysiological recording

Whole-cell recordings in the voltage-clamp mode (~70 mV) were made with patch pipettes containing (in mM): 132.5 Cs-glucanate, 17.5 CsCl, 2 MgCl₂, 0.5 EGTA, 10 HEPES, 4 ATP, and 5 QX-314, with pH adjusted to 7.3 by CsOH. Patch pipettes (resistance, 8–10 MΩ) were pulled from 1.5 mm thin-walled borosilicate glass (Sutter Instruments, Novato, CA) on a horizontal puller (model P-97; Sutter Instruments, Novato, CA). Basal synaptic responses were evoked at 0.05 Hz by electrical stimulation of the Schaffer collateral afferents using concentric bipolar electrodes. CA1 neurons were viewed under upright microscopy (FN-1, Nikon Instruments, Melville, NY) and recorded with Axopatch-700B amplifier (Molecular Devices, San Jose, CA). Data were low-pass filtered at 2 kHz and acquired at 5 to 10 kHz. The series resistance (Rs) was consistently monitored during recording in case of reseal of ruptured membrane. Cells with Rs >20 MΩ or with Rs deviated by >20% from initial values were excluded from analysis. Excitatory postsynaptic currents (EPSCs) were recorded in ACSF containing 15 μM bicuculline methiodide to block GABA-A receptors. The stimulation intensity was adjusted to evoke EPSCs that were 40% of the maximal evoked amplitudes ("test intensity"). 5 to 10 min after membrane rupture, EPSCs were recorded for 7 min at a test stimulation intensity that produced currents of ~40% maximum. For recording of paired-pulse ratio (PPR), paired-pulse stimuli with 50 ms or 200 ms interpulse interval were given. The PPR was calculated as the ratio of the second EPSC amplitude to the first. For recording of AMPA/NMDA ratio, the membrane potential was firstly held at ~70 mV to record only AMPAR current for 20 sweeps with 20 s intervals. Then the membrane potential was turned to +40 mV to record NMDAR current for 20 sweeps with perfusion of 5 μM NBQX to block AMPAR. Mini EPSCs were recorded by maintaining neurons at ~70 mV with ACSF containing 1 μM TTX and 15 μM bicuculline methiodide to block action potentials and GABA-A receptors, respectively. mEPSCs were recorded for 5 to 10 min for analysis. Data were collected and analyzed using the Axopatch 700B amplifiers and pCLAMP10 software (Molecular Devices), and mEPSCs are analyzed using mini Analysis Program.

Mice brain preparation and synaptoneurosome preparation

Mouse brain fractions were prepared as follows. Brains were homogenized (w/v = 100 mg tissue/1 ml buffer) in buffer A (5 mM HEPES, 1 Mm MgCl₂, 0.5 mM CaCl₂, PH7.4) supplemented with protease/phosphatase inhibitor (Thermo Fisher Scientific 78440) on ice. Homogenate was centrifuged at 1400g for 10 min. The supernatant was collected and labeled as S1 fraction. S1 was centrifuged at 13,800g for 10 min to generate pellet. The pellet was resuspended in 300ul buffer B (0.32 M sucrose, 6 mM Tris, pH 8.0), layered a discontinuous sucrose gradient (0.85, 1.0 and 1.2 M sucrose, 6 mM Tris, pH 8.0), and centrifuged at 83,000g for 2 h. SP fraction was present as a white band at the 1.0/1.2 M interface. The SP fraction was lysed with Buffer T (5% Triton X-100, 6 mM Tris, pH 8.0) to achieve final Triton-X 100 concentration to 0.5%. After 15 min shaking on ice, samples were centrifuged at 20,000g for 20 min. The separated supernatant was collected and labeled as Triton soluble (SPs) fraction. The pellet was resuspended in 50 ml Buffer T and was labeled as Triton insoluble (SPI) fraction.

scRNA-seq analysis

Full project data was downloaded from the Mouse Development Atlas (32). The full expression values and metadata were downloaded in loom format and imported to Seurat for additional analyses. Cells expression values were normalized using scran and clustered following the standard workflow (33). Cluster markers were transferred from the development atlas to ensure purity with cell classifications. For cell coexpression of molecules, a normalized value of 1 was considered positive for the query gene of interest, and the expression of Itm2b was pulled for each positive cell for comparative visualization.

Plasmids

Constructs of Flag-human full-length BRI2 (ITM2bF-WT) in pcDNA3.1 were previously described (24). Flag-FBD mutant BRI2 (ITM2bF-D) and Flag-FDD mutant BRI2 (ITM2bF-D) were
both obtained by mutagenesis of $ITM2b^{F-WT}$. $ITM2b^{F-B}$ expressed the extra C-terminal 11 amino acids long sequence “RTVKKNIIEEN”. In $ITM2b^{F-D}$, the last amino acid, a Ser (S), is replaced by “FNLFLNSQEKHY.”

**Cell culture and transfection**

Neuro-2A (N2A) cells (ATCC CCL-131) and Hela cells (ATCC CCL-2) were maintained in Eagle’s Minimum Essential Medium (EMEM) (Gibco 11095-098) supplemented with 10% fetal bovine serum and Antibiotic-Antimycotic (Gibco 15240112). Both Hela cells and N2A cells were transfected with indicated plasmids via Fugene kit (Promega, E2311) as previously described (34–36).

**Immunocytochemistry and cell counting**

The N2A cells were plated on coverslips precoated with poly L-lysine (Sigma, P4707). Twenty-four hours after transfection, N2A cells were differentiated by 0.1% FBS and 2 mM retinoic acid for 24 h. Differentiated N2A cells were washed with PBS, fixed by 4% PFA, permeabilized with 0.1% Triton X-100 in PBS, blocked with 10% BSA in PBS, incubated with Anti-Flag M2 FITC (Sigma, F4049), and visualized on confocal microscope (Nikon A1R). For cell counting, we replicated $n = 4$ independent transfection and took ten nonoverlapped images for each transfection. An average of 250 cells were counted for each transfection.

**Biotinylation, streptavidin precipitation**

Biotinylation experiments were performed as described (9). Twenty-four hours after transfection, HeLa cells or N2A cells were washed three times with cold PBS plus $Ca^{2+}$ and $Mg^{2+}$ (PBS-CM) and labeled for 30 min on ice in 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) dissolved in PBS-CM. Media was removed by washing three times with PBS-CM containing 0.1% BSA. The cells were lysed in RIPA buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 140 mM NaCl). The lysates were cleared by centrifuging at 20,000g for 10 min and were mixed with streptavidin agarose beads (Sigma S1638). After collecting unbound lysate (U), the beads were washed four times with the RIPA buffer and were boiled in 2× SDS buffer (B). Comparable volume of the samples was subjected to Western immunoblot. The streptavidin precipitants correspond to five times of total lysates or unbound proteins.

Figure 6. Modeling wild-type and mutant BRI2 maturation, trafficking, and degradation. A, BRI2 is synthesized in the ER as an immature precursors (BRI2-Bri23) that is cleaved in the Golgi apparatus at the C-terminus by proprotein convertase to produce the mature (mBRI2) and the COOH-terminus peptide Bri23. mBRI2 is transported to the plasma membrane where it can undergo an additional cleavage by ADAM10 in its ectodomain. This cleavage releases sBRI2 and the membrane-bound BRI2-NTF. Both mBRI2 and BRI2-NTF are endocytosed and degraded by the lysosomes. B, the Danish mutation changes the last amino acid of BRI2 and adds 11 additional amino acids at the COOH-terminus. The British mutations add 11 extra amino acids, which are different from those added by the Danish mutation, at the COOH-terminus as well. A fraction of immature mutant Danish (BRI2-ADan) and British (BRI2-ABri) BRI2 traffic to the Golgi where convertase cleavage generates mBRI2 and two distinct 34 amino acid long peptides, called ADan and ABri, respectively. ADan and ABri can form amyloid fibrils. It is important noting that mBRI2 produced by these pathogenic precursors is identical to the wild-type mBRI2. Large fractions of BRI2-ADan and BRI2-ABri are targeted for ERLAD. It is likely that the ADan and ABri sequences may cause misfolding of BRI2-ADan and BRI2-ABri in the ER.
Table 1  
List of antibodies used

| Antibody      | Brand               | Cat. # | Host    | Type       | Usage note                   |
|---------------|---------------------|--------|---------|------------|------------------------------|
| α-tubulin     | Sigma-Aldrich       | T6199  | Mouse   | monoclonal | 1:1000                       |
| Br2-N terminus| D'Adamo's lab       |        |         |            |                              |
| NMDA R2B      | Cell Signaling Technology | 4212 | Rabbit  | polyclonal | 6 μg/ml                      |
| PSD95         | Cell Signaling Technology | 2507 | Rabbit  | polyclonal | 1:1000                       |
| Vamp2         | Synaptic Systems    | 104,211| Mouse   | monoclonal | 1:1000                       |
| Flag-hrp      | Sigma-Aldrich       | A8592  | Mouse   | monoclonal | 1:1000                       |
| Flag-6x-c     | Sigma-Aldrich       | F4049  | Mouse   | monoclonal | 1:1000                       |
| LC3A          | Cell Signaling Technology | 4599 | Rabbit  | monoclonal | 1:1000                       |
| LC3B          | Cell Signaling Technology | 2775 | Rabbit  | polyclonal | 1:1000                       |
| GAPDH         | Sigma-Aldrich       | G9545  | Rabbit  | polyclonal | 1:1000                       |

Primary hippocampus neuron culture

Mouse hippocampus neurons were derived from Itm2b<sup>WT/WT</sup>, Itm2b<sup>KO/KO</sup>, Itm2b<sup>B/B</sup>, and Itm2b<sup>D/D</sup> postnatal day 0 mouse pups. Briefly, after the removal of adherent meninges, the hippocampi were collected in dissection media (HBSS w/o Mg and Ca, 1 mM sodium pyruvate, 0.1%glucose, 0.01 M HEPES). The hippocampi were dissected into single cell suspension with trituration following 15 min 37 °C 0.25% trypsin digestion. Cells were subsequently applied 0.1% Dnase (Sigma, dn25) and enriched in plating media (BME, 10% FBS, 0.09% Glucose, 1 mM Sodium Pyruvate, 2 mM Glutamine, 1× Pen/Strep). Cells were passed through Falcon 70 μm nylon cell strainer and counted with Hemacytometer. 300k dissociated cells were plated in each single well in Poly L lysine pretreated 12-well-plate in 1 ml of maintenance media (Neurobasal media, 1× B-27, 2 mM glutamine, 1× Pen/Strep). Cells were refeed with 0.5 ml of maintenance media every 2 days. Cells were used for further treatment at DIV 14.

Pharmacological treatment and sample preparation

Primary neurons were treated with 50 mM Chloroquine (Cell signaling, 14774s) or same amount of PBS (Veh) for 18 h. Treated cell were washed with PBS and lysed in RIPA buffer with protease/phosphatase inhibitor for 15 min on ice. Lysed samples were centrifuged at full speed for 15 min. The supernatants were mix with LDS buffer supplemented with 10% β-mercapto-ethanol. Samples were boiled for 2 min before loading to a gel.

Western immunoblot analysis

Protein content quantified by Bradford analysis. Ten micromgrams from each fraction was brought to 10 μl with PBS and LDS buffer supplemented with 10% β-mercapto-ethanol and 8 M Urea. Denatured samples were loaded on a 4 to 12% Bis-Tris polyacrylamide gel (Bio-Rad, 3450125) and run at constant voltage (140 V). Proteins were transferred onto nitrocellulose membrane at 25 V for 7 min using Trans-blot Turbo system (Bio-Rad) and visualized by red Ponceau staining. Membranes were blocked with 5% nonfat dry milk (Bio-Rad, 1706404), washed with PBS/Tween-20 (0.05%), and applied primary antibody (see list and information about the dilution used in Table 1), which is diluted in blocking solution (Thermo Fisher Scientific, 37573) overnight at 4 °C. Membranes were washed 3 × 10 min and subsequently against by either HRP linked antimouse (SouthernBiotech, OB103105) or anti-rabbit (1:1 mix of SouthernBiotech, OB405005 and Cell Signaling Technology, 7074) at 1:1000 dilution for 45 min with shaking in room temperature. Membranes were developed with West Dura ECL reagent (Thermo Fisher Scientific, PI34076) and visualized on a ChemiDoc MP Imaging System (Bio-Rad). Signal intensity was quantified with Image Lab software (Bio-Rad). Data were analyzed using Prism software and represented as mean ± SD.

Statistics

All the experiments mentioned in the paper were analyzed by one-way ANOVA or two-way ANOVA by indication. Data showing statistical significance by one-way ANOVA or two-way ANOVA were subsequently analyzed either by Tukey’s multiple comparisons test or by Sidak’s multiple comparisons. All statistical analyses were performed using Prism 8 (GraphPad) software.

Data availability

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments—All the authors read and approved the final manuscript.

Author contributions—L. D. generated the animals; T. Y. performed the biochemical and molecular experiments; W. Y. performed the electrophysiology experiments; A. D. analyzed the scRNA-Seq data; all the authors designed the experiments; L. D. and T. Y. wrote the paper.

Funding and additional information—L. D. was funded by the NIH/NIA R01AG052286, RF1AG064821-01, RF1AG064821-01, 1R01AG033007-01A109. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: EMEM, Eagle’s Minimum Essential Medium; ERLAD, ER-to-lysosomes-associated degradation; FBD, familial British dementia; FDD, familial Danish
Itm2b and excitatory transmission

- dementia; ISI, interstimulus interval; KL, knock-in; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic currents; PPF, paired-pulse facilitation; PPR, paired-pulse ratio.

References

1. Choi, S. I., Vidal, R., Frangione, B., and Levy, E. (2004) Axonal transport of British and Danish amyloid peptides via secretory vesicles. *FASEB J.* **18**, 373–375
2. Vidal, R., Revesz, T., Rostagno, A., Kim, E., Holton, J. L., Bek, T., Bojen-Moller, M., Braenggaard, H., Plant, G., Ghiso, J., and Frangione, B. (2000) A decamer duplication in the 3' region of the BRI gene originates an amyloid peptide that is associated with dementia in a Danish kindred. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4920–4925
3. Vidal, R., Frangione, B., Rostagno, A., Mead, S., Révész, T., Plant, G., and Ghiso, J. (1999) A stop-codon mutation in the BRI gene associated with familial British dementia. *Nature* **399**, 776–781
4. Yao, W., Yin, T., Tambini, M. D., and D'Adamio, L. (2019) The Familial dementia gene Itm2b/BRI2 facilitates glutamate transmission via both presynaptic and postsynaptic mechanisms. *Sci. Rep.* **9**, 4862
5. Tamayev, R., Matsuda, S., Fa, M., Arancio, O., and D'Adamio, L. (2010) Danish dementia mice suggest that loss of function and not the amyloid cascade causes synaptic plasticity and memory defects. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 20822–20827
6. Tamayev, R., Giliberto, L., Li, W., d’Abramo, C., Arancio, O., Vidal, R., and D'Adamio, L. (2010) Memory deficits due to familial British dementia BRI2 mutation are caused by loss of BRI2 function rather than amyloidosis. *J. Neurosci.* **30**, 14915–14924
7. Giliberto, L., Matsuda, S., Vidal, R., and D'Adamio, L. (2009) Generation and initial characterization of FDD knock in mice. *PLoS One* **4**, e7900
8. Zucker, R. S., and Regehr, W. G. (2002) Short-term synaptic plasticity. *Annu. Rev. Physiol.* **64**, 355–405
9. Matsuda, S., Matsuda, Y., Snapp, E. L., and D'Adamio, L. (2008) Regulated intramembrane proteolysis of Bri2 (Itm2b) by ADAM10 mediates synaptic plasticity and memory de
10. Martin, L., Fluhrer, R., Reiss, K., Kremmer, E., Saftig, P., and Haass, C. (2004) Axonal transport of British and Danish amyloid peptides via secretory vesicles. *FASEB J.* **18**, 373–375
11. Fregno, I., and Molinari, M. (2019) Proteasomal andlysosomal clearance of faulty secretory proteins: ER-associated degradation (ERAD) and ER-to-lysosome-associated degradation (ERLAD) pathways. *Crit. Rev. Biochem. Mol. Biol.* **54**, 153–163
12. Fanutza, T., Del Prete, D., Ford, M. J., Castillo, P. E., and D'Adamio, L. (2015) APP and APLP2 interact with the synaptic release machinery and facilitate transmitter release at hippocampal synapses. *Elife* **4**, e09743
13. Fogel, H., Frere, S., Segev, O., Bharill, S., Shapira, L., Gazit, N., O'Malley, T., Slomowitz, E., Berdichovsky, Y., Walsh, D. M., Isacoff, E. Y., Hirsch, J. A., and Slutsky, I. (2014) APP homodimers transduce an amyloid-beta-mediated increase in release probability at excitatory synapses. *Cell Rep.* **7**, 1560–1576
14. Yao, W., Tambini, M. D., Liu, X., and D'Adamio, L. (2019) Tuning of glutamate, but not GABA, release in a intrasynaptic vesicles APP domain whose function can be modulated by beta- or alpha-secretase cleavage. *J. Neurosci.* **39**, 6992–7005
15. Tamayev, R. and D'Adamio, L. (2011) JNK-1 independent of, yet dependent. *J. Neurochem.* **119**, 1000–100054
16. Scheinfeld, M. H., Roncarati, R., Vito, P., Lopez, P. A., Abdallah, M., and D’Adamio, L. (2002) Jun NH2-terminal kinase (JNK) interacting protein 1 (JIP) binds the cytoplasmic domain of the Alzheimer’s beta-amyloid precursor protein (APP). *J. Biol. Chem.* **277**, 3767–3775
17. Scheinfeld, M. H., Matsuda, S., and D’Adamio, L. (2003) JNK-interacting protein-1 promotes translocation of A beta protein precursor but not A beta precursorlike proteins, mechanistically different than Fe65. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 1729–1734
18. Scheinfeld, M. H., Ghersi, E., Davies, P., and D’Adamio, L. (2003) Amyloid beta protein precursor is phosphorylated by JNK-1 independent of, yet facilitated by, JNK-interacting protein (JIP)-1. *J. Biol. Chem.* **278**, 42058–42063
19. Zhang, D., Zhang, C., Ho, A., Kirkwood, A., Sudhof, T. C., and Shen, J. (2010) Inactivation of presenilins causes pre-synaptic impairment prior to post-synaptic dysfunction. *J. Neurochem.* **115**, 1215–1221
20. Xia, D., Watanabe, H., Wu, B., Lee, S. H., Li, Y., Tsvetkov, E., Bolshakov, V. Y., Shen, J., and Kelleher, R. J., 3rd (2015) Presenilin-1 knockin mice reveal loss-of-function mechanism for familial Alzheimer’s disease. *Neuron* **85**, 967–981
21. Ren, S., Yao, W., Tambini, M. D., Yin, T., Norris, K. A., and D’Adamio, L. (2020) Microglia. *Elife* **9**, e57513
22. Matsuda, S., Giliberto, L., Matsuda, Y., Davies, P., McGowan, E., Pickford, F., Ghiso, J., Frangione, B., and D’Adamio, L. (2005) The familial dementia BRI2 gene binds the Alzheimer gene amyloid-beta precursor protein and inhibits amyloid-beta production. *J. Biol. Chem.* **280**, 28912–28916
23. Fotinopoulou, A., Tsachaki, M., Vlavaki, M., Pouloupolous, A., Rostagno, A., Frangione, B., Ghiso, J., and Efthimiopoulos, S. (2005) BRI2 interacts with amyloid precursor protein (APP) and regulates amyloid beta (Abeta) production. *J. Biol. Chem.* **280**, 30768–30772
24. Matsuda, S., Giliberto, L., Matsuda, Y., McGowan, E. M., and D’Adamio, L. (2008) BRI2 inhibits amyloid beta-peptide precursor protein processing by interfering with the docking of secretases to the substrate. *J. Neurosci.* **28**, 8668–8676
25. Matsuda, S., Tamayev, R., and D’Adamio, L. (2011) Increased AβPP processing in familial Danish dementia patients. *J. Alzhei. Dis.* **27**, 385–391
26. Tamayev, R., Matsuda, S., Giliberto, L., Arancio, O., and D’Adamio, L. (2011) APP heterozygosity averts memory deficit in knockin mice expressing the Danish dementia BRI2 mutant. *EMBO J.* **30**, 2501–2509
27. Tamayev, R., and D’Adamio, L. (2012) Inhibition of γ-secretase worsens memory deficits in a genetically congruous mouse model of Danish dementia. *Mol. Neurodegener.* **7**, 19
28. Tamayev, R., and D’Adamio, L. (2012) Memory deficits of British dementia knock-in mice are prevented by Aβ-precursor protein haploinsufficiency. *J. Neurosci.* **32**, 5481–5485
29. Tamayev, R., Matsuda, S., Arancio, O., and D’Adamio, L. (2012) - but not γ-secretase proteolysis of APP causes synaptic and memory deficits in a mouse model of dementia. *EMBO Mol. Med.* **4**, 171–179
30. Lombino, F., Biundo, F., Tamayev, R., Arancio, O., and D’Adamio, L. (2013) An intracellular threonine of amyloid-β precursor protein mediates synaptic plasticity deficits and memory loss. *PLoS One* **8**, e57120
31. Biundo, F., Ishiowari, K., Del Prete, D., and D’Adamio, L. (2016) Deletion of the γ-secretase subunits Apl1/B/C impairs memory and worsens the deficits of knock-in mouse modeling the Alzheimer-like familial Danish dementia. *Oncotarget* **7**, 11923–11944
32. Zeinel, A., Hochgermher, L., Lünnerberg, P., Johnsson, A., Menic, F., van der Zwan, J., Häring, M., Braun, E., Born, L. E., La Manno, G., Code-luppi, S., Furlan, A., Lee, K., Skene, N., Harris, K. D., et al. (2018) Molecular architecture of the mouse nervous system. *Cell* **174**, 999–1014. e1022
33. Hafemeister, C., and Satija, R. (2019) Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* **20**, 296