A familial Danish dementia rat shows impaired presynaptic and postsynaptic glutamatergic transmission

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Familial British dementia and familial Danish dementia are neurodegenerative disorders caused by mutations in the gene integral membrane protein 2B (ITM2b) encoding BRI2, which tunes excitatory synaptic transmission at both presynaptic and postsynaptic termini. In addition, BRI2 interacts with and modulates proteolytic processing of amyloid-β precursor protein (APP), whose mutations cause familial forms of Alzheimer’s disease (AD) (familial AD). To study the pathogenic mechanisms triggered by the Danish mutation, we generated rats carrying the Danish mutation in the rat Itm2b gene (Itm2bD rats). Given the BRI2/APP interaction and the widely accepted relevance of human amyloid β (Aβ), a proteolytic product of APP, to AD, Itm2bD rats were engineered to express two humanized App alleles and produce human Aβ. Here, we studied young Itm2bD rats to investigate early pathogenic changes in these diseases. We found that periadolescent Itm2bD rats not only present subtle changes in human Aβ levels along with decreased spontaneous glutamate release and α-amino-3-hydroxy-5-methyl-4-isoxazoleproionic acid receptor-mediated responses but also had increased short-term synaptic facilitation in the hippocampal Schaeffer-collateral pathway. These alterations in excitatory interneuronal communication can impair learning and memory processes and were akin to those observed in adult mice producing rodent Aβ and carrying either the Danish or British mutations in the mouse Itm2b gene. Collectively, the data show that the pathogenic Danish mutation alters the physiological function of BRI2 at glutamatergic synapses across species and early in life. Future studies will determine whether this phenomenon represents an early pathogenic event in human dementia.

Model organisms that reproduce the pathogenesis of human diseases are useful to dissect disease mechanisms, identify therapeutic targets, and test therapeutic strategies. Because genetic manipulation has been easier in mice, mice have overtaken rats as the major rodent-based model organism in neurodegeneration research. Thus, to study familial Danish dementia (FDD) and familial British dementia (FBD), 15 years ago, we generated mice carrying the pathogenic Danish and British dementia mutations (integral membrane protein 2B [ITM2b]; Itm2bB rats and Itm2bD mice) into the Itm2b mouse gene (1–3). We choose a knock-in (KI) approach rather than the more common transgenic overexpression approach for several reasons. KIs mimic the genetic of FDD and FBD and make no assumption about pathogenic mechanisms (except the unbiased genetic one), whereas the transgenic approach aims to reproduce pathology (plaques, neurofibrillary tangles [NFTs], etc.), under the assumption that this “pathology” is pathogenic. In KI models, expression of mutant genes is controlled by endogenous regulatory elements, avoiding issues related to overexpression of disease proteins in a nonphysiological quantitative–spatial–temporal manner. Finally, potential confounding “insertion” effects of transgenes are avoided.

Because rats are better suited to study neurodegenerative diseases, we took advantage of recent developments in gene-editing technologies and introduced the familial Danish mutation into the genomic Itm2b rat locus (Itm2bD rats). The rat was the organism of choice for most behavioral, memory, and cognitive research, which is critical when studying neurodegenerative diseases—because physiological processes are similar in rats and humans and the rat is an intelligent and quick learner (4–7).

Several procedures that are important in dementia research are more easily performed in rats as compared with mice because of the larger size of the rat brain. Cannulas—to administer drugs, biologics, viruses, and others—and microdialysis probes—for sampling extracellular brain levels of neurotransmitters, amyloid β (Aβ), soluble tau, and others—can be accurately directed to individual brain regions, causing less damage and increasing specificity. In vivo brain imaging techniques, such as MRI (8) and PET (9–11), can assess the extent and course of neurodegeneration with better spatial resolution in rats. Moreover, rats are large enough for convenient in vivo electrophysiological recordings or serial sampling of cerebrospinal fluid for detection of biomarkers.

Finally, gene-expression differences suggest that rats may be advantageous model of neurodegenerative diseases over mice. For example, alternative spicing of tau (12–15), which forms NFTs and is mutated in frontotemporal dementia (16–23), leads to expression of tau isoforms with three or four...
microtubule-binding domains (3R and 4R, respectively). Adult human and rat brains express both 3R and 4R tau isoforms (24): in contrast, adult mouse brains express only 4R tau (25), suggesting that the rat may be a better model organism for dementias with tauopathy, such as FDD and FBD.

BRI2 physically interacts with and modulates processing of amyloid-β precursor protein (APP), which bears relevance to Alzheimer’s disease (AD) pathogenesis (26–30). In addition, APP processing mediates long-term potentiation and memory deficits of Danish and British KI mice (31–36). Aggregated forms of Aβ, a product of APP processing, are by and large considered the main pathogenic molecule in AD. Rat and human APP differ by three amino acids in the Aβ region: given that human Aβs are believed to have higher propensity to form toxic Aβ species as compared with rodent Aβs, we produced rats carrying the humanized Aβ sequence (App<sup>h</sup>) rats (37, 38).

Thus, to study possible interactions between the Danish mutation and human Aβ, Itm2b<sup>D</sup> rats were backcrossed to App<sup>h</sup> rats. Hence, all rats used in this study produce human and not rodent Aβ species.

Here, we studied periadolescent Itm2b<sup>D</sup> rats, with the purpose of investigating early dysfunctions that may underlie initial pathogenic mechanisms leading to dementia later in life.

Results

Generation of Itm2b<sup>D</sup> KI rats carrying humanized App<sup>h</sup> alleles

The KI founder F0-Itm2b<sup>D</sup> rat, which is carrying FDD mutation on Itm2b rat gene, was generated by CRISPR/Cas–mediated genome engineering as described in the Experimental procedures section and Supporting information. The F0-Itm2b<sup>D</sup> rat, which is a chimera for the Itm2bw<sup>w</sup> gene, was crossed to WT (Itm2bw<sup>w/w</sup>) Long–Evans rats to generate F1-Itm2b<sup>D/w</sup> rats. F1-Itm2b<sup>D/w</sup> rats were crossed to WT Long–Evans to generate F2-Itm2b<sup>D/w</sup> rats. These crossings were repeated three more times to obtain F5-Itm2b<sup>D/w</sup> rats. The probability that F5 rats carry unidentified off-target mutations (except those, if present, on chromosome 15) is ~1.5625%. Male and female F5-Itm2b<sup>D/w</sup> rats were crossed to obtain Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup>, and Itm2b<sup>w/w</sup> rats.

The FDD mutation consists of a ten nucleotides duplication one codon before the normal stop codon (39). This produces a frameshift in the BRI2 sequence generating a precursor protein 11 amino acids larger than normal (Fig. 1A). To verify that the Danish mutation was correctly inserted into Itm2b exon 6, we amplified by PCR the Itm2b gene exon 6 Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup>, and Itm2b<sup>w/w</sup> rats. Sequencing of the PCR products shows that the Danish mutation was correctly inserted in the Itm2b gene exon 6 (Fig. 1B) and encoded for the COOH terminus of the Danish BRI2 mutant. When we generated FDD KI mice, we humanized the mouse COOH-terminal region of BRI2 by introducing an alanine (A) substituted for threonine (T) at codon 250 (3). Since that humanization did not result into deposition of ADan peptides in amyloid plaques in KI mice (3), that modification was not repeated in rats.

To generate Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup>, and Itm2b<sup>w/w</sup> rats on a background in which rat App has a humanized Aβ region, Itm2b<sup>D/w</sup> and App<sup>h/w</sup> rats were crossed to generate Itm2b<sup>D/w</sup>; App<sup>h/w</sup> rats. The App<sup>w</sup> allele was removed in subsequent crosses. Henceforth, Itm2b<sup>D/D</sup>, Itm2b<sup>D/w</sup>, and Itm2b<sup>w/w</sup> rats used in this study have an App<sup>h/w</sup> background and produce human and not rodent Aβ species.

To determine whether Itm2b expression is disrupted by the introduced mutations, we examined Itm2b mRNA levels in p21 Itm2b<sup>D/D</sup> and Itm2b<sup>w/w</sup> rats by standard RNA-Seq analysis on total brain RNA. The mRNA expression of Itm2b shows no significant difference between Itm2b<sup>D/D</sup> and Itm2b<sup>w/w</sup> rats (Fig. 1C).

The Itm2b<sup>D</sup> allele encodes for a longer Bri2 precursor protein (Bri2-ADan) that accumulates in primary neurons

BRI2 is a type II membrane protein that is synthesized as an immature precursor (imBRI2). imBRI2 is cleaved at the COOH terminus by proprotein convertase to produce the NH<sub>2</sub>-terminal mature Bri2 (mBRI2). When we generated FDD KI mice, we humanized the mouse COOH-terminal region of Bri2 by introducing an alanine (A) substituted for threonine (T) at codon 250 (3). Since that humanization did not result into deposition of ADan peptides in amyloid plaques in KI mice (3), that modification was not repeated in rats.

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![Figure 1. Characterization of Itm2b<sup>D</sup> KI rats. A, sequences of the COOH terminus of Bri2–23 (WT) and Bri2-ADan (Danish). B, PCR amplification and sequencing of the Itm2b gene exon 6 from Itm2b<sup>w/w</sup> and Itm2b<sup>D/D</sup> rats shows that the Danish mutation was correctly inserted in the Itm2b exon 6 of Itm2b<sup>D/D</sup> rats. This mutation causes the predicted frameshift in the BRI2 sequence generating a precursor protein 11 amino acids larger-than-normal coding for the Bri2-ADan mutant protein (partial DNA sequences of WT and Danish exon 6 are shown). Inserted nucleotides are highlighted in black, and the amino-acid sequences are indicated above for the Danish mutant allele and below for the WT allele—the DNA sequences. C, levels of Itm2b mRNA in brains of 21-day-old Itm2b<sup>w/w</sup> and Itm2b<sup>D/D</sup> rats were determined by Standard-RNA-Seq analysis. No significant differences between Itm2b<sup>w/w</sup> and Itm2b<sup>D/D</sup> rats were evident. Data are represented as mean ± SD. Data were analyzed by Student’s t test. N = 4 rats per genotype. KI, knock in.](image-url)
Danish patients, a frameshift caused by a ten nucleotide duplication 5’ to the stop codon leads to the synthesis of a BRI2 precursor protein 11 amino acids larger than normal (39). Convertase-mediated cleavage of Danish imBRI2 generates a WT-like mBRI2 and a 34 amino acid–long peptide called ADan, which codeposits with Aβ species in amyloid fibrils in patients. For clarity, we will refer to the WT imBri2 as Bri2–Bri23 and to the Danish mutant imBri2 as Bri2-ADan.

To determine whether the Itm2bD allele codes for Bri2-ADan, we examined Bri2 expression in total neuronal lysates isolated from male and female 2-month-old Itm2bD/w, Itm2bD/D, and Itm2bw/w rats. However, the Bri2 antibody tested identified many nonspecific bands (Fig. S1), making a rigorous assessment of Bri2 expression in rat brains challenging.

Analysis of mouse Itm2bw/w and Itm2bD/D primary neurons showed that the mBri2/Bri2–Bri23 primary neurons was significantly higher than the mBri2/Bri2-ADan ratio in Itm2bw/w primary neurons (41). In addition, lysosomal inhibition caused accumulation of mBri2 but not Bri2–Bri23 in Itm2bw/w primary neurons; in contrast, both mBri2 and Bri2-ADan accumulated in Itm2bD/D primary neurons (41). These observations indicated that the Danish mutation reduced maturation of the mutant precursor Bri2 in mouse neurons. Based on these observations, we probed whether primary neurons could be used to assess mBri2, Bri2–Bri23, and Bri2-ADan expression in KI rats. Primary neurons are a simpler system compared with total brain; this, per se, may reduce the number of nonspecific bands identified by anti-Bri2 antibodies. Moreover, inhibition of lysosome-mediated degradation of Bri2 species in primary neurons may help identify specific Bri2 molecules. Thus, primary neurons derived from Itm2bw/w and Itm2bD/D rats were treated with the lysosomal inhibitor chloroquine and analyzed by Western blot. The anti-Bri2 antibody identified a band of ~34 kDa in all samples, which was increased by chloroquine (Fig. 2, A and C). These observations are consistent with the ~34 kDa corresponding to mBri2. A second band of ~36 kDa was detected in Itm2bw/w primary neurons (Fig. 2A). In contrast, a slightly

Figure 2. Distinct degradation pathways of Bri2–23 and Bri2-ADan in Itm2bw/w and Itm2bD/D primary neurons. WB analysis of Bri2 (A) and LC3A/B (B) from primary hippocampal neurons isolated from Itm2bw/w (+) and Itm2bD/D (−) P1 pups treated with either the vehicle PBS (−) or 50 μM chloroquine (+) for 18 h. C, quantification of LC3A/B and Bri2 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. Detailed statistical analysis results are shown in Table 1. WB, Western blotting.
larger second band (~37 kDa) that was increased by chloroquine treatment was detected in Itm2b<sup>D/D</sup> primary neurons (Fig. 2, A and C). These observations are consistent with the ~36 and ~37 kDa bands corresponding to Bri2–Bri23 and Bri2-ADan, respectively.

Without treatment, the levels of Bri2-ADan in Itm2b<sup>D/D</sup> primary neurons were significantly higher than the levels of Bri2–Bri23 in Itm2bw<sup>w/w</sup> primary neurons (Fig. 2, A and C), and the mBri2/Bri2–Bri23 ratio in Itm2bw<sup>w/w</sup> primary neurons was significantly higher than the mBri2/Bri2-ADan ratio in Itm2b<sup>D/D</sup> primary neurons (Fig. 2, A and C).

LC3A and LC3B are autophagosome membrane proteins: the LC3A I/LC3A II and LC3B I/LC3B II ratios for each provides an independent measure of the impact of chloroquine on lysosomal degradation. Chloroquine significantly reduced the LC3A I/LC3A II and LC3B I/LC3B II ratios (Fig. 2, B and C), confirming inhibition of lysosome-mediated degradation.

**Subtle increase in Aβ42 levels in young Itm2b<sup>D</sup> KI rats**

Sequential processing of APP by α-, β-, and γ-secretase (26–30), we determined the steady-state levels of several of these APP metabolites in the central nervous system of young male and female Itm2b<sup>D</sup> KI rats. Full-length APP, α-CTF, and β-CTF were measured by Western blot; soluble APPs (sAPPα/sAPPβ) were detected by ELISA, and human Aβ species (Aβ38, Aβ40, Aβ42, and Aβ43) were detected by human Aβ-specific ELISA. These measurements have previously been used for other KI rats generated in our laboratory (38, 42, 43).

Neither the levels of full-length APP, CTFs, Aβ38, Aβ40, Aβ42, sAPPα, and sAPPβ were unchanged in 8-week-old Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup>, and Itm2bw<sup>w/w</sup> rats (Fig. 3, A–C) nor was the Aβ43/Aβ42 ratio altered (Fig. 3C). In contrast, there was a slight but significant increase in Aβ42 as well as the Aβ42/ Aβ40 ratio in Itm2b<sup>D/D</sup> compared with Itm2bw<sup>w/w</sup> rats (Fig. 3C). Small but statistically significant decreases in both Aβ43 and Aβ43/Aβ42 ratios were evident in Itm2b<sup>D/D</sup> as compared with Itm2bw<sup>w/w</sup> rats (Fig. 3C). Overall, these data indicate a gene dosage–dependent minor increase in steady-state levels of Aβ42, and decrease in Aβ43, in periadolescent Itm2bw<sup>w</sup> rats. Analysis of older rats will be needed to determine whether the Danish mutation in Itm2b alters APP processing in KI rats and whether these alterations may more robustly change the steady-state levels of APP metabolites with aging.

### Table 1

| Source of variation | Interaction | Treatment | Genotype | Sidak’s multiple comparisons test | Summary | Adjusted p |
|---------------------|-------------|-----------|----------|----------------------------------|---------|------------|
| imBri2              | F (DFn, Dfd) | p         |          |                                  |         |            |
|                     | F (1, 20) = 272.2 |          |          |                                  |         | <0.0001    |
|                     | F (1, 20) = 354.3 |          |          |                                  |         | <0.0001    |
|                     | F (1, 20) = 1966 |          |          |                                  |         | <0.0001    |
| Interaction         | F (1, 20) = 207.4 |          |          |                                  |         | <0.0001    |
| Treatment           | F (1, 20) = 186.9 |          |          |                                  |         | <0.0001    |
| Genotype            | F (1, 20) = 228.5 |          |          |                                  |         | <0.0001    |
| mBri2               | F (DFn, Dfd)   | p         |          |                                  |         |            |
|                     | F (1, 20) = 354.3 |          |          |                                  |         | <0.0001    |
|                     | F (1, 20) = 1966 |          |          |                                  |         | <0.0001    |
| Interaction         | F (1, 20) = 207.4 |          |          |                                  |         | <0.0001    |
| Treatment           | F (1, 20) = 186.9 |          |          |                                  |         | <0.0001    |
| Genotype            | F (1, 20) = 228.5 |          |          |                                  |         | <0.0001    |
| LC3A/I/I            | F (DFn, Dfd)   | p         |          |                                  |         |            |
|                     | F (1, 20) = 353.5 |          |          |                                  |         | <0.0001    |
|                     | F (1, 20) = 361.6 |          |          |                                  |         | <0.0001    |
| Interaction         | F (1, 20) = 37.36 |          |          |                                  |         | <0.0001    |
| Treatment           | F (1, 20) = 353.5 |          |          |                                  |         | <0.0001    |
| Genotype            | F (1, 20) = 361.6 |          |          |                                  |         | <0.0001    |
| mBri2/mBri2         | F (DFn, Dfd)   | p         |          |                                  |         |            |
| mBri2/mBri2         | F (1, 20) = 207.4 |          |          |                                  |         | <0.0001    |
| Treatment           | F (1, 20) = 186.9 |          |          |                                  |         | <0.0001    |
| Genotype            | F (1, 20) = 228.5 |          |          |                                  |         | <0.0001    |

Abbreviation: NS, not significant.

**** indicates p < 0.0001.
It has been postulated that toxic forms of Aβ are oligomeric (44). Thus, we tested whether toxic oligomers are augmented in periadolescent Itm2bD rats. To this end, we used the prefibrillar oligomer-specific antibody A11 to perform dot blots (45). We found no evidence supporting an increase in neurotoxic brain oligomer levels in periadolescent Itm2bD/w and Itm2bD/D rats as compared with Itm2bw/w rats (Fig. 4). However, Aβ oligomers appeared to be significantly increased in Itm2bD/w rats compared with Itm2bD/D animals (Fig. 4). Analysis of older rats will be needed to clarify the relevance of this odd observation.

Glutamatergic synaptic transmission at hippocampal SC–CA3>CA1 synapses is impaired in periadolescent Itm2bD rats

Bri2 modulates glutamatergic synaptic transmission at both presynaptic and postsynaptic termini of Schaeffer-collateral (SC) pathway–CA3>CA1 synapses (46). This function is compromised in both adult Itm2bD and Itm2bD KI mice (41). Here, we analyzed glutamatergic transmission at SC–CA3>CA1 synapses in young periadolescent Itm2bD male and female rats. First, we analyzed miniature excitatory postsynaptic currents (mEPSCs), the frequency of which is determined, in part, by the probability of release (Pr) of glutamatergic synaptic vesicles release (47). Thus, mEPSC frequency is regulated mostly by presynaptic mechanisms. As shown in Figure 5, A–C, the Danish Itm2b mutation caused a significant reduction in the frequency of mEPSC: this reduction is gene dosage dependent (Itm2bD/w versus Itm2bD/D, p = 0.003; Itm2bD/w versus Itm2bD/w, p < 0.0001; and Itm2bD/D versus Itm2bD/D, p = 0.0051) and suggests a decrease in Pr of glutamatergic synaptic vesicles.

The amplitude of mEPSC is instead dependent on postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) responses. AMPAR-mediated mEPSC
responses amplitude was also significantly decreased in Itm2bD rats (Fig. 5, A, D, E, and G). Also in this case, the reduction is gene dosage dependent (Itm2bw/w versus Itm2bD/w, p = 0.0016; Itm2bw/w versus Itm2bD/D, p = 0.0005). Decay time of mEPSC was not significantly affected in Itm2bD rats compared with littermate controls (Fig. 5, A, F, and G).

Since mEPSC AMPAR-mediated responses are reduced in amplitude, we measured the AMPAR/N-methyl-D-aspartic acid (NMDAR) peak current ratio in evoked responses. Consistent with the hypothesis that the Danish Itm2b mutation impairs AMPAR-mediated responses, the AMPAR/NMDAR peak current ratio was reduced in Danish KI rats (Fig. 5H). This difference was statistically different only between Itm2bw/w and Itm2bD/D rats, with Itm2bD rats showing an intermedia phenotype.

Finally, we examined the effect of the pathogenic Danish mutation on paired-pulse facilitation (PPF). PPF is a form of short-term synaptic plasticity that is in part determined by changes in Pr of glutamatergic synaptic vesicles (41, 43, 47). Facilitation at both 50 and 200 ms interstimulus interval (ISI) was significantly increased in Itm2bD/D (Fig. 5J). Even in this case, the changes were gene dosage dependent (50 ms ISI: Itm2bw/w versus Itm2bD/D, p < 0.0001; Itm2bw/w versus Itm2bD/D, p = 0.0031; 200 ms ISI: Itm2bw/w versus Itm2bD/D, p = 0.0205). Interestingly, also an increase in PPF is consistent with a decrease in Pr, just like a decrease in mEPSC frequency. Overall, our data indicate that the pathogenic Danish Itm2b mutation alters glutamatergic synaptic transmission at excitatory hippocampal SC–CA3>CA1 synapses in periadolescent KI rats. These alterations are like those seen in Itm2b KO and Itm2bD/Itm2bB KI adult mice.

### Discussion

The choice of the genetic approach and the model organisms used to model human diseases have major implications on the phenotypic expression of disease-associated genetic mutations. For the last 13 years, our laboratory has modeled AD and AD-like neurodegenerative disorders in mice, using a KI approach (3, 48–51). The KI approach was preferred because it generates models genetically faithful to human diseases and makes no preconceived assumption about pathogenic mechanisms (except the unbiased genetic one). We have recently extended our KI modeling of familial and sporadic forms of AD and AD-related disorders to rats (38, 42, 43, 52, 53) because the rat is better suited for behavioral tests and other procedures that are important when studying neurodegenerative diseases. In addition, gene expression differences suggest that rats may be advantageous model of neurodegenerative diseases over mice. Alternative splicing of Mapt (12–15), which forms NFTs and is mutated in frontotemporal dementia (16–23), leads to expression of tau isoforms with three or four microtubule-binding domains (3R and 4R, respectively). Adult human and rat brains express both 3R and 4R tau isoforms (24); in contrast, adult mouse brains express only 4R tau (25), suggesting that the rat may be a better model organism for dementias with tauopathy.

To explore early dysfunctions that may underlie initial mechanisms leading to dementia, we studied young KI rats...
Figure 5. Glutamatergic synaptic transmission is reduced at hippocampal SC–CA3–CA1 synapses of Itm2b<sup>D</sup> KI rats. 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 the following animals: Itm2bw/w N = 11 (4M/6, 3F/5, indicating that six recordings were obtained from the four males and five recordings from the three females), Itm2bw/D/D N = 15 (3M/7, 4F/8), Itm2bw/D/D N = 10 (3M/5, 3F/5). A, representative recording traces of mEPSC at SC–CA3–CA1 synapses. B, the Danish mutation causes a significant decrease in mEPSC frequency (ANOVA summary, F(2, 33) = 20.66, p < 0.0001****; post hoc Tukey’s multiple comparisons test: Itm2bw/w versus Itm2bw/D/D, p = 0.0033**, Itm2bw/w versus Itm2bw/D/D, p = 0.0001****). C, cumulative probability of AMPAR-mediated mEPSC frequency interevent intervals. D, the Danish mutation causes a significant decrease in mEPSC amplitude (ANOVA summary, F(2, 33) = 10.78, p = 0.0002***; post hoc Tukey’s multiple comparisons test: Itm2bw/w versus Itm2bw/D/D, p = 0.0016**; Itm2bw/w versus Itm2bw/D/D, p = 0.0005***; Itm2bw/D/D versus Itm2bw/D/D, p = 0.6691). E, cumulative probability of AMPAR-mediated mEPSC amplitude. F, in contrast, decay time of mEPSC was not significantly changed (ANOVA summary, F(2, 33) = 1.292, p = 0.2882). G, average mEPSC of Itm2bw/w, Itm2bw/D/D, and Itm2bw/D/D rats. H, AMPAR/NMDAR peak current ratio is significantly decreased in Itm2bw/D/D rats (ANOVA summary, F(2, 16) = 8.417, p = 0.0032**; post hoc Tukey’s multiple comparisons test: Itm2bw/w versus Itm2bw/D/D, p = 0.2393, Itm2bw/w versus Itm2bw/D/D, p = 0.0023**; Itm2bw/D/D versus Itm2bw/D/D, p = 0.0818). Representative traces are shown on top of the
carrying the ITm2bD FDD mutation. Consistent with the findings in ITm2bD/D mouse KIs (41), we found that Bri2-ADan maturation is altered and accumulates in ITm2bD/D primary neurons (Fig. 2). Analysis of APP metabolism in periadolescent ITm2bD KI rats (Figs. 3 and 4) only showed subtle but significant changes in Aβ42 and Aβ43 steady-state levels, which were slightly increased and decreased, respectively (Fig. 3).

We have previously shown that the Danish and British ITM2b mutations lead to reduced glutamatergic neurotransmitter release and AMPAR-mediated responses in adult ITm2bD and ITm2bD mice. These reductions are like those seen in adult ITm2b KO mice (41, 46). Interestingly, we detected identical, gene dosage–dependent, presynaptic and postsynaptic glutamatergic transmission changes in the SC pathway of periadolescent ITm2bD rats (Fig. 5). More specifically, the frequency of mEPSC and PPF is significantly decreased and increased, respectively, in ITm2bD rats, suggesting a presynaptic reduction of the PPF of glutamatergic synaptic vesicles. In addition, mEPSC amplitude and the AMPAR/NMDAR peak current ratio were both decreased in ITm2bD rats, suggesting a postsynaptic reduction of AMPAR-mediated responses. Collectively, these data together with our previously published observations indicate that the synaptic transmission alteration caused by Danish mutation occurs early in life and are neither species nor gene-editing technology specific. These studies underlie the potential relevance of our studies to functional changes caused by the pathogenic ITM2b mutations in humans.

Given the functional and pathological interaction between APP and BRII2 (26–36), it is possible that the presence of human Aβ in the rat model may lead to an earlier manifestation of synaptic plasticity deficit in rat as compared with mice, which express rodent Aβ. Moreover, the evidence that both APP and BRII2 tune excitatory synaptic transmission, and that these functions are altered by pathogenic mutations in both APP and BRII2 (37, 41, 46, 54–56), suggests that early alterations in glutamatergic transmission may underlie initial pathogenic mechanisms in dementia. Future studies will be needed to test these hypotheses.

Experimental procedures

Rats and ethics statement

Rats were handled according to the National Institutes of Health Ethical Guidelines for Treatment of Laboratory Animals. The procedures were described and approved by the Institutional Animal Care and Use Committee at Rutgers University (Institutional Animal Care and Use Committee; protocol number: PROTO201702513).

Generation of rats expressing the Danish ITm2b mutation (ITm2bD rats)

The rat ITm2b gene (GenBank accession number: NM_001006963.1; Ensembl: ENSRNOG00000016271) is located on rat chromosome 15. It comprises six exons, with ATP start codon in exon 1 and TGA stop codon in exon 6. The FDD mutation (TTTAATGTATGGAGCTCACAGCTCAAGAAAACATTAT) KI site in oligo donor was introduced into exon 6, which is the target site by homology-directed repair. A silent mutation (GTG to GTC) was also introduced to prevent the binding and recutting of the sequence by Cas9 after homology-directed repair. The detailed procedures are reported in the supporting information file.

Standard RNA-Seq analysis

Total brain RNA from 21-day-old ITm2bD/D and ITm2bD/D rats (two male and two females per each genotype) was extracted with RNeasy RNA Isolation kit (Qiagen). Standard RNA-Seq procedures and data analysis were performed by Genewiz following proprietary methods (https://cdn2.hubspot.net/hubfs/3478602/NGS/RNA-Seq/GENEWIZ_RNA-Seq_Technical_Specifications_US.pdf). Student’s t test was used for all analyses, with data presented as mean ± SD.

Protein preparation, Western blots, and ELISA of rat brain

These procedures were performed as previously described (32, 53). Briefly, rats were anesthetized with isoflurane and perfused via intracardiac catheterization with ice-cold PBS. Brains were extracted and homogenized with a glass-tellon homogenizer in 250 mM sucrose, 20 mM Tris base, pH 7.4, 1 mM EDTA, 1 mM EGTA plus protease and phosphatase inhibitors (Thermo Fisher Scientific). All steps were carried out on ice. Homogenates were solubilized with 1% NP-40 for 30 min rotating and spun at 20,000g for 10 min. Supernatants were collected, and protein content was quantified by Bradford.

For Western blot analyses, proteins were diluted with PBS and LDS sample buffer—10% β-mercaptoethanol (Invitrogen; NP0007) and 4.5 M urea to 1 μg/μl, loaded on a 4 to 12% Bis–Tris polyacrylamide gel (Bio-Rad; 3450125), and transferred onto nitrocellulose at 25 V for 7 min using the Trans-blot Turbo system (Bio-Rad). Blotting efficiency was visualized by red Ponceau staining on membranes. For dot-blot analysis, 2.5 μg of material was directly spotted with a p20 pipette on a nitrocellulose membrane. Dot membrane was also visualized
by red Ponceau after it was totally dried. Membranes were blocked in 5% milk (Bio-Rad; 1706404) for 30 min and washed in PBS/Tween-20 to 0.05%. Primary antibodies were applied in dilution in blocking solution (Thermo; 37573). The following antibodies were used: polyclonal anti-Bri2 serum bleeds provided by Cell Signaling Technology was used at 1:500 with overnight shaking at 4 °C. APP-Y188 (Abcam; 32136), Oligomer Aβ A11 (shared by Rakez Kayed’s laboratory), LC3A (CST; 4599), and LC3B (CST; 2775) were used at 1:1000 with same other condition. Secondary antibodies (either antimouse [Southern Biotech; 1031-05] or a 1:1 mix of anti-rabbit [Southern Biotech; OB405005] and anti-rabbit [Cell Signaling; 7074]) were diluted 1:1000 in 5% milk and used against either mouse or rabbit primary antibodies for 1 h at room temperature, with shaking. Membranes were washed with PBS/Tween-20 to 0.05% (three times, 10 min each time), developed with West Dura ECL reagent (Thermo: 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 (GraphPad Software, Inc) and represented as mean ± SD.

For analysis of human Aβ peptides and sAPPα/sAPPβ, brain lysates were diluted at 4 ng/μl. Aβ38, Aβ40, and Aβ42 were measured with V-PLEX Plus Aβ Peptide Panel 1 6E10 (K15200G; Meso Scale Discovery), and sAPPα/sAPPβ were measured with sAPPα/sAPPβ (K15120E; Meso Scale Discovery). Plates were read on a MERO QuickPlex SQ 120. Aβ43 was quantified using the IBL human Aβ43 Assay Kit #27710.

Primary hippocampal neuron culture

Rat hippocampal neurons were prepared from Itm2bw/w and Itm2bw/D/D postnatal day 1 pups. Briefly, after removal of meninges, the hippocampi were collected in Hank’s balanced salt solution without magnesium and calcium, 1 mM sodium pyruvate, 0.1% glucose, and 10 mM Heps. Hippocampi were dissected into single cell by trituration followed by 15-min incubation at 37 °C in 0.25% trypsin. Cells were subsequently treated with 0.1% DNase (Sigma; dn25) in plating media (beta-mercaptoethanol, 10% fetal bovine serum, 0.09% glucose, 1 mM sodium pyruvate, 2 mM glutamine, and 1× penicillin/streptomycin). Cells were filtered through a Falcon 70 μm nylon cell strainer and were plated in poly-D-lysine–pretreated 12-well plate (300,000 cells/well) in neurobasal media, 1× B-27, 2 mM glutamine, and 1× penicillin/streptomycin. Half of the culture media was changed every 2 days. The six Itm2bw/w and Itm2bw/D/D cultures shown in Figure 2 were derived from one P1 pup. For each animal, equal amounts of cells were cultured into two wells. For each biological replicate, one well was treated with PBS and one well with chloroquine.

Pharmacological treatment and sample preparation

After 9 days in culture, primary neurons were treated with 50 μM chloroquine (Cell Signaling; 14774s) or PBS (Veh) for 18 h. After treatment, cells were washed with PBS and lysed in radioimmunoprecipitation buffer with protease/phosphatase inhibitor for 15 min on ice. Lysed cells were centrifuged at full speed for 15 min. Cell lysates were quantified and analyzed by Western blot as described earlier for brain lysates.

Electrophysiological recording

These procedures were performed as previously described (52). Briefly, rats were anesthetized with isoflurane and perfused intracardially with an ice-cold cutting solution containing (in millimolar) 120 choline chloride, 2.6 KCl, 26 NaHCO3, 1.25 Na2HPO4, 0.5 CaCl2, 7 MgCl2, 1.3 ascorbic acid, and 15 glucose, prebubbled with 95% O2/5% CO2 for 15 min. The brains were rapidly removed from the skull, and 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 artificial cerebrospinal fluid (ACSF) containing (in millimolar): 126 NaCl, 3 KCl, 1.2 Na2HPO4; 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 hemislices 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 deg. C.

Whole-cell recordings in the voltage-clamp mode (~70 mV) were made with patch pipettes containing (in millimolar): 132.5 Cs gluconate, 17.5 CsCl, 0.5 EGTA, 10 Heps, 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) on a horizontal puller (model P-97; Sutter Instruments). Basal synaptic responses were evoked at 0.05 Hz by electrical stimulation of the SC afferents using concentric bipolar electrodes. CA1 neurons were viewed under upright microscopy (FN-1; Nikon Instruments) and recorded with Axopatch-700B amplifier (Molecular Devices). 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. EPSCs were recorded in ACSF containing the gamma aminobutyric acid-A receptors inhibitor bicculline methiodide (15 μM). The stimulation intensity was adjusted to evoke EPSCs that were 40% of the maximal evoked amplitudes (“test intensity”). About 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, paired-pulse stimuli with 50 or 200 ms interpulse interval were given. The paired-pulse ratio was calculated as the ratio of the second EPSC amplitude to the first. For recording of AMPAR/NMDAR peak current ratio, the membrane potential was first 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 action potential blocker (1 μM tetrodotoxin) and GABA-A receptor inhibitors (15 μM bicuculline methiodide). mEPSCs were recorded for ~10 min. Data were collected with Axopatch 700B amplifiers and analyzed with pCLAMP10 software (Molecular Devices). mEPSCs are analyzed using Mini Analysis Program.

**Statistics**

All the experiments mentioned in the article were analyzed by one-way ANOVA or two-way ANOVA as indicated. Data showing statistical significance by one-way ANOVA or two-way ANOVA were subsequently analyzed by either Tukey’s multiple comparisons test or Sidak’s multiple comparisons. All statistical analyses were performed using Prism 9 software.

**Data availability**

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

**Supporting information**—This article contains supporting information.

**Acknowledgments**—All authors read and approved the final article.

**Author contributions**—L. D. conceptualization; L. D. methodology; T. Y. and L. D. validation; T. Y., W. Y., and L. D. formal analysis; T. Y., W. Y., K. A. N., and L. D. investigation; K. A. N. resources; T. Y. administration; L. D. funding acquisition.

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

**Abbreviations**—The abbreviations used are: Aβ, amyloid β; ACSF, artificial cerebrospinal fluid; AD, Alzheimer’s disease; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid receptor; APP, amyloid-β precursor protein; FBD, familial British dementia; FDD, familial Danish dementia; imBR1, immature BR1; ISI, interstimulus interval; ITM2b, integral membrane protein 2B; KI, knock in; mBR1, mature BR1; mEPSC, miniature excitatory postsynaptic current; NFT, neurofibrillary tangle; NMDAR, N-methyl-D-aspartic acid; PPF, paired-pulse facilitation; Pr, probability of release; Rs, series resistance; SC, Schaeffer-collateral.

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