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- Familial Danish dementia young Knock-in rats expressing humanized APP and human Aβ show impaired pre and postsynaptic glutamatergic transmission

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Running title: Danish dementia and excitatory transmission

Keywords: Familial Danish Dementia, Amyloid precursor protein (APP), Amyloid β, neurodegeneration, synaptic plasticity, rat, animal model, glutamate, Familial Danish Dementia, BRI2, Integral membrane protein 2B (ITM2b)

ABSTRACT

Familial British and Danish dementia (FBD and FDD) are two neurodegenerative disorders caused by mutations in the Integral membrane protein 2B (ITM2b). BRI2, the protein encoded by ITM2b, tunes excitatory synaptic transmission at both pre- and post-synaptic terminus. Too, BRI2 interacts with and modulates proteolytic processing of Amyloid-β precursor Protein (APP), whose mutations cause familial forms of Alzheimer disease (FAD). To study pathogenic mechanism triggered by the Danish mutation we generated rats carrying the Danish mutation into the rat Itm2b gene (Itm2b⁰ rats).

Given the BRI2/APP interaction and the widely accepted relevance of human Aβ, a proteolytic product of APP, to AD, Itm2b⁰ rats were engineered to express two humanized App alleles, to produce human Aβ. Here, we studied young Itm2b⁰ rats to investigate early pathogenic changes. We found that peri-adolescent Itm2b⁰ rats present subtle changes in human Aβ levels along with decreased spontaneous glutamate release and AMPAR-mediated responses but increased short-term synaptic facilitation in the hippocampal Schaeffer-collateral pathway. These changes are like those observed in adult mice producing rodent Aβ and carrying either the Danish or British mutations into the mouse Itm2b gene. Collectively, the data show that the pathogenic Danish mutation alters the physiological function of BRI2 at glutamatergic synapses; these functional alterations are detected across species and occur early in life. Future studies will be needed to determine whether this phenomenon represents an early pathogenic event in human dementia.

INTRODUCTION

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 FDD and FBD, fifteen years ago we generated mice carrying the pathogenic Danish and British dementia mutations (Itm2b⁰ and Itm2b⁰)
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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), while 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 over-expression of disease-proteins in a non-physiological 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 (Itm2b\(^{\text{D}}\) 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 to mice due to the larger size of the rat brain. Cannulas -to administer drugs, biologics, viruses etc.- and microdialysis probes –for sampling extracellular brain levels of neurotransmitters, A\(\beta\), soluble tau etc.- 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 splicing 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 APP, which bears relevance to AD pathogenesis (26-30). In addition, APP processing mediates LTP and memory deficits of Danish and British KI mice (31-36). Aggregated forms of A\(\beta\), a product of APP processing, are by and large considered the main pathogenic molecule in AD. Rat and human APP differ by 3 amino-acids in the A\(\beta\) region: given that human A\(\beta\) are believed to have higher propensity to form toxic A\(\beta\) species as compared to rodent A\(\beta\), we produced rats carrying the humanized A\(\beta\) sequence (App\(^{\text{h}}\) rats) (37,38). Thus, to study possible interactions between the Danish mutation and human A\(\beta\), Itm2b\(^{\text{D}}\) rats were backcrossed to App\(^{\text{h}}\) rats. Hence, all rats used in this study produce human and not rodent A\(\beta\) species.

Here, we studied peri-adolescent Itm2b\(^{\text{D}}\) rats, with the purpose of investigating early dysfunctions that may underlie initial pathogenic mechanisms leading to dementia later in life.

RESULTS

Generation of Itm2b\(^{\text{D}}\) KI rats carrying humanized App\(^{\text{h}}\) alleles.

The knock-in founder F0-Itm2b\(^{\text{D}}\) rat, which is carrying FDD mutation on Itm2b rat gene, was generated by CRISPR/Cas-mediated genome engineering as described in Experimental Procedures and Supporting Information. The F0-Itm2b\(^{\text{D}}\) rat, which is a chimera for the Itm2b gene, was crossed to WT (Itm2b\(^{\text{Dow}}\)) Long-Evans rats to generate F1-Itm2b\(^{\text{Dow}}\) rats. F1-Itm2b\(^{\text{Dow}}\) rats were crossed to WT Long-Evans to generate F2-Itm2b\(^{\text{Dow}}\) rats. These crossing were repeated three more
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times to obtain F5-Itm2b<sup>h/w</sup> rats. The probability that F5 rats carry unidentified off- target mutations (except those, if present, on Chr. 15) is ~1.5625%. Male and female F5- 176 Itm2b<sup>D/w</sup> rats were crossed to obtain Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup> and Itm2b<sup>h/w</sup> rats.

The FDD mutation consist of a 10 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 (Figure 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>h/w</sup> rats. Sequencing of the PCR products shows that the Danish mutation was correctly inserted in the Itm2b gene exon 6 (Figure 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) was substituted for threonine (T) at codon 250 (3). Since that humanization 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>h/w</sup> rats on a background in which rat App has a humanized Aβ region, Itm2b<sup>D/w</sup> and Itm2b<sup>h/w</sup> rats were crossed to generate 179 Itm2b<sup>D/D</sup>, Itm2b<sup>D/w</sup> and Itm2b<sup>h/w</sup> rats. The <sup>D</sup> allele was removed in subsequent crosses. Henceforth, Itm2b<sup>D/D</sup>, Itm2b<sup>D/w</sup> and Itm2b<sup>h/w</sup> rats used in this study have an App<sup>D</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>h/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>h/w</sup> rats (Figure 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 NH2-terminal mature BRI2 (mBRI2) and the 23 amino acid-long COOH-terminal peptide called Bri23 (40). As noted above, in the Danish patients, a frameshift caused by a 10 nucleotides 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 immature Danish BRI2 generates a WT-like mBRI2 and a 34 amino acid long peptide called ADan, which co-deposits with Aβ species in amyloid fibrils in patients. For clarity, we will refer to the wild type imBri2 as Bri2-Bri23, and to the Danish mutant imBri2 as Bri2-ADan.

To determine whether the Itm2b<sup>D</sup> allele codes for Bri2-ADan we examined Bri2 expression in total neuronal lysates isolated from male and female 2 months old Itm2b<sup>D/w</sup>, Itm2b<sup>D/D</sup> and Itm2b<sup>h/w</sup> rats. However, the Bri2 antibody tested identified many non-specific bands (Figure S1), making a rigorous assessment of Bri2 expression in rat brains challenging.

Analysis of mouse Itm2b<sup>h/w</sup> and Itm2b<sup>D/D</sup> primary neurons showed that the mBri2/Bri2-Bri23 ratio in Itm2b<sup>h/w</sup> primary neurons was significantly higher than the mBri2/Bri2-ADan ratio in Itm2b<sup>D/D</sup> primary neurons (41). In addition, lysosomal inhibition caused accumulation of mBri2 but not Bri2-Bri23 in Itm2b<sup>h/w</sup> primary neurons; in contrast, both mBri2 and Bri2-ADan accumulated in Itm2b<sup>D/D</sup> 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 to total brain; this, per se’, may reduce the number of non-specific 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 Itm2b<sup>h/w</sup> and Itm2b<sup>D/D</sup> rat were treated with the lysosomal inhibitor chloroquine and analyzed by Western
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blot. The anti-Bri2 antibody identified a band of ~34 kDa in all samples, which was increased by chloroquine (Figure 2A and 2C). These observations are consistent with the ~34 kDa band corresponding to mBri2. A second band of ~36 kDa was detected in \(\text{Itm2b}^{D/D}\) primary neurons (Figure 2A). In contrast, a slightly larger second band (~37 kDa) that was increased by chloroquine treatment, was detected in \(\text{Itm2b}^{D/D}\) primary neurons (Figure 2A and 2C). These observations are consistent with the ~36 kDa and ~37 kDa bands corresponding to Bri2-Bri23 and Bri2-ADan, respectively.

Without treatment, the levels of Bri2-ADan in \(\text{Itm2b}^{D/D}\) primary neurons were significantly higher than the levels of Bri2-Bri23 in \(\text{Itm2b}^{D/D}\) primary neurons (Figure 2A and 2C) and the mBri2/Bri2-Bri23 ratio in \(\text{Itm2b}^{D/D}\) primary neurons was significantly higher than the mBri2/Bri2-ADan ratio in \(\text{Itm2b}^{D/D}\) primary neurons (Figure 2A and 2C). Chloroquine significantly reduced the LC3A/I/LC3A II and LC3B I/LC3B II ratios (Figure 2B and 2C), confirming inhibition of lysosome-mediated degradation.

**Subtle increase in A\(\beta\)42 levels in young \(\text{Itm2b}^D\) KI rats.**

Sequential processing of APP by \(\alpha-/\gamma\)-secretase and \(\beta-/\gamma\)-secretase generate the following APP metabolites: sAPP\(\beta\), sAPP\(\alpha\), \(\beta\)-CTF, \(\alpha\)-CTF, AID/AICD, P3 and A\(\beta\).

Since Bri2 interacts with APP and modulates APP processing by \(\alpha\)-, \(\beta\)-, and \(\gamma\)-secretase (26-30), we determined the steady-state levels of several of these APP metabolites in the central nervous system (CNS) of young male and female \(\text{Itm2b}^D\) KI rats. Full length APP, \(\alpha\)-CTF and \(\beta\)-CTF were measured by Western blot: soluble APPs (sAPP\(\alpha\)/sAPP\(\beta\)) were detected by ELISA, and human A\(\beta\) species (A\(\beta\)38, A\(\beta\)40, A\(\beta\)42 and A\(\beta\)43) were detected by human A\(\beta\) specific-ELISA. These measurements have previously been used for other KI rats generated in our lab (38,42-44).

Levels of full-length APP, CTFs, A\(\beta\)38, A\(\beta\)40, A\(\beta\)43, sAPP\(\alpha\) and sAPP\(\beta\) were unchanged in 8 weeks old \(\text{Itm2b}^{D/D}\), \(\text{Itm2b}^{D/D}\) and \(\text{Itm2b}^{D/D}\) rats (Figure 3A-C), nor was the A\(\beta\)43/A\(\beta\)42 ratio altered (Figure 3C). In contrast, there was a slight but significant increase in A\(\beta\)42 as well as the A\(\beta\)42/A\(\beta\)40 ratio in \(\text{Itm2b}^{D/D}\) compared to \(\text{Itm2b}^{D/D}\) rats (Figure 3C). Small but statistically significant decreases in both A\(\beta\)43 and A\(\beta\)43/A\(\beta\)42 ratio were evident in \(\text{Itm2b}^{D/D}\) as compared to \(\text{Itm2b}^{D/D}\) rats (Figure 3C). Overall, these data indicate a gene dosage-dependent minor increase in steady-state levels of A\(\beta\)42, and decrease in A\(\beta\)43, in peri-adolescent \(\text{Itm2b}^D\) rats. Analysis of older rats will be needed to determine whether the Danish mutation in \(\text{Itm2b}\) alters APP processing in KI rats and whether these alterations may more robustly change the steady-state levels of APP metabolites with aging.

It has been postulated that toxic forms of A\(\beta\) are oligomeric (45). Thus, we tested whether toxic oligomers are augmented in peri-adolescent \(\text{Itm2b}^D\) rats. To this end, we used the prefibrillar oligomer-specific antibody A11 to perform dot blots (46). We found no evidence supporting an increase in neurotoxic brain oligomer levels in peri-adolescent \(\text{Itm2b}^{D/D}\) and \(\text{Itm2b}^{D/D}\) rats as compared with \(\text{Itm2b}^{D/D}\) and \(\text{Itm2b}^{D/D}\) rats (Figure 4).

However, A\(\beta\) oligomers appeared to be significantly increased in \(\text{Itm2b}^{D/D}\) rats compared to \(\text{Itm2b}^{D/D}\) animals (Figure 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 peri-adolescent \(\text{Itm2b}^D\) rats.**

Bri2 modulates glutamatergic synaptic transmission at both pre- and post-synaptic termini of Schaeffer-collateral pathway (SC)–CA3>CA1 synapses (47). This function is compromised in both adult \(\text{Itm2b}^D\) and \(\text{Itm2b}^D\) KI mice (41). Here, analyzed glutamatergic transmission at SC–CA3>CA1 synapses in young peri-adolescent \(\text{Itm2b}^D\) male and female rats. First, we analyzed miniature excitatory postsynaptic currents (mEPSC), the frequency of which is determined, in part, by the probability of release (Pr) of glutamatergic synaptic vesicles release (48). Thus, mEPSC frequency is regulated mostly by pre-synaptic mechanisms: As shown in Figure 5A, B,C, the Danish \(\text{Itm2b}\) mutation...
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caused a significant reduction in the frequency of mEPSC: this reduction is gene-dosage dependent (Itm2b<sup>−/−</sup> vs. Itm2b<sup>+/+</sup>), P=0.003; Itm2b<sup>−/−</sup> vs. Itm2b<sup>+/−</sup>, P<0.0001; Itm2b<sup>−/−</sup> vs. Itm2b<sup>−/+</sup>, P=0.0051) and suggests a decrease in Pr of glutamatergic synaptic vesicles.

The amplitude of mEPSC is instead dependent on post-synaptic AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-receptor, AMPAR) responses. AMPAR-mediated mEPSC responses amplitude was also significantly decreased in Itm2b<sup>−/−</sup> rats (Figure 5A, D, E, G). Also in this case, the reduction is gene-dosage dependent (Itm2b<sup>−/−</sup> vs. Itm2b<sup>+/−</sup>, P=0.0016; Itm2b<sup>−/−</sup> vs. Itm2b<sup>−/+</sup>, P=0.0005). Decay time of mEPSC was not significantly affected in Itm2b<sup>−/−</sup> rats compared to littermate controls (Figure 5A, F, G).

Since mEPSCs AMPAR-mediated responses are reduced in amplitude, we measured the AMPA/NMDA ratio in evoked responses. Consistent with the hypothesis that the Danish Itm2b mutation impairs AMPAR-mediated responses, the AMPA/NMDA ratio was reduced in Danish Ki rats (Figure 5H).

This difference was statistically different only between Itm2b<sup>−/−</sup> and Itm2b<sup>+/−</sup> rats, with Itm2b<sup>−/−</sup> 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 (48)(41). Facilitation at both 50ms and 200ms interstimulus interval (ISI), was significantly increased in Itm2b<sup>+/−</sup> (Figure 41). Even in this case the changes were gene-dosage-dependent (50ms ISI: Itm2b<sup>−/−</sup> vs. Itm2b<sup>+/−</sup>, P<0.0001; Itm2b<sup>−/−</sup> vs. Itm2b<sup>−/+</sup>, P=0.0033; 200ms ISI: Itm2b<sup>−/−</sup> vs. Itm2b<sup>−/+</sup>, 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 peri-adolescent KI rats. These alterations are like those seen in Itm2b KO and Itm2b<sup>+/−</sup>/Itm2b<sup>−/−</sup> 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 (3,49-52). The KI approach was preferred because it generates models genetically faithful to human diseases and make 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 (38,42,44,53,54), because the rat is better suited for behavioral tests and other procedures that are important when studying neurodegenerative diseases. In addition, genetic expression differences suggest that rats may be advantageous model of neurodegenerative diseases over mice. Alternative spicing of Mapt (12-15), which forms NFTs and is mutated in FTD (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 carrying the Itm2b<sup>−/−</sup> Familial Danish dementia mutation. Consistent with the findings in Itm2b<sup>+/−</sup> mouse KIs (41), we found that B12-ADan maturation is altered and accumulates in Itm2b<sup>+/−</sup> primary neurons (Figure 2). Analysis of APP metabolism in peri-adolescent Itm2b<sup>−/−</sup> KI rats (Figure 3 and 4) only showed subtle but significant changes in Aβ42 and Aβ43 steady-state levels, which were slightly increased and decreased, respectively (Figure 3).
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We have previously shown that the Danish and British ITM2b mutations lead to reduced glutamatergic neurotransmitter release and AMPAR-mediated responses in adult Itm2b\(^{b}b\) and Itm2b\(^{b}b\) mice (41). These reductions are like those seen in adult Itm2b\(^{b}b\) knock-out mice (41, 47). Interestingly, we detected identical, gene dosage-dependent, pre-and post-synaptic glutamatergic transmission changes in the SC pathway of peri-adolescent Itm2b\(^{b}b\) rats (Figure 5). More specifically, the frequency of mEPSC and the PPF are significantly decreased and increased, respectively, in Itm2b\(^{b}b\) rats, suggesting a pre-synaptic reduction of the Pr of glutamatergic synaptic vesicles. In addition, mEPSCs’ amplitude and the AMPA/NMDA ratio were both decreased in Itm2b\(^{b}b\) rats, suggesting a post-synaptic reduction of AMPAR-mediated responses. Collectively, these data together with our previously published observations, indicate that the synaptic transmission alteration caused by Danish mutation occur early in life, and are neither species nor gene-editing technology-specific. These studies underline 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 BRI2 (26-36), it is possible that the presence of human A\(\beta\) in the rat model may lead to an earlier manifestation of synaptic plasticity deficit in rat as compared to mice, which express rodent A\(\beta\). Moreover, 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 (37, 41, 47, 55-57) suggest 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 NIH Ethical Guidelines for Treatment of Laboratory Animals. The procedures were described and approved by the Institutional Animal Care and Use Committee (IACUC) at Rutgers (IACUC, protocol number PROTO210702513).

**Generation of rats expressing the Danish Itm2b mutation (Itm2\(^{D}\) rats).** The rat Itm2b gene (GenBank accession number: NM_001006963.1; Ensembl: ENSRNOG0000016271) is located on rat chromosome 15. It comprises 6 exons, with ATP start codon in exon 1 and TGA stop codon in exon 6. The FDD mutation (GTG to GTC) was also 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 re-cutting 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 days old Itm2b\(^{bD}\) and Itm2b\(^{wD}\) rats (2 male and 2 females per each genotype) was extracted with RNeasy RNA Isolation kit (Qiagen). Standard RNA-Seq procedures and data analysis was 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.

**Rats brain proteins preparation, Western blots and ELISA** - These procedures were performed as previously described (42, 54).

Briefly, rats were anesthetized with isoflurane and perfused via intracardiac catheterization with ice-cold PBS. Brains were extracted and homogenized with a glass-teslin homogenizer in 250 mM Sucrose, 20 mM Tris-base pH 7.4, 1 mM EDTA, 1mM EGTA plus protease and phosphatase inhibitors (ThermoScientific).

All steps were carried out on ice.

Homogenates were solubilized with 1% NP-40 for 30 min rotating and spun at 20,000 g for 10 minutes. 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)
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NP0007) and 4.5M urea to 1µg/µl, loaded on a 4-12% Bis-Tris polyacrylamide gel. Blotting efficiency was visualized by red Ponceau staining on membranes. For Dot-blot analysis 2.5 µg of material was spotted with a p20 pipette on a nitrocellulose membrane. Dot membrane was also washed in PBS/Tween20-0.05%. Primary antibodies were applied dilution in blocking solution (Thermo 37573). The following antibodies were used: Polyclonal anti-Bri2 serum test bleeds provided by Cell Signaling and LC3B antibodies (Abcam32136), were diluted 1:1000 in 5% milk and used against brain lysates were diluted at 1:1000 in 5% milk and used against either mouse or rabbit primary antibodies for 1 hour at RT, with shaking. Membranes were washed with PBS/Tween20-0.05% (3 times, 10 minutes each time), developed with West Dura ECL reagent (Thermo, PI34076) and visualized on a ChemiDoc MP Imaging System (Biorad). Signal intensity was quantified with Image Lab software (Biorad). Data were analyzed using Prism software and represented as mean ± SD. For analysis of human Aβ peptides and sAPPα/sAPPβ, brain lysates were diluted at 4µg/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 MESCO QuickPlex SQ 120. Aβ43 was quantified using the IBL human Aβ43 Assay Kit #27710.

627 Primary hippocampal neuron culture- Rat
628 hippocampal neurons were prepared from Itm2β−/− and Itm2β+/− post-natal day 1 pups. Briefly, after removal of meninges, the hippocampi were collected in HBSS without magnesium and calcium, 1mM sodium pyruvate, 0.1%glucose, 10mM HEPES. Hippocampi were dissected into single cell by trituration followed by 15 minutes incubation at 37°C in 0.25% trypsin. Cells were subsequently treated with 0.1% DNAse (Sigma, dn25) in plating media (BME, 10% FBS, 0.09% Glucose, 1mM Sodium Pyruvate, 2mM Glutamine, 1x Pen/Strep). Cells were filtered through a Falcon 70µm nylon cell strainer and were plated in Poly L lysine pretreated 12-well-plate (300,000 cells/well) in Neurobasal media, 1x B-27, 2mM glutamine, 1x Pen/Strep. Half of the culture media was changed every 2 days.

647 Pharmacological treatment and sample preparation- After 9 days in culture, primary neurons were treated with 50µM Chloroquine (Cell signaling, 14774s) or PBS (Veh) for 18 hours. After treatment, cells were washed with PBS and lysed in RIPA buffer with protease/phosphatase inhibitor for 15 minutes on ice. Lysed cells were centrifuged at full speed for 15 minutes. Cell lysates were quantified and analyzed by Western blot as described earlier for brain lysates.

658 Electrophysiological recording- These procedures were performed as previously described (44). Briefly, rats were anesthetized with isoflurane and perfused intracardially 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, 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 (350µm 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
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at 30°C for 1 hr 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 ± 0.1°C.

Whole-cell recordings in the voltage-clamp mode (~70 mV) were made with patch pipettes containing (in mM): 132.5 Cs-glucuronate, 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 5–10 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 the GABA-A receptors inhibitor bicuculline methiodide (15 μM). The stimulation intensity was adjusted to evoke EPSCs that were 40% of the maximal evoked amplitudes (“test intensity”). 5–10 min after membrane rupture, EPSCs were recorded for 7 minutes 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 inter-pulse 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 potentials blocker (1 μM TTX) and GABA-A receptors inhibitors (15 μM bicuculline methiodide). mEPSCs were recorded for ~10 mins. 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 paper 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 (GraphPad) software.

Acknowledgement- All authors read and approved the final manuscript.

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

Conflicts of interest- The authors declare that they have no competing interests.

Author Contributions- LD generated the ideas, designed the experiments; LD and TY wrote the paper. L.D. was funded by the NIH/NIA R01AG063407, RF1AG064821, 1R01AG033007

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**Figure legends**

**Figure 1.** Characterization of *Itm2b* 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 *Itm2bw/w* and *Itm2bD/D* rats shows that the Danish mutation was correctly inserted in the *Itm2b* exon 6 of *Itm2bD/D* 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 days old *Itm2bw/w* and *Itm2bD/D* rats were determined by Standard-RNAseq analysis. No significant differences between *Itm2bw/w* and *Itm2bD/D* rats were evident. Data are represented as mean ± SD. Data were analyzed by Student’s t-test. N=4 rats per genotype.
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**Figure 2. Distinct degradation pathways of Bri2-23 and Bri2-ADan in *Itm2b^w/w* and *Itm2b^D/D***

**A** Primary neurons. WB analysis of Bri2 (A) and LC3A/B (B) from primary hippocampal neurons isolated from *Itm2b^w/w* and *Itm2b^D/D* P1 pups treated with 50µM chloroquine for 18h. (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. ANOVA summary: LC3A: F_interact (1,20)=37.36, P<0.0001, F_treatment (1,20)=353.5, P<0.0001, F_genotype (1,20)=36.16, P<0.0001. Post-hoc Sidak’s multiple comparisons test: *Itm2b^w/w*: vehicle (veh) vs Chlor, P<0.0001****. *Itm2b^D/D*: veh vs Chlor, P<0.0001****. LC3B: F_interact (1,20)=8.11, P<0.01, F_treatment (1,20)=26.75, P<0.0001, F_genotype (1,20)=8.283, P<0.01, post-hoc Sidak’s multiple comparisons test: *Itm2b^w/w*: veh vs Chlor, P=0.2185, *Itm2b^D/D*: veh vs Chlor, P<0.0001****. mBri2: F_interact (1,20)=272.2, P<0.0001, F_treatment (1,20)=354.3, P<0.0001****, F_genotype (1,20)=1966, P<0.0001****, post-hoc Sidak’s multiple comparisons test: *Itm2b^w/w*: veh vs Chlor, P=0.5229, *Itm2b^D/D*: veh vs Chlor, P<0.0001****. No treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P<0.0001****. Chlor treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P<0.0001****, *Itm2b^w/w*: veh vs Chlor, P<0.0001****, *Itm2b^D/D*: veh vs Chlor, P=0.9966. No treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P=0.9969 ns. Chlor treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P<0.0001****. mBri2/mBri2 Ratio: F_interact (1,20)=108.3, P<0.0001, F_treatment (1,20)=104.2, P<0.0001, F_genotype (1,20)=627.1, P<0.0001, post-hoc Sidak’s multiple comparisons test: *Itm2b^w/w*: veh vs Chlor, P<0.0001, *Itm2b^D/D*: veh vs Chlor, P<0.0001****. No treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P<0.0001****, Chlor treatment: *Itm2b^w/w* vs *Itm2b^D/D*, P<0.0001****.
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Figure 3. APP metabolite levels in Itm2b<sup>−/−</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 statistically significant differences. We analyzed 8 weeks old rats, and 5 female and 5 male rats per genotype. (A) Levels of full-length APP, αCTF, and βCTF, were determined by Western analysis of brain lysate of Itm2b<sup>+/+</sup>, Itm2b<sup>−/+</sup> and Itm2b<sup>−/−</sup> male and female rats. (B) Quantitation of Western blots. Signal intensity of APP metabolites were normalized to red ponceau staining of nitrocellulose membranes. ANOVA summary: mAPP, F<sub>(2, 27)</sub> = 0.7931, P=0.4627; imAPP, F<sub>(2, 27)</sub> = 1.367, P=0.2720; α-CTF, F<sub>(2, 27)</sub> = 0.6075, P=0.5520; β-CTF, F<sub>(2, 27)</sub> = 1.614, P=0.2177). (C) Levels of sAPP<sub>α</sub>, Aβ38, Aβ40, Aβ42 and Aβ43 were determined by ELISA of brain lysate from the same Itm2b<sup>+/+</sup>, Itm2b<sup>−/+</sup> and Itm2b<sup>−/−</sup> male and female rats. [ANOVA summary: sAPP<sub>α</sub>, F<sub>(2, 27)</sub> = 0.1084, P=0.8977; sAPP<sub>β</sub>, F<sub>(2, 27)</sub> = 0.7666, P=0.4744; Aβ38, F<sub>(2, 27)</sub> = 0.1121, P=0.8943; Aβ40, F<sub>(2, 27)</sub> = 2.030, P=0.1509; Aβ42, F<sub>(2, 27)</sub> = 4.764, P=0.0169 (post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/+</sup>, P=0.0019; Itm2b<sup>−/+</sup> vs Itm2b<sup>+/+</sup>, P=0.0059); Aβ43, F<sub>(2, 26)</sub> = 2.654, P=0.0893; Aβ42/Aβ40, F<sub>(2, 26)</sub> = 4.031, P=0.0299 (post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/+</sup>, P=0.0802, Itm2b<sup>−/+</sup> vs Itm2b<sup>+/+</sup>, P=0.0347; Itm2b<sup>−/+</sup> vs Itm2b<sup>−/−</sup>, P=0.09137); Aβ43/Aβ42, F<sub>(2, 26)</sub> = 3.281, P=0.0558]. P<0.05*; P<0.01**; P<0.001***; P<0.0001****.
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**Figure 4. Levels of human Aβ oligomeric species in the brain of peri-adolescent *Itm2b**

**D/D**, **D/w** and **w/w** male and female rats. (A) We analyzed material from the same rats analyzed in Figure 3. Quantitation of dot-bLOTS using the oligomer-specific antibody A11. Before immunoblot analysis, membranes were stained with Ponceau red. Quantitative analysis of A11 blot was normalized to the Ponceau red quantitative analysis. 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 statistically significant differences. ANOVA summary: $F_{(2, 27)} = 4.593, P=0.0192^*$; post-hoc Tukey's multiple comparisons test: *Itm2b**w/w** vs **Itm2b**D/w**, $P=0.6406$; *Itm2b**w/w** vs **Itm2b**D/D**, $P=0.1195$; *Itm2b**D/w** vs **Itm2b**D/D**, $P=0.0170^*$.
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Figure 5. Glutamatergic synaptic transmission is reduced at hippocampal SC–CA3>CA1 synapses of Itm2b<sup>−/−</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: Itm2b<sup>−/−</sup> N=11 (4M/6, 3F/5, indicating that 6 recordings were obtained from the 4 males and 5 recordings from the 3 females), Itm2b<sup>+/−</sup> N=15 (3M/7, 4F/8), Itm2b<sup>−/−</sup> N=10 (3M/3,3F/3). (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<sub>(2, 33)</sub> = 20.66, P < 0.0001**; post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/−</sup>, P=0.003**, Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P<0.0001****; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.0051**]. (C) Cumulative probability of AMPAR-mediated mEPSC frequency inter event intervals. (D) [ANOVA summary, F<sub>(2, 33)</sub> = 10.78, P<0.0001***; post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/−</sup>, P=0.0016***, Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P<0.0005***; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, 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<sub>(2, 33)</sub> = 1.292, P=0.2882]. (G) Average mEPSC of Itm2b<sup>−/−</sup>, Itm2b<sup>+/−</sup> and Itm2b<sup>−/−</sup> rats. (H) AMPA/NMDA ratio is significantly decreased in Itm2b<sup>−/−</sup> rats [ANOVA summary, F<sub>(2, 16)</sub> = 8.417, P=0.0032***; post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/−</sup>, P=0.2393; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.0023***; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.0818]. Representative traces are shown on top of the graph (traces are averaged from 20 sweeps). Animals used: Itm2b<sup>−/−</sup> N=7 (4M/4, 3F/3), Itm2b<sup>+/−</sup> N=6 (3M/3,3F/3), Itm2b<sup>−/−</sup> N=6 (3M/3,3F/3). (I) Average PPF at 50ms (left panel) and 200ms (right panel) Inter stimulus Interval (ISI) shows that PPF is increased in Itm2b<sup>−/−</sup> rats [ANOVA summary, F<sub>(1, 60)</sub> = 11.89, P<0.0001****; post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>+/−</sup>, P=0.2315; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P<0.0001***; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P<0.0031**]. 200ms ISI PPF ANOVA summary: F<sub>(1, 60)</sub> = 3.802, P=0.0275*; post-hoc Tukey's multiple comparisons test: Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.4504; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.0205*; Itm2b<sup>−/−</sup> vs Itm2b<sup>−/−</sup>, P=0.2462]. Representative traces are shown on top of the panels. P<0.05*, P<0.01**, P<0.001***, P<0.0001****. Animals used: Itm2b<sup>−/−</sup> N=18 (4M/10,3F/8), Itm2b<sup>+/−</sup> N=20 (3M/10,4F/10), Itm2b<sup>−/−</sup> N=17 (3M/8,3F/9).