Modeling the β secretase cleavage site and humanizing Amyloid-Beta Precursor Protein in rat and mouse to study Alzheimer Disease.

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Abstract

Background

Three amino acid differences between rodent and human APP affect medically important features including β-secretase cleavage of APP and aggregation of the Aβ peptide (1–3). Most rodent models for Alzheimer’s disease (AD) are therefore based on the human APP sequence expressed from artificial mini-genes randomly inserted in the rodent genome. While these models mimic rather well biochemical aspects of the disease such as Aβ-aggregation, they are also prone to overexpression artifacts and to complex phenotypical alterations due to genes affected in or close to the insertion sites of the mini-genes (4,5). Knock-in strategies introducing clinical mutants in a humanized endogenous rodent APP sequence (6) represent useful improvements, but need to be compared with appropriate humanized wild type (WT) mice.

Methods

Computational modelling of the human β-CTF bound to BACE1 was used to study the differential processing of rodent and human APP. We humanized the three pivotal residues G676R, F681Y and R684H (labeled according to the human APP770 isoform) in the mouse as well as in the rat by a CRISPR-Cas9 approach. These new models, termed mouse and rat App hu/hu, express APP from the endogenous promoter. We also introduced the early-onset familial Alzheimer’s disease (FAD) mutation M139T into the endogenous Rat Psen1 gene.

Results

We show that the three amino acid substitutions in the rodent sequence lower the affinity of APP substrate for BACE1 cleavage. The effect on β-secretase processing was confirmed as both humanized rodent models produce three times more (human) Aβ compared to their WT rodent original strain. These models represent suitable controls or starting points for studying the effect of transgenes or knock-in mutations on APP processing (6). We introduced the early-onset familial Alzheimer disease (FAD) mutation M139T into the endogenous Rat Psen1 gene and provide an initial characterization of Aβ processing in this novel rat AD model.
Conclusion

The different humanized APP models (rat and mouse) expressing human Aβ and PSEN1 M139T are valuable controls to study APP processing in vivo and allow to implement the use of human Aβ Elisa which is more sensitive than their rodent counterpart. These animals will be made available to the research community.
Background

Alterations in Aβ generation and aggregation are central to AD(7–9). More than 177 transgenic models overexpressing mutated human APP and/or PSEN are currently available(10). While these models lack tangles and dementia symptoms, they remain good models to investigate amyloid accumulation. Drawbacks of these models are protein overexpression (for instance overexpression of APP might block GABAergic neurotransmission(11)) and the random integration of transgenes which could disturb the expression of adjacent genes (5,12). Recent improvements in genome editing make it relatively straightforward to introduce subtle disease-causing mutations or to humanize genes rather than overexpressing mutant mini-genes(13).

APP knock-in models are available that express clinical AD mutations (KM 670/671NL, E693G, I716F) in the endogenous mouse sequence(6,14). These models develop amyloid pathology and interesting phenotypes without the burden of APP overexpression. However, the WT control (humanized APP without these mutations) is not readily available. The three amino acid substitutions (G676R, F681Y and R684H) needed to humanize the mouse Aβ sequence have a profound effect on Aβ generation (1).

In the current study we show how these substitutions affect the interaction with BACE1 using in silico modeling. We used CRISPR-Cas9 technology to scarless humanize the Aβ sequence in the mouse and rat App genes(15) and investigate the effects on APP processing. We also created a PSEN1 knock-in mutation to generate a rat model for AD.
Material and methods

Mice

Mice App<sup>em1Bdes</sup> with a humanized Aβsequence G676R, F681Y, R684H were generated using CRISPR/Cas9 technology to target the exon 16 of the mouse App gene. RNA guides were selected using the CRISPOR web tool. Guide 5'-GCAGAAUUCGGACAGAUUC-3' and 5'-GUCCGCCAUCAAAAACUGGU-3' were selected and tested in Mouse Embryonic Fibroblast cells for cleaving efficiency. To promote homologous recombination directed repair we made use of a ssODN repair template to mutate the target amino acids and to introduce two silent nucleotide substitutions. The first silent substitution destroys an EcoRI restriction cleaving site, facilitating genotyping. The second silent substitution prevents Cas9 cleaving the modified locus.

Ribonucleoproteins (RNPs) containing 0.3 μM purified Cas9HiFi protein (Integrated DNA Technologies, IDT), 0.6 μM CRISPR RNAcrRNA, 0.6µM trans activating crRNA (IDT) and 10 ng/µl ssODN (5’-tactttgtttgacgcagGTCTGGGACTCAAGACGGAAGAGATCGGAACGAGATTGAGATGGATGCAGAATTtaGACATGATTCAGGATaTGAAGTCCaCCATCAgAAACTGgtaggcaaaaataaactgcctctccccgagattgcgtctggccagatgaatacgtggcacctcgtggcttgtcctgtgt-3') were injected into the pronucleus of 72 C57Bl6J embryos by microinjection in the Mouse Expertise Unit of KU Leuven. One pup was identified by PCR and restriction analysis. Sanger sequencing of the exon 16 region of the App gene and the 5 most predicted off target sites by the CRISPOR web tools confirmed the correct targeting (Additional file3) and absence of off targeting events in the other sites. The founder mouse was backcrossed over two generations with C57BL6J mice before a homozygous colony was established named App<sup>hu/hu</sup>. The strain is maintained on the original C57Bl6J background by backcrosses every 5th generation.

Standard genotyping is performed by PCR with primers 5’-taggtggttgaatggttaattatg-3’ and 5’-cgtagctgcaaccttggtgttcatggtttggtttggtttg-3’, digestion with EcoRI.

App<sup>tm3.1Tcs</sup> (6) also known as App NL-G-F and Tg(Thy1-MAPT)<sup>22</sup>schd (35) also known as Thy-Tau22 mice were used as positive controls during histological examination. Mice are kept on a C57Bl6J background.
background and both females and males were included in the study. Mice are housed in cages enriched with wood wool and shavings as bedding, and given access to water and food ad libitum. All experiments were approved by the Ethical Committee on Animal Experimentation of the University of Leuven (KU Leuven).

Rats

As the rat is one of the most studied model organism (36) and until recently no knock-in rat models to study AD were available (27), we embarked to humanize the Aβ sequence in rats using a similar strategy as used for the mice. Two gRNAs (Additional file3), GUGAAGAUGGAUGCGGAGUU and UUUUGCAUACCAGUUUUUGA, Cas9 mRNA and an oligo donor with targeting sequence, flanked by 120 bp homologous sequences combined on both sides were co-injected into zygotes of Long Evans rats. We also introduced the early-onset familial Alzheimer disease (FAD) mutation M139T (23) into the endogenous Rat Psen1 gene. To target Psen1 Cas9 mRNA, sgRNA GAUGACACUGAUCAUGAUGG and an oligo donor containing the ATG/ACC substitution with 120 bp homologous sequences were co-injected into zygotes (additional file4). F0 rats were genotyped after weaning using PCR and Sanger sequencing. Founder rats carrying the humanized Aβ sequence and M139T mutant allele were crossed twice with WT Long Evans rats (Charles River). Homozygous rats for the humanized APP KI were obtained after crossing heterozygous offsprings. A breeding colony homozygous for the humanized Aβ sequence and heterozygous Psen1M139T allele was established named App^{hu/hu;Psen1M139T}. Standard genotyping for the APP KI mutation is done with forward primer 5’-caTGATTcAGGCTaCGAAGTCCat-3’ and common reverse primer 5’-CTCAGTGGTAAATACGcCTGCCTAGC-3’. 5’-TGATTcAGGCTtCGAAGTCCgc-3’ is the forward primer for amplification of the wild type App allele. For Psen1 genotyping is performed by PCR with WT specific forward primer 5’- cgatcttgatgcccgccatcg-3’ or M139T specific forward primer 5’-cgatcttgatgcccgccatcacc-3’ with common reverse primer 5’-ctgcacatgtacactctggcaag-3’. Rats are kept on a Long Evans background with every fifth generation crossed back to WT Long Evans rats. Both females and males were included in the study. Rats are housed in cages enriched with wood wool and shavings as bedding,
and given access to water and food ad libitum. All experiments were approved by the Ethical Committee on Animal Experimentation of the University of Leuven (KU Leuven).

Human brain tissue samples were resected from the lateral temporal neocortex and were obtained from patients who underwent amygdalohippocampectomy for medial temporal lobe seizures. Samples were collected at the time of surgery and immediately transferred to the laboratory for tissue processing. All procedures were conducted according to protocols approved by the local Ethical Committee (protocol number S61186).

**Sample collection and protein analysis**

Three female and 3 male mice and rats, 14 weeks old, of the indicated genotype, were euthanized by carbon dioxide overdose, followed by intracardial perfusion of ice-cold phosphate buffered saline.

Brains were removed from the skull, and cerebrum was snap frozen on liquid nitrogen and stored at -70°C until further processing. Half hemisphere was weighted and homogenized in a bead mill using 5 times volume of buffer containing 20 mM Tris, 250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.4 supplemented with cOmplete™ protease inhibitor cocktail (Roche) and PhosSTOP™ (Sigma). This homogenate was divided in three fractions of 250 µl. One fraction was used to extract 0.4% DAE soluble Aβ (30 minutes at 4°C), after high spin clearing at 100,000g, 4°C for 1h the sample was neutralized by adding 1/10 volume of 0.5 M Tris-HCl, pH 6.8 and analyzed by ELISA. Aβ38, Aβ40 and Aβ42 levels were quantified on Meso Scale Discovery (MSD) 96-well plates by ELISA using end-specific antibodies provided by Dr. Marc Mercken (Janssen Pharmaceutica). Monoclonal antibodies JRFcAβ38/5, JRFcAβ40/28 and JRFcAβ42/26, which recognize the C terminus of Aβ species terminating at amino acid 38, 40 or 42, respectively, were used as capture antibodies. JRF/rAβ/2 (rodent specific antibody) or JRFAβN/25 (human specific antibody) labeled with sulfo-TAG were used as the detection antibodies. Human Aβ43 was measured using the Amyloid-beta (1-43) high sensitive ELISA kit from IBL. To measure APP protein in the brain sample 250 µl of homogenate was supplemented with 1% Triton X100, incubated for 30 minutes on ice and cleared for 30 minutes at
For the extraction of soluble MAPT protein, 250 µl of a buffer containing 300 mM NaCl, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2% NP-40, 0.5 % sodium deoxycholate, pH 7.5, supplemented with cOmplete™ protease inhibitors cocktail (Roche) and PhosStop™ (Sigma) was added to 250 µl of brain homogenate. The sample was sonicated and incubated for 30 minutes on ice and cleared by centrifugation at 14000g, 4°C for 30 minutes. When indicated, cell lysates were dephosphorylated after dialysis against Tris-HCl (50mM) pH7.6 using Calf Intestine Phosphatase (Bioke). Total protein content of the cell lysates was measured using a protein assay kit of Biorad.

Fifty microgram of protein was loaded in reducing and denaturing conditions on NuPAGE™ (Thermo) and analyzed by western blotting on Nitrocellulose. Membranes were blocked with nonfat milk 5% in TRIS buffered saline, containing 0.1% Tween 20, and incubated with the indicated primary antibodies, washed, and incubated with horseradish peroxidase conjugated secondary antibodies (Biorad). Blots were developed using the ECL Renaissance kit (Perkin Elmer). Primary antibodies used in this study were B63 (against the C-terminal amino acids of APP(37)), 82E1 (IBL), JRF/rAβ/2 (Janssen), anti-human TAU (Dako), anti 3RTAU (Millipore), anti 4RTAU (CosmoBio) and Anti Actb clone AC-15 (Sigma). Intensities of the bands were quantified with Aida/2D densitometry software.

All data are presented as mean ± SD, and were analyzed by GraphPad Prism 8. Unpaired two way Student t-test and one-way ANOVA were used for group comparisons. P < 0.05 was considered statistically significant.

Histology

Rats and control mice were terminally anesthetized with an overdose of carbon dioxide and transcardially perfused with PBS, brain tissue was harvested and postfixed in 4% PFA in PBS, overnight. Brains were cut in serial sections of 40 µm thickness with a vibrating microtome (Leica). For each sample, six series of sections were sequentially collected in free-floating conditions and permeabilized for 30 minutes with PBST (0.2% Triton X-100 in PBS) and blocked for 2 hours with 5% normal donkey serum in PBST. Antigen retrieval was performed by boiling the sections for 1 minute in PBS containing 0.01% tween 20 and 10 mM sodium citrate, pH6 in a microwave. After three rinses
with PBS–0.1% Tween 20, sections were incubated for 20 minutes with 10µM X34 (Sigma, 0.1g% NaOH made in 40% Ethanol. After washes with PBS–0.1% Tween 20, sections were counterstained with TO-PRO (Thermo Fisher) and after final three washes mounted on super frost microscope slides. Sections were visualized on a Nikon A1R Eclipse confocal system.

Computational Model of the humanized β-CTF bound to BACE1
We have modelled the humanized β-CTF into the binding site of Bace1, using as template the co-crystalized peptide in the BACE1 crystal structure (PDB ID 5MCQ)(16). The sequence of the humanized β-CTF was aligned to the peptide in 5MCQ, aligning residue E11 from the humanized β-CTF sequence to the STA query (Threonine) of the peptide crystalized (Fig1).

Results
Humanized β-CTF provides a better substrate for BACE1
Three amino acid substitutions (G676R, F681Y and R684H) in the Aβ sequence decrease BACE1 processing of rodent compared to human APP(1). We superposed the rodent and humanized β-CTF on a 21 amino acid peptide inhibitor at the binding site of BACE1 (Fig1) using the crystal structure PDB id:5MCQ(16). The modeled binding modes reveal two important extra interactions of the humanized β-CTF with BACE1. G676R replacing the glycine in the rodent sequence with arginine, provides a large positive charge that interacts with glutamate E326 of BACE1. F681Y introduces an extra OH group which acts as a hydrogen bond donor to N294 in BACE1. The two amino acids E326 and N294 of BACE1 require no reorganization or conformational change to make the interactions with the humanized β-CTF (Fig1).
We validated the different APP processing of human, mouse and rat brain by western blot analysis (Fig1). The ratio of β-CTF over full length APP in human samples is 4.8 and 7.8 times higher compared to mouse and rat, respectively, confirming that human APP is a better substrate for BACE1.

Processing of humanized Aβ APP in the rodent brain
We humanized the rodent APP genes (additional files 1 and 2) to APP
\textsuperscript{hu/hu} and analyzed brain homogenates by western blotting (Fig2, Fig3). APP full length and total APP-CTF protein levels were
unchanged in humanized rat and mice, but APP-β-CTF signals increased 2.5 fold (p<0.0001) and 4.2 fold (p=0.009) in App<sup>hu/</sup>hu mice and rats, respectively. The Aβ40 levels in brain tissue rose from 2,091±130 pg/g in WT to 4,827±257 pg/g App<sup>hu/</sup>hu mouse, p<0.0001 and from 3,302±1,256 pg/g in WT to 9,292±516 pg/g in App<sup>hu/</sup>hu rats (p<0.0001). The higher Aβ40 levels correlate with the fact that APP-β-CTF in App<sup>hu/</sup>hu rats and basal Aβ generation in WT rats are also higher than in the mice (Fig2, Fig3). Soluble extracted total Aβ measured as the sum of Aβ38+40+42 raised to 7,187±1,022 pg/g in App<sup>hu/</sup>hu mouse and 12,615±1,511 pg/g in rat which is approximately half of the concentration 21,141±8,712 pg/g measured in control human brain.

**M139T mutation in PSEN1 results in a shift in Aβ profile**

The M139T FAD mutation(17) alters Aβ38/Aβ42 and Aβ42/Aβ40 ratios without affecting the endopeptidase cleaving activity which releases the APP intracellular fragment(18). This mutation is predicted not to interfere with Notch signaling(19) which is confirmed as App<sup>hu/</sup>hu rats homozygous for the M139T FAD mutation are fertile and are indistinguishable from their PSEN WT littermates. Brain homogenates of App<sup>hu/</sup>hu;Psen1M139T<sup>+/−</sup> mice were analyzed (Fig3). The amounts of APP-CTF, APP-β-CTF and total Aβ measured as Aβ38+40+42+43 (11,898±486 pg/g in App<sup>hu/</sup>hu compared to 12,834±676 pg/g in App<sup>hu/</sup>hu;Psen1M139T<sup>+/−</sup>) are unaffected by the mutation. However the mutation causes a relative shift towards more Aβ42, a very small amount of Aβ43 and less Aβ40 (Fig3C). This results in an increased Aβ42/Aβ40 ratio (from 0.10±0.01 to 0.27±0.01) and a decreased Aβ38/Aβ42 ratio (2.60±0.33 to 1.48±0.25 to) indicating less efficient carboxypeptidase like activity of the γ-secretase, which agrees with our previous in vitro work (18). Unexpectedly Aβ43 was 2.5 times reduced in the brain of homozygous App<sup>hu/</sup>hu;Psen1M139T<sup>+/−</sup> rats, resulting in an increased Aβ40/Aβ43 ratio (42.96±6.39 to 92.41±15.02). As total Aβ is unaffected by the mutation, the reduction of Aβ43 is not due to intracellular aggregation, indicating that the M139T mutation shifts APP processing towards the Aβ42 pathway(20). Disappointingly, even the oldest rat available at age 2 did not show amyloid plaque pathology.

**MAPT expression profile in rat is more complex compared to mouse**
One of the reasons to generate a rat model for AD is that MAPT is claimed to be more similar to human MAPT, especially at the level of alternative splicing (21, 22). 3RMAPT is indeed very low in mice (Fig2) and detectable in rats. 4RMAPT is abundant in both species and higher mobility bands indicate the presence of 1N4R, 2N4R splice variants (Fig3), better visible after dephosphorylation (Additional file3). Rodent MAPT, which is smaller, migrates faster compared to the human MAPT reference ladder. The estimated ratio of 3R to 4R MAPT is 1:13 in the rat brain, and thus very remote from the 1:1 ratio in human brain. No tangle or plaque formation was observed until the age of 2 years; in single and double KI rats total MAPT and 4RMAPT protein levels are unchanged over time, while the 3RMAPT isoform decreases and the Alzheimer’s disease-relevant AT100 phosphorylation increases over age (Additional file4). It seems unlikely that the rats will develop amyloid plaque or tangle pathology at a later age.

Discussion

We created mice and rats harboring a humanized Aβ sequence in the endogenous App gene. The models produce about three times more (human) Aβ compared to their WT rodent original strain but still two times less compared to human. The rats and mice are suitable controls or starting points to study the effect of transgenes or knock-in mutations on APP processing. One of the biggest advantages of these new models is that human Aβ ELISA can be used to study the different Aβ species.

We hereby provide a first example model by introducing the FAD mutation M139T into the endogenous Rat Psen1 gene. The PSEN1M139T(23) has a mean age of onset of disease between 39-51 years old. Preclinical carriers have relatively high levels of Aβ42 in the cerebrospinal fluid (24). in vitro this mutant affects the production rates of Aβ38 and Aβ40 while endopeptidase activity is not altered (18). Our new data confirm these effects but the strong lowering of Aβ43 and increase in Aβ40/Aβ43 ratio in the brain of the rat indicate that in vivo the mutation mainly works via a selective shift towards the Aβ42 product pathway and less via destabilization of the enzyme-substrate complex (25).
While our work was ongoing the group of LaFerla generated App\textsuperscript{tm1.1Aduci} mice via homologous recombination introducing the humanized A\textbeta{} sequence in the endogenous locus (JAX, Stock No: 03203). Recently also a knock-in rat model was described by D’Adamio and colleagues (26,27) with a humanized A\textbeta{} sequence and a KI of PSEN1L435F mutation. The L435F mutation affects endopeptidase activity and therefore Notch signaling. Surprisingly, while the mutation is embryonically lethal in mice (28), homozygous rats are viable. No explanation for this interspecies difference is available (27). These rats also did not develop amyloid plaques although only data of two week old rats were reported.

The fact that our humanized rat model, incorporating a homozygous PSEN mutation, is not developing symptoms of AD despite highly pathological A\textbeta{} ratios at an age of two year old, raises some tantalizing questions with regard to the many overexpressing models generated over the last thirty years to study AD. The amyloid plaques in these models seem critically dependent on strong overexpression of APP (and sometimes Presenilin). Such overexpression of proteins can lead to all kind of artefacts. The fact that the simple introduction of the PSEN FAD mutations in the rodent homologues does not cause amyloid plaque formation despite clear alterations in A\textbeta{} ratio’s suggests that molecular and cellular events upstream and downstream of amyloid plaque formation in humans are lacking in rodents. Aging might be one of them, as the generation of amyloid plaques in humans spans several decades. However, the transition from biochemical accumulation of plaques to disease involves complex feedback loops between glial cells and amyloid plaques, which might be only partially mimicked in rodent brain (29).

\textbf{Conclusion}

In conclusion, the field should move away from the use of artificial mini-genes and protein overexpression when creating next generation models. Furthermore, knock-in of AD mutations in primates, or the use of human stem cells to generate organoids (30), or to transplant into mouse-human chimaera (31–33), might provide ways forward to dissect the human molecular and cellular aspects of the disease.
List of abbreviations

APP amyloid precursor protein
Aβ amyloid beta peptide
AD Alzheimer disease
WT Wildtype
CTF carboxy terminal fragment
BACE beta-site amyloid precursor protein cleaving enzyme
CRISPR clustered Regularly Interspaced Short Palindromic Repeats
Hu humanized
FAD early-onset familial Alzheimer disease
PSEN Presenilin
ELISA enzyme linked immunosorbent assays
ssODN single-stranded oligodeoxynucleotides
RNP Ribonucleoprotein
PCR Polymerase Chain Reaction
MAPT Microtubule-Associated Protein Tau
sgRNA single guide Ribonucleic Acid
KI Knock-in
PFA Paraformaldehyde
PBS Phosphate-buffered saline
BDL below detection limit

Declarations

Ethics approval and consent to participate

All animal experiments were conducted according to protocols approved by the local Ethical Committee of Laboratory Animals of the KU Leuven (governmental license LA1210579) following
governmental and EU guidelines. All procedures with human brain tissue were conducted to
protocols approved by the local Ethical Committee (protocol number S61186).

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

App$^{hu/hu}$ mice and App$^{hu/hu;}Psen1M139T$ rats will be made available upon request.

Competing interests

The authors report no competing interests. Bart de Strooper is or has been consultant for
pharmaceutical companies including Jansen Pharmaceuticals and Eisai.

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B.D.S. is holder of the Bax-Vanluffelen Chair for Alzheimer’s disease.
Authors’ contributions

LS, DT, and BDS conceived and designed the study and wrote the manuscript. LS and DT performed experiments. PBL performed Computational Modelling. TT provided human brain tissue from epilepsy surgery specimens. All authors read and approved the final manuscript.

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Authors’ information

Not applicable
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Figures

Figure 1

A

Peptide inhibitor 5MCQ: G G G Y P Y F I P DLY G DLY G E V N STA V A E
Rodent β-CTF: - - - - - - D A E F G H D S G F E V R H
Humanized β-CTF: - - - - - - D A E F R H D S G Y E V H H

B

C

D

E

Fig 1: Model of humanized β-CTF bound to BACE1 and western blot analysis of mouse, rat and human APP. (A) Sequence alignment between the peptide inhibitor used in the crystal structure 5MCQ and the humanized β-CTF. Green: the three amino acids different between human and rodent sequence. (B) The BACE structure is presented with its flap (16) in yellow and the 10S loop in orange and a model of the rodent Aβ peptide is fitted. (C) Shows the same structure but now modelled with the humanized β-CTF. Two extra interactions between the G676R of the peptide and residue E326
and F681Y and residue N294 of BACE can be noted. (D) Western blot analysis of APP protein in cerebrum of WT mouse (M), rat (R) and human (H), (n=6). B63 antibody against the C-terminal part of APP stains APP full length (FL APP) and the C-terminal fragments (CTF, longer exposure). β-ACTIN is the loading control. (E) Quantification of APP protein in relative intensities, human=1 (n=6, mean±SE, **p=0.0063 α-CTF/FL APP, **p=0.0023 CTF/FL APP, *** p=0.0002, ****p<0.0001, One-Way ANOVA, Turkey post-hoc test).
Figure 2

**Fig2. Humanization of Aβ sequence in the mouse affects APP processing.** (A) Western blot analysis of APP protein in cerebrum of 14 weeks old WT and App^hu/hu^ mice (n=6). B63 stains APP full length (APP FL) and the C-terminal fragments (CTF, longer exposure). 82E1 antibody stains the human Aβ (1-16) sequence confirming the authenticity of human β-CTF. β- ACTIN is the loading control. (B)
Quantification of APP protein in relative intensities (n=6, mean±SD, *** p<0.0001 two tailed t-test).

(C) ELISA analysis of soluble Aβ expressed as pg/g tissue. Below detection limit (BD), Not determined (ND), (n=6, mean±SD, ***p<0.0001 two tailed t-test). (D) Immunoblot of total MAPT in mouse cerebrum with 3Rtau-specific antibody RD3, 4Rtau-specific antibody RD4 and total Tau. The MAPT ladder shows recombinant human MAPT (0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R). Notice that mouse MAPT proteins are migrating faster compared to the corresponding human splice variants. β-ACTIN is the loading control. (E) The quantification of MAPT relative to WT samples ((n=6, mean±SD).
Fig3. Humanization of Aβ sequence in the rat affects APP processing while M139T mutation in PSEN1 results in increased Aβ42 concentrations in the rat brain. (A) Western blot analysis of APP protein in cerebrum of 14 weeks old WT, Apphu/hu and Apphu/hu;Psen1M139T/+ rats (n=6). B63 binds to APP full length (FL APP) and the C-terminal fragments (CTF, longer exposure). 82E1 confirms human β-CTF; rAβ/2 confirms the authenticity of rodent β-CTF. β- ACTIN is the loading control. (B)
Quantification of APP protein in relative intensities (n=6, mean±SD, ** p=0.009, * p=0.017, One-Way ANOVA, Turkey post-hoc test). (C) ELISA analysis of soluble Aβ expressed as pg/g tissue. Below detection limit (BD), Not determined (ND), (n=6, mean±SD, *** p<0.0001, * p=0.012, One-Way Anova, Turkey post-hoc test). (D) Aβ ratios for App<sup>hu/hu</sup> and App<sup>hu/hu</sup>,Psen1M139T<sup>+/+</sup> harboring the M139T mutation show impairment in γ-secretase cleavage (n =6, mean±SD, ***p<0.0001 two tailed t-test). (E) Immunoblot of total MAPT in rat cerebrum with 3Rtau-specific antibody RD3, 4Rtau-specific antibody RD4 and total Tau. The MAPT ladder shows recombinant human MAPT (0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R). Notice that rat MAPT proteins are migrating faster compared to the corresponding human splice variant. β-ACTIN is the loading control. (F) The quantification of MAPT relative to rat WT samples (n=6, mean±SD).
**Additional file1. Generation of APP KI mice and rats by CRISPR Cas (A)** The top panel displays an alignment between human Aβ and rodent Aβ peptide. Differences are indicated in red, boxes represent transmembrane domains. The lower panel depicts the genomic organization and exon 16 sequence of the mouse and App gene. Exons are indicated as black boxes, arrows denote primers used for genotyping and sequencing. Underlined sequences are CRISPR guides, ssODN represent the template used for homologous recombination. Nucleotides and amino acids indicated in red are the target sequences, nucleotides in green are silent mutations introduced to prevent Cas9 to recut after homologous recombination. **(B)** Sanger sequencing results indicating the introduction of the point mutations in one strand as indicated above the chromatograms. **(C)** PCR analysis of APP KI mice. Digestion with EcoRI is indicative for KI allele as the restriction site is destroyed after gene editing.
Additional file2

(A) Partial sequence of exon 5 of the mouse and \textit{Psen1} gene. Underlined sequences indicate the CRISPR guide, ssODN represent the template used for homologous recombination. Nucleotides and amino acids indicated in red are the target sequences.

(B) Sanger sequencing results indicating the introduction of the point mutations (underlined).

\begin{verbatim}
PSEN1 Exon5 5'-...CTG CAC TCG ATC TTG AAT GCC GCC ATC ATG ATC AGT GTC ATC GTC GTT ATG ACC ATC CTC...'-3'
sscDN     5'-...CTG CAC TCG ATC TTG AAT GCC GCC ATC ACC ATC AGT GTC ATC GTC GTT ATG ACC ATC CTC...'-3'
\end{verbatim}
Comparing MAPT splice isoforms between Mouse and Rat. Analysis of MAPT isoforms after dephosphorylation with alkaline phosphatase (AP). The extracts of cerebrum were treated with (+) or without (−) alkaline phosphatase (AP) at 37 °C for 1h and immunoblotted with RD3, RD4, Total MAPT. The middle lane is recombinant human MAPT (ladder). Mouse express mainly 0N4R splice variant compared to the more complex expression pattern in the rat brain lysates showing all 6 splice forms. The estimated ratio 3R/4R MAPT = 1/13.
Additional file 4. MAPT protein analysis in two year old rats. Immunoblot of MAPT in rat cerebrum with total Tau antibody, 3Rtau-specific antibody RD3, 4Rtau-specific antibody RD4, AT270 and AT100 of two year old rats (n=2). LE rats* are cerebrum samples from wildtype Long Evans rats aged 14 weeks. MAPT ladder is recombinant human MAPT (0N3R, 0N4R, 1N3R, 1N4R, 2N3R, 2N4R). Notice that mouse MAPT proteins are migrating faster compared to the corresponding human splice variant.