An Intracellular Threonine of Amyloid-β Precursor Protein Mediates Synaptic Plasticity Deficits and Memory Loss

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Abstract
Mutations in Amyloid-β Precursor Protein (APP) and BRI2/ITM2b genes cause Familial Alzheimer and Danish Dementias (FAD/FDD), respectively. APP processing by BACE1, which is inhibited by BRI2, yields sAPPβ and β-CTF. β-CTF is cleaved by gamma-secretase to produce Aβ. A knock-in mouse model of FDD, called FDDKI, shows deficits in memory and synaptic plasticity, which can be attributed to sAPPβ/β-CTF but not Aβ. We have investigated further the pathogenic function of β-CTF focusing on Thr⁶⁶⁸ of β-CTF because phosphorylation of Thr⁶⁶⁸ is increased in AD cases. We created a knock-in mouse bearing a Thr⁶⁶⁸Ala mutation (APP⁶⁶⁸ mice) that prevents phosphorylation at this site. This mutation prevents the development of memory and synaptic plasticity deficits in FDDKI mice. These data are consistent with a role for the carboxyl-terminal APP domain in the pathogenesis of dementia and suggest that averting the noxious role of Thr⁶⁶⁸ is a viable therapeutic strategy for human dementias.

Introduction

Familial dementias are caused by mutations in APP [1] and genes that regulate APP processing. These include the PSEN1/2 genes, which code for the catalytic component of the gamma-secretase, and the BRI2/ITM2b gene, whose protein product BRI2 binds APP and inhibits APP processing [1–8]. Cases caused by APP/PSEN mutations are classified as FAD and those caused by mutations in BRI2/ITM2b as FDD or Familial British dementia (FBD). The prevailing pathogenic model for these dementias posits that amyloid peptides trigger dementia. In AD, the amyloid peptide Aβ is a part of APP; in FDD and FBD, the amyloidogenic peptides, called ADan and Aβri, are generated from the mutant BRI2 proteins [2,3]. FDD patients present mixed amyloid plaques containing both Aβ and ADan. However, recent data suggest that these dementias share pathogenic mechanisms involving synaptic-toxic APP metabolites distinct from Aβ [9,10].

In FDD, a 10-nucleotide duplication in the BRI2/ITM2b gene leads to the synthesis of a longer BRI2 protein [8]. In normal individuals, BRI2 is synthesized as an immature type-II membrane protein (imBRI2) that is cleaved at the C-terminus into mature BRI2 and a 23aa soluble C-terminal fragment [11]. In FDD patients, cleavage of the BRI2 mutant protein leads to the release of the longer ADan peptide [8]. To model FDD we generated FDDKI mice that like FDD patients [8], carry one wild type Brie2/Im2b allele and the other one has the Danish mutation [12]. FDDKI mice develop synaptic and memory deficits due to loss of Brie2 protein, but do not develop amyloidosis [13]. Brie2 binds to APP and inhibits cleavage of APP by secretases [4–7]. Owing to the loss of Brie2, processing of APP is increased in FDD [14,15]. Memory and synaptic deficits of FDDKI mice require APP [14], and are mediated by sAPPβ and/or β-CTF produced during synaptic plasticity and memory acquisition. Inhibition of γ-secretase, the enzyme that processes β-CTF to yield Aβ, worsens memory deficits and is associated with an accumulation of β-CTF [10,16,17]. In addition, caspase-9 in activated in FDDKI mice and caspase-9 activity mediates memory/synaptic plasticity deficits [18]. Overall, these results suggest that β-CTF, rather than Aβ, is a major toxic species causing dementia. Here, we have investigated further the pathogenic role of the carboxyl-terminal region of APP and especially the role of residue Thr⁶⁶⁸.
Results

Thr^{668} of APP Mediates Object Recognition Deficits found in FDDKI Mice

Recent findings suggest that products of BACE1-processing of APP (predominantly β-CTF) trigger several pathological features related to human dementias both in a mouse model of FDD [10,16] and human neurons derived from familial and sporadic AD [9]. Thus, we decided to probe in more detail the pathogenic function of the carboxyl-terminal region of APP, focusing on the intracellular Thr^{668} residue (following the numbering of the APP^{695} isoform). The phosphorylation status of Thr^{668} either creates or destroys docking sites for intracellular proteins that interact with APP [19–22]. In addition, phosphorylation at Thr668 is increased in AD cases [23] suggesting potential pathogenic implications. We generated mice expressing APP with a Thr^{668}Ala mutation, called APP^{T668A} [24]. Western blot analysis of hippocampal synaptosomes from either APP^{WT/WT} or APP^{T668A/T668A} mice shows that the Thr^{668}Ala mutation abolishes phosphorylation at Thr^{668} (Figure 1a).

Thus, the APP^{T668A} mice are an ideal genetic tool to study the role of Thr^{668} and its phosphorylation in the pathogenesis of dementia. To this end, we utilized FDDKI mice, which develop severe age-dependent memory and synaptic plasticity deficits that first become measurable at ~5 months of age [13]. Most importantly, these deficits are prevented when FDDKI mice lack one allele of APP, reducing the APP protein load [14], and require production of APP β-CTF [10,16]. Thus, since memory and synaptic deficits of FDDKI mice are dependent on endogenous APP, we can test the pathogenic role of Thr^{668} by introducing this APP mutation on the FDDKI background.

By crossing FDDKI/APP^{TA/TA} to APP^{T668A/T668A} mice we generated littermates of the following 6 genotypes: WT, FDDKI, FDDKI/APP^{TA/TA}, FDDKI/APP^{TA/WT}, FDDKI/APP^{TA/TA} and APP^{T668A/T668A}. To test memory, six-month-old mice were subjected to the novel object recognition (NOR) task, which is a non-aversive task that relies on the mouse’s natural exploratory behavior. Open field studies showed that FDDKI, FDDKI/APP^{TA/TA}, FDDKI/APP^{TA/WT}, APP^{T668A/T668A} and APP^{T668A/T668A} mice have no defects in habituation and locomotor behavior, sedation, risk assessment and anxiety-like behavior in novel environments (Figure 1b and c). During the training session, mice of all genotypes spent the same amount of time exploring the identical objects during the training phase (Figure 1d). The following day, when a novel object was introduced, FDDKI spent the same amount of time exploring the two objects as if they were both novel to them, while the WT, APP^{T668A/T668A} and APP^{T668A/WT} mice still spent more time exploring the novel object (Figure 1e). Notably, FDDKI/APP^{TA/TA} and FDDKI/APP^{TA/WT} mice behaved like the WT mice and explored preferentially the novel object (Figure 1e), demonstrating a prevention of the defect of the FDDKI mice. We subjected the mice to the NOR task at 9 months, and also at 12 months to confirm that this is a true prevention of deficits and not a delay. We found similar data to the data at 6 months with the FDDKI mice showing no preference between the two objects on the second day, while the FDDKI/APP^{TA/TA}, FDDKI/APP^{TA/WT}, APP^{T668A/T668A} and APP^{T668A/WT} mice all behaved similar to the WT mice (Figure 1f and 1g). These data confirm that memory is impaired in FDDKI mice upon aging in an ethologically relevant, non-aversive behavioral context; remarkably, development of this deficit is fully prevented by changing the Thr^{668} residue on the intracellular region of APP to an Alanine.

Thr^{668} of APP Mediates Short-term Memory Deficits Found in FDDKI Mice

To further test memory, WT, FDDKI, FDDKI/APP^{TA/TA}, FDDKI/APP^{TA/WT}, APP^{T668A/T668A}, APP^{T668A/WT} mice were subjected at 5.5 months of age to the radial arm water maze (RAWM) task, a spatial working memory test that depends upon hippocampal function [25]. This task tests short-term memory, which is the memory affected in early stages of AD. The six genotypes were required to learn and memorize the location of a hidden platform in one of the arms of a maze with respect to spatial cues. WT, APP^{T668A/TA}, and APP^{T668A/WT} mice were able to acquire (A) and retain (R) memory of the task. FDDKI mice showed severe abnormalities during both acquisition and retention of the task (Figure 2a), confirming that FDDKI mice have severe impairment in short-term spatial memory for platform location during both acquisition and retention of the task. This defect was due to a deficit in memory per se and not to deficits in vision, motor coordination or motivation because testing with the visible platform showed no difference in the swimming speed and the time needed to find the platform between the FDDKI and WT mice (Figure 2c and d). Both the FDDKI/APP^{TA/TA} and the FDDKI/APP^{TA/WT} mice showed no defects in the memory test (Figure 2a), showing that mutating the intracellular APP residue Thr^{668} to an alanine prevented the RAWM deficit of FDDKI mice, and confirming the data seen in NOR. To ensure that this was not simply a delay of the deficit, the mice were re-tested at 9 months in the RAWM task, and once again the FDDKI/APP^{TA/TA} and the FDDKI/APP^{TA/WT} mice did not show the deficit seen in the FDDKI mice (Figure 2b).

Discussion

In this manuscript, we have pinpointed an intracellular residue of APP that is required for memory and synaptic plasticity deficits. FDDKI mice allow for a genetic analysis of pathogenic pathways on a genetic background that is congruous to the human disease. We showed that haploinsufficiency in APP prevented all FDDKI mice’s deficits at all ages. Now we take this further by showing that mutation in just one residue of APP, the intracellular amino acid Thr^{668}, can also prevent the memory and synaptic deficits.

We studied the functional relevance of Thr^{668} of APP because APP^{T668A} is enriched in AD patients [23], suggesting a pathogenic role for phosphorylation at this residue, and because it has profound effects on APP protein/protein interactions and...
Figure 1. A Thr<sup>668</sup>Ala mutation on APP prevents the object recognition memory deficit of FDDK<sub>1</sub> mice. (a) Western blot analysis of hippocampal synaptosomal preparations shown that the Thr to Ala mutation abolishes phosphorylation of Thr<sup>668</sup> (APP<sup>pThr<sub>668</sub></sup>). Interestingly, only the mature form of APP (mAPP) and not the immature (imAPP), is found phosphorylated on this Thr in hippocampal synaptic fractions of WT mice. (b and c) Open field is a sensorimotor test for habituation, exploratory, emotional behavior, and anxiety-like behavior, in novel environments. The percent of time in the center (b) and the number of entries into the center (c) are indicators of anxiety levels. The more the mouse enters the center and explores it, the lower the level of anxiety-like behavior. Since the FDDK<sub>1</sub>, FDDK<sub>1</sub>/APPTA/TA, FDDK<sub>1</sub>/APPTA/WT, APPTA/TA, APPTA/WT mice are similar to the WT animals there is no deficit or excess of anxiety. (d) All six genotypes (WT, FDDK<sub>1</sub>, FDDK<sub>1</sub>/APPTA/TA, FDDK<sub>1</sub>/APPTA/WT, APPTA/TA, APPTA/WT) mice spent similar amounts of time exploring the two identical objects on day 1. (e) FDDK<sub>1</sub>/APPTA/TA and FDDK<sub>1</sub>/APPTA/WT mice behaved similarly to WT mice and prevented the deficit in the NOR tests found in FDDK<sub>1</sub> mice at 6 months of age (FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/TA P = 0.011; FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/WT P = 0.0083; FDDK<sub>1</sub> versus WT P = 0.001), (f) 9 months of age (FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/TA P = 0.01; FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/WT P = 0.347; FDDK<sub>1</sub> versus WT P = 0.000995), and (g) 12 months of age (FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/TA P = 0.0003; FDDK<sub>1</sub> versus FDDK<sub>1</sub>/APPTA/WT P = 0.0002; FDDK<sub>1</sub> versus WT P < 0.0001). Thus the APPTA point mutation prevented the novel object recognition deficit of FDDK<sub>1</sub> mice.

doi:10.1371/journal.pone.0057120.g001
APP biology. For example, Thr668 phosphorylation impairs APP/Fe65 interaction [20,21] but promotes Pin1 binding [22]. In addition, this phosphorylation regulates trafficking of APP and APP derived metabolites [26]. Previous studies in mice suggested a protective role for phosphorylation of Thr668 in the pathogenesis of AD by showing that Pin1 decreases APP processing and Aβ production by binding APP phosphorylated on Thr668 [27]. However, analysis of the APPTA mice has shown that preventing phosphorylation by mutating Thr668 into an Ala does not change Aβ levels in vivo [28,29].

If Aβ were a major neuro-toxic peptide in dementia, FDDKI/APPTA mice should either have deficits comparable to FDDKI mice based on the evidence that the Thr668Ala mutation does not change Aβ levels [28,29], or should present with a worsened phenotype. This tyrosine is comprised in the intracellular 682YENPTY687 sequence of APP, a docking region for numerous APP-binding proteins that regulate processing and functions of APP [19,30–35]. Phosphorylation of Tyr682 is consequential. Some proteins, such as Grb2 [36], Shc [37,38], Grb7 and Crk [39] interact with APP only when Tyr682 is phosphorylated; others, like Fe65, Fe65L1 and Fe65L2 only when this tyrosine is not seen in a short-term memory test, such as the RAWM task, and also in an ethologically relevant, non-aversive behavioral context, such as the NOR task. The memory deficits were prevented at their start and no deficits could be found even as late as 9–12 months of age. The same was true for the synaptic plasticity at 12 months old. The FDDKI mice show strong synaptic defects in the Schaffer collateral pathway, however, FDDKI/APPTA mice showed no such deficits.

In this context, it is worth noting that mutation of another phosphorylated amino acid present in the APP intracellular region, namely Tyr682, results in a different (almost opposite) phenotype. This tyrosine is comprised in the intracellular 682YENPTY687 sequence of APP, a docking region for numerous APP-binding proteins that regulate processing and functions of APP [19,30–35]. Phosphorylation of Tyr682 is consequential. Some proteins, such as Grb2 [36], Shc [37,38], Grb7 and Crk [39] interact with APP only when Tyr682 is phosphorylated; others, like Fe65, Fe65L1 and Fe65L2 only when this tyrosine is not
phosphorylated [40], suggesting that phosphorylation–dephosphorylation on Tyr682 modulates APP functions. To test the in vivo function of Tyr682 we have created mice with Tyr682 replaced by a Gly. This knock-in mutation alters the function of APP in memory formation, development/aging [41,42] and changes APP processing, leading to a significant decrease in Aβ levels [29]. Thus, while the Thr668Ala mutation on APP, which does not reduce Aβ production, prevents memory deficits of FDD KI mice, the Tyr682Gly mutation, which reduces Aβ production, causes cognitive defects on its own [41]. These data show that the intracellular region of APP has a fundamental role in memory formation, a role that is not linked to Aβ.

New evidence points to β-derived metabolites of APP, especially β-CTF, as the synaptic-toxic APP fragments mediating synaptic and memory impairments. The data presented here suggest that the synaptic-toxic activity of β-CTF requires Thr668 (Figure 4a–c). It is possible that this synaptic-toxic activity necessitates or is enhanced by phosphorylation of Thr668 (Figure 4d–f), which is abolished by the Thr668Ala mutation. It is interesting to note that this mutation does not alter essential biological functions of APP during development [24], suggesting that targeting the role of Thr668, and perhaps its phosphorylation, in dementia may be an effective and safe therapeutic approach for dementia. Since the APP<sup>T<sub>A</sub></sup> mutation prevents memory and synaptic deficits in heterozygosis, a partial reduction of the noxious pathogenic functions mediated by Thr<sup>668</sup> will be therapeutically efficient.

**Methods**

**Mouse Handling**

The animals used for these studies were backcrossed to C57Bl6/J mice for at least 14 generations. Mice were handled according to the Ethical Guidelines for Treatment of Laboratory Animals of Albert Einstein College of Medicine. The procedures were described and approved in animal protocol number 200404. The Institutional Animal Care and Use Committee (IACUC) approved this protocol. IACUC is a federally mandated committee that oversees all aspects of the institution’s animal care and use program, facilities and procedures. The regulations of the USDA and PHS require institutions using animals to appoint an IACUC. The members of the IACUC are appointed by the Dean of Albert Einstein College of Medicine of Yeshiva University (Einstein).

**Synaptosomes Preparations**

Hippocampi were homogenized in H buffer [5 mM Heps/NaOH pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.32 M sucrose, plus phosphatase/protease inhibitors at 10% (w/v) and centrifuged at 800 g for 10 min. The supernatant (S1) was separated to supernatant (S2) and pellet (P2) by spinning at 9,200 g for 15 min. P2 represents crude synaptosomal fraction.

**Antibodies**

The following antibodies were used: anti-APP (Chemicon), anti-APP-CTF (Invitrogen), anti-APP<sup>Thr<sub>686</sub></sup> and : anti-Akt (Cell Signaling). Secondary antibodies conjugated with horse-radish-peroxidase are from Southern Biotechnology.
Electrophysiology and Behavior

Only male mice were used to avoid variations due to hormonal fluctuations during the estrous female cycle, which influence severely behavioral and electrophysiological tests.

Spatial Working Memory

A six-armed maze was placed into white tank filled with water (24–25°C) and made opaque by the addition of nontoxic white paint. Spatial cues were presented on the walls of the testing room. At the end of one of the arms was positioned a clear 10 cm submerged platform that remained in the same location for every trial in 1 d but was moved approximately randomly from day to day. On each trial, the mouse started the task from a different randomly chosen arm. Each trial lasted 1 min, and errors were counted each time the mouse entered the wrong arm or needed more than 10 s to reach the platform. On each trial, the mouse started the task from a different randomly chosen arm. Each trial lasted 1 min, and errors were counted each time the mouse entered the wrong arm or needed more than 10 s to reach the platform. After each error, the mouse was pulled back to its starting position. After four consecutive acquisition trials, the mouse was placed in its home cage for 30 min, then returned to the maze and administered a fifth retention trial. The scores for each mouse on the last 3 days of testing were averaged and used for statistical analysis.

Visible Platform Testing

Visible platform training to test visual and motor deficits was performed in the same pool as in the RAWM; however, the arms of the maze were removed. The platform was marked with a black flag and positioned randomly from trial to trial. Time to reach the platform and speed were recorded with a video tracking system (HVS 2020; HVS Image).

Open Field and Novel Object Recognition

After 30 min to acclimate to the testing room, each mouse was placed into a 40 cm x 40 cm open field chamber with 2 ft high opaque walls. Each mouse was allowed to habituate to the normal open field box for 10 min, and repeated again 24 hours later, in which the video tracking system (HVS 2020; HVS Image) quantifies the number of entries into and time spent in the center of the locomotor arena. Novel object recognition was performed as previously described [43]. Results were recorded as an object discrimination ratio (ODR), which is calculated by dividing the time the mice spent exploring the novel object, divided by the total amount of time exploring the two objects.

Electrophysiology

Transverse hippocampal slices (400 μm) were transferred to a recording chamber where they were maintained at 29°C and perfused with artificial cerebrospinal fluid (ACSF) continuously bubbled with 95% O₂ and 5% CO₂. The ACSF composition in mM was: 124 NaCl, 4.4 KCl, 1 Na₂HPO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgSO₄, and 10 glucose. CA1 field-excitatory-post-synaptic potentials (fEPSPs) were recorded by placing both the stimulating and the recording electrodes in CA1 stratum radiatum. For LTP experiments, a 30 min baseline was recorded every minute at an intensity that evoked a response approximately 35% of the maximum evoked response. LTP was induced using a tetra-burst stimulation (four pulses at 100 Hz, with bursts repeated at 5 Hz and each tetanus including one ten-burst train). Responses were recorded for 90 min after tetanization and plotted as percentage of baseline fEPSP slope.
Conceived and designed the experiments: LD. Performed the experiments: LD FL FB RT. Analyzed the data: LD FL FB RT. Contributed reagents/materials/analysis tools: OA. Wrote the paper: LD.

References

1. Betran L, Lill CM, Tzuri RE (2010) The genetics of Alzheimer disease: back to the future. Neuron 60: 270–281.
2. Vidal R, Frangione B, Rostagno A, Mead S, Revesz T, et al. (1999) A stop-codon mutation in the BRII gene associated with familial British dementia. Nature 399: 776–778.
3. St George-Hyslop PH, Petit A (2005) Molecular biology and genetics of Alzheimer’s disease. C R Biol 328: 119–130.
4. Matsuda S, Gilbertso L, Matsuda Y, Davies P, McGowan E, et al. (2005) The familial dementia BRII gene binds the Alzheimer gene amyloid-beta precursor protein and inhibits amyloid-beta production. J Biol Chem 280: 20812–20816.
5. Fotinoopoulos A, Tsachaki M, Vlacki M, Poulopoulos A, Rostagno A, et al. (2005) BRII2 interacts with amyloid precursor protein (APP) and regulates amyloid beta (Abeta) production. J Biol Chem 280: 30768–30772.
6. Matsuda S, Matsuda Y, Snapp EL, D’Adamio L (2011) Maturation of BRII generates a specific inhibitor that reduces APP processing at the plasma membrane and in endoctic vesicles. Neurobiol Aging 32: 1400–1408.
7. Matsuda S, Giliberto L, Matsuda Y, McGowan EM, D’Adamio L (2008) BRII inhibits amyloid beta-peptide precursor protein processing by interfering with the docking of secretases to the substrate. J Neurosci 28: 8668–8676.
8. Vidal R, Revesz T, Rostagno A, Kim E, Holton J, et al. (2000) A decamer duplication in the 3′ region of the BRII gene originates an amyloid peptide that is associated with dementia in a Danish kindred. Proc Natl Acad Sci U S A 97: 4920–4925.
9. Israel MA, Yuan SH, Bardy C, Reyna SM, Mu Y, et al. (2012) Probing sporadic and familial Alzheimer’s disease using induced pluripotent stem cells. Nature 481: 216–220.
10. Tamayev R, Matsuda S, Arancio O, D’Adamio L (2012) beta- but not gamma-secretase proteolysis of APP causes synaptic and memory deficits in a mouse model of dementia. EMBO Mol Med 4: 171–179.
11. Garringer HJ, Murrell J, D’Adamio L, Ghetti B, Vidal R (2009) Modeling familial British and Danish dementia. Brain Struct Funct.
12. Giliberto L, Matsuda S, Vidal R, D’Adamio L (2009) Generation and Initial Characterization of FDD Knock In Mice. PLoS One 4: e7900.
13. Tamayev R, Matsuda S, D’Adamio L (2003) JNK-interacting protein-1 mediates a specific inhibitor that reduces APP processing at the plasma membrane and in endoctic vesicles. Neurobiol Aging 32: 1400–1408.
14. Tamayev R, Matsuda S, Arancio O, D’Adamio L (2010) Finnish familial dementia mice suggest that loss of function and not the amyloid cascade causes synaptic plasticity and memory deficits. Proc Natl Acad Sci U S A 107: 20822–20827.
15. Matsuda S, Matsuda Y, D’Adamio L (2003) Amyloid beta protein precursor (AbetaPP), but not AbetaPP-like protein 2, is bridged to the kinesin light chain by the scaffold protein JNK-interacting protein 1. J Biol Chem 278: 38961–38966.
16. Tamayev R, Matsuda S, D’Adamio L (2010) Essential Role of APP-Thr668 in Neurodegeneration. PLoS One 5: e15503.
17. Barbagallo AP, Weldon R, Tamayev R, Zhou D, Giliberto L, et al. (2010) Tyr62 in the Intraeural Domain of APP Regulates Amyloidogenic APP Processing In Vivo. PLoS One 5: e15503.
18. Barbagallo AP, Weldon R, Tamayev R, Zhou D, Giliberto L, et al. (2010) Tyr62 in the intracellular domain of APP regulates amyloidogenic APP processing in vivo. PLoS One 5: e15503.