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The db mutation improves memory in younger mice in a model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease, while obesity is a major global public health problem associated with the metabolic disorder type 2 diabetes mellitus (T2DM). Chronic obesity and T2DM have been identified as invariant risk factors for dementia and late-onset AD, while their impacts on the occurrence and development of AD remain unclear. As shown in our previous study, the diabetic mutation (db, $Lepr^{db/db}$) induces mixed or vascular dementia in mature to middle-aged $APP^{\Delta NL/\Delta NL}$ x $PS1^{P264L/P264L}$ knock-in mice (db/AD). In the present study, the impacts of the db mutation on young AD mice at 10 weeks of age were evaluated. The db mutation not only conferred young AD mice with severe obesity, impaired glucose regulation and activated mammalian target of rapamycin (mTOR) signaling pathway in the mouse cortex, but lead to a surprising improvement in memory. At this young age, mice also had decreased cerebral Aβ content, which we have not observed at older ages. This was unlikely to be related to altered Aβ synthesis, as both β- and γ-secretase were unchanged. The db mutation also reduced the cortical IL-1β mRNA level and IBA1 protein level in young AD mice, with no significant effect on the activation of microglia and astrocytes. We conclude that the db mutation could transitorily improve the memory of young AD mice, a finding that may be partially explained by the relatively improved glucose homeostasis in the brains of db/AD mice compared to their counterpart AD mice, suggesting that glucose regulation could be a strategy for prevention and treatment of neurodegenerative diseases like AD.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder that is clinically characterized by a progressive cognitive decline and dementia, and pathologically characterized by the development of extracellular neuritic plaques containing beta-amyloid (Aβ) protein and intracellular neurofibrillary tangles composed of the microtubule-associated protein tau [1–3]. AD is caused by complex interactions among multiple genetic, epigenetic, and environmental factors, such as lifestyle (e.g., diet and exercise) [2,3]. While the *apolipoprotein ε4* allele has been genetically linked to late-onset (> 60 years) familial and sporadic AD, mutations in three genes, *amyloid precursor protein* (*APP*), *presenilin-1* and *presenilin-2*, cause early-onset (< 60 years) autosomal-dominant AD [3]. The two forms of AD ultimately exhibit similar pathologies caused by the combination of Aβ and tau accumulation that promote progressive synaptic failure and neuronal loss, leading to memory loss

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and cognitive impairment [1–4].

Obesity is a major global public health problem and is associated with the metabolic disorder type 2 diabetes mellitus (T2DM) that is characterized by the progressive loss of glucose homeostasis, development of insulin resistance and pancreatic β-cell degeneration, leading to insufficient insulin secretion from β-cells to meet the demands of increased peripheral insulin resistance in the late stages of the disease [5,6]. Prior to the development of overt T2DM, a prediabetic state is identified based on impaired fasting glucose levels or impaired glucose tolerance after a glucose tolerance test (GTT) [7], accompanied by compensatory increases in the β-cell number and alterations in β-cell functions in human and animal models [8–10]. Leptin suppresses energy intake and stimulates energy expenditure, leading to a reduction in body fat storage [11]. Leptin-resistance mice (db, $Lepr^{db/db}$) are a recognized mouse model of T2DM that exhibit a lack of leptin signaling due to a point mutation in the leptin receptor [12,13].

Diabetes and AD are two highly prevalent pathological conditions worldwide, while the interplay between diabetes and AD remains controversial. Numerous studies have suggested that obesity at midlife and T2DM are potentially modifiable risk factors for dementia and late-onset Alzheimer's disease [14–17]. Our previous study also showed an acceleration of the development of tau pathology in obese and diabetic $Lepr^{db/db}$ mice in adulthood (~23 weeks old) [18]. Other studies suggested that the extent of AD pathology is essentially unchanged in patients with a history of T2DM, while cerebrovascular pathology increases [19,20]. Due to improved treatments, patients with T2DM are living longer, placing them at increased risk of developing age-related complications, particularly vascular aging-associated cerebrovascular dysfunction and vascular dementia. For a better understanding of the interaction between T2DM and dementia, a novel mouse model has been created by crossing the $Lepr^{db/db}$ mice with the $APP^{\Delta NL/\Delta NL} \times PS1^{P264L/P264L}$ knock-in model of AD [4]. The resulting mice were morbidly obese, glucose intolerant, insulin resistant, and displayed a unique model of mixed dementia characterized by both AD-related and vascular pathologies at 10–14 months of age, along with profound cognitive impairments and marked cerebrovascular abnormalities that did not appear to be driven by Aβ deposition [4].

In the present study, we characterized the effects of the db mutation on the development of pathology in the brain of AD mice at 6–10 weeks of age, when mice are not predicted to have significant histopathology or cognitive problems. Combined with our previous results obtained from older mice, we aimed to obtain a better understanding of the evolution of the interplay between obesity/diabetes and AD during the initiation and development of AD.

2. Materials and methods

2.1. Animals and dietary treatments

All animal experiments were approved by the Institutional Animal Care and Use Committee of Pennington Biomedical Research Center.

Both the AD mouse model ($APP^{\Delta NL/\Delta NL}$ x $PS1^{P264L/P264L}$) and diabetic mouse model (db, $Lepr^{db/db}$) used in this study have been described in our previous studies [4,18,21–23]. In the knock-in AD mice, the endogenous APP and PS1 genes were replaced with mutated, humanized APP and PS1 genes (ΔNL and P264L, respectively), which remain under the control of their endogenous, murine promoters. The C57BLKS/J heterozygous $Lepr^{db/+}$ mice were purchased from the Jackson Laboratory (#000697, Bar Harbor, ME, USA). The $Lepr^{db/+}$/AD mice were obtained by crossing AD mice with db mice. As the homozygous db mice ($Lepr^{db/db}$) are infertile, the experimental cohorts used in this study were male and female AD mice ($APP^{\Delta NL/\Delta NL}$ x $PS1^{P264L/P264L}$) and db/AD mice ($Lepr^{db/db} \times APP^{\Delta NL/\Delta NL}$ x $PS1^{P264L/P264L}$) obtained by crossing $Lepr^{db/+}$/AD mice, with the aim of examining the impacts of obesity and diabetes on the young AD mice at 10 weeks of age.

Mice were housed in groups of two to three mice per standard cage on a 12:12 light/dark cycle and were provided ad libitum access to water and their diet.

2.2. Body composition measurement and glucose tolerance test

At 6 weeks of age, the body weight, total body fat and body muscle content of 8 AD and 7 db/AD littermates were measured using nuclear magnetic resonance spectroscopy (Minispec, Brucker Optics, Billerica, MA).

At 10 weeks of age, these mice were subjected to the glucose tolerance test (GTT), all glucose measurements were obtained via tail bleed using a WBM Bayer Contour NEXT EZ Blood Glucometer and test strips (Bayer, Tarrytown, NY). For this, all animals were fasted overnight, a baseline measurement was obtained after which the GTT was initiated by intraperitoneal injection of D-(+)-glucose (0.5 g/kg body weight). Subsequent measurements were recorded at 30, 60, and 120 min post-injection.

2.3. Serum and tissue collection

After the GTT test, the 8 AD mice and 7 db/AD mice at 10 weeks of age were then weighed, euthanized under isoflurane anesthesia, exsanguinated via cardiac puncture, perfused with phosphate-buffered saline (pH 8.0) and decapitated. Brains were collected and divided into hemispheres, one for formalin fixation and one frozen for biochemical measurements.

2.4. Serum analysis

Blood collected at euthanasia via cardiac puncture was allowed to clot overnight at 4 °C and then centrifuged. Serum was isolated and analyzed using ELISAs to detect free fatty acids (#K612-100, BioVision, Inc., Milpitas, CA, USA), insulin (#90080, Crystal Chem. Inc., Downers Grove, IL, USA), total cholesterol (#439-17501, WAKO Diagnostics, Osaka, Japan), and leptin (#DY498, R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturers' instructions.

2.5. Histology and immunohistochemistry

The PBS-perfused mouse brain tissues were stored in formalin for 24–48 h and then embedded in paraffin. Samples were sectioned at a thickness of 5 μm, and immunohistochemical (IHC) staining was performed as described previously in studies by our laboratory without modifications [24,25]. The primary anti-IBA1 antibody (#019-19741) was purchased from Wako (Osaka, Japan); the anti-GFAP (#ab48050) and anti-Collagen IV (#ab6586) antibodies were purchased from Abcam (Cambridge, MA, USA).

2.6. Aβ analysis

The amount of Aβ in mouse brain tissues was determined using a three-step serial extraction from increasingly insoluble fractions of the mouse brain, phosphate-buffered saline (PBS), 2% sodium dodecyl sulfate (SDS), and finally, 70% formic acid (FA), followed by the quantitative measurement of Aβ concentrations using an ELISA. One hemisphere of the mouse brain was homogenized and used in this experiment. A detailed description of the methodology and antibodies used has been outlined previously in numerous studies from our laboratory and collaborators [4,21,23,26,27].

2.7. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) experiments were performed as described in previous studies from our laboratory [28,29]. Total RNA was extracted from 30 mg of mouse cortex using an RNeasy Mini Kit

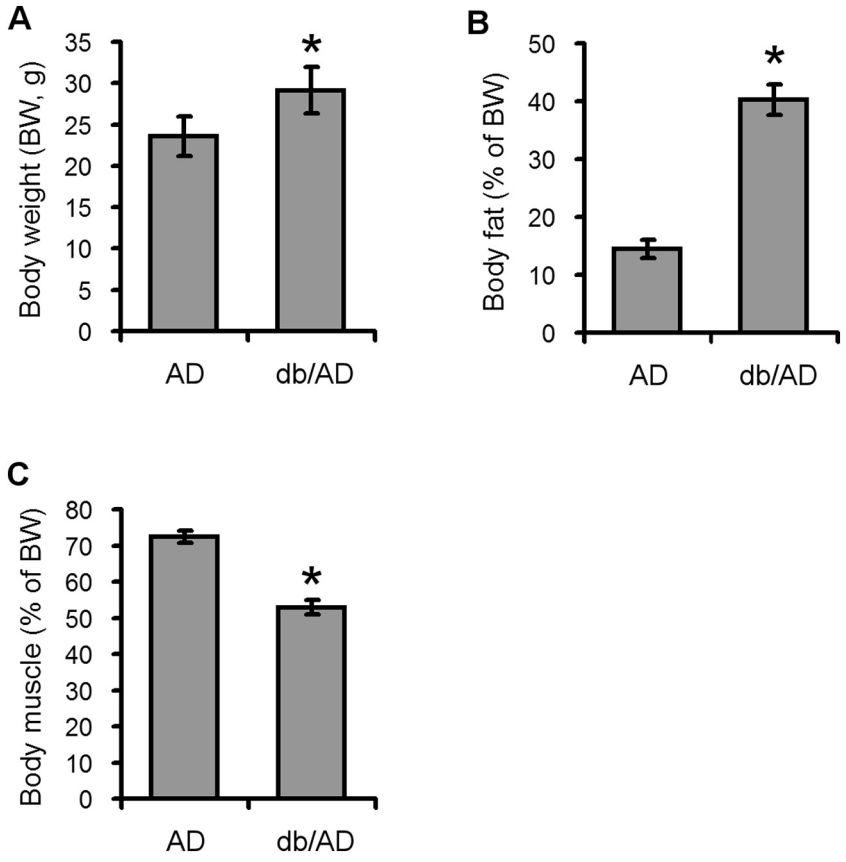

Fig. 1. Mouse NMR test at 6 weeks of age. At 6 weeks of age, db/AD mice were significantly heavier (A), and contained more body fat (B) but less body muscle (C) than AD mice, as expressed as percentage of BW. (Bars represent means ± SEM; n = 8 AD mice, n = 7 db/AD mice; *$p < 0.05$ compared with AD mice).

(Qiagen, Valencia, CA) according to the manufacturer's instructions. The corresponding cDNAs were prepared from 2 μg of extracted total RNA with M-MuLV transcriptase (New England Biolabs, Ipswich, MA) using 20 μL of the reverse transcription system, according to the manufacturer's instructions. For qPCR, aliquots of cDNA templates were subjected to qPCR in 20 μL of 1× Brilliant II QPCR & QRT-PCR Reagents (Agilent Technologies, Santa Clara, CA), 1× primers and TaqMan probe (6-FAM/ZEN/IBFQ mode) and 10 ng of cDNA templates. Primers and probes for IL-1β, IL-6, TNFα and Gapdh were ordered from the PrimeTime Pre-designed qPCR Assays system of Integrated DNA Technologies (IDT, Coralville, IA). Each sample was loaded in triplicate, and negative and positive controls were included. Gadph was amplified as an internal reference gene. The following PCR amplification conditions were employed using an ABI PRISM 7000 sequence detector according to the manufacturer's instructions (Applied Biosystems, Foster City, CA): 50 °C for 2 min, 95 °C for 15 min, and 40 cycles each with 95 °C for 15 s and 60 °C for 45 s. The $\Delta\Delta C_t$ method was used to analyze the data. The relative mRNA expression of each gene is reported as the mean and standard deviation of 6 independent total RNA extractions and real-time PCR analyses.

2.8. Western blotting

The anti-BACE1 antibody (#BAF931) was purchased from R&D Systems (Minneapolis, MN, USA), and the anti-BACE2 antibody (#ab8025) was purchased from Abcam (Cambridge, MA, USA). The anti-β-actin antibody (#sc-47778) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against p70 S6 Kinase (#2708), phospho-p70 S6 Kinase (#9234), mTOR (#2983), phospho-mTOR (#5536S), Rictor (#9476), Raptor (#2280S), 4EBP1 (#9644), phospho-4EBP1 (#9451), and γ-Secretase subunits, including Nicastrin (#5665), PEN2 (#5451), Presenilin 1 (#5643) and Presenilin 2 (#2192) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Protein lysates from the mouse cortex were prepared in RIPA buffer using a tissue homogenizer, and the amount of protein in lysates was estimated using BCA reagent (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were separated using SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was then probed with antibodies as described previously. All electrophoresis and immunoblotting reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The HRP-conjugated secondary antibodies were purchased from the Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

2.9. Fear conditioning test

Testing was performed in the PBRC Animal Metabolism and Behavior Core (https://www.pbrc.edu/research-and-faculty/core-services/?serviceid=61).

Mice were evaluated in an automated video-recorded fear conditioning system (Med Associates). Fear conditioning on day 1 consisted of 5 min of acclimation, followed by five stimuli of a 30 s tone (85 dB, 4 kHz) that co-terminated with a shock (0.5 mA × 1 s). On day 2, the mice were returned to the same chambers, but no stimuli were applied. The activity level (percentage of freezing behaviors) measured in 30 s intervals reflects contextual fear. On day 3, the mice were returned to the chambers that had been modified. A flat floor overlay the usual grid, an insert modified the chamber dimensions, and an acetic acid odor was used to reinforce a novel environment. After habituating the mice to the chambers for 5 min, a continuous tone (85 dB, 4 kHz) was applied for 5 min. The percentage of freezing behaviors was recorded.

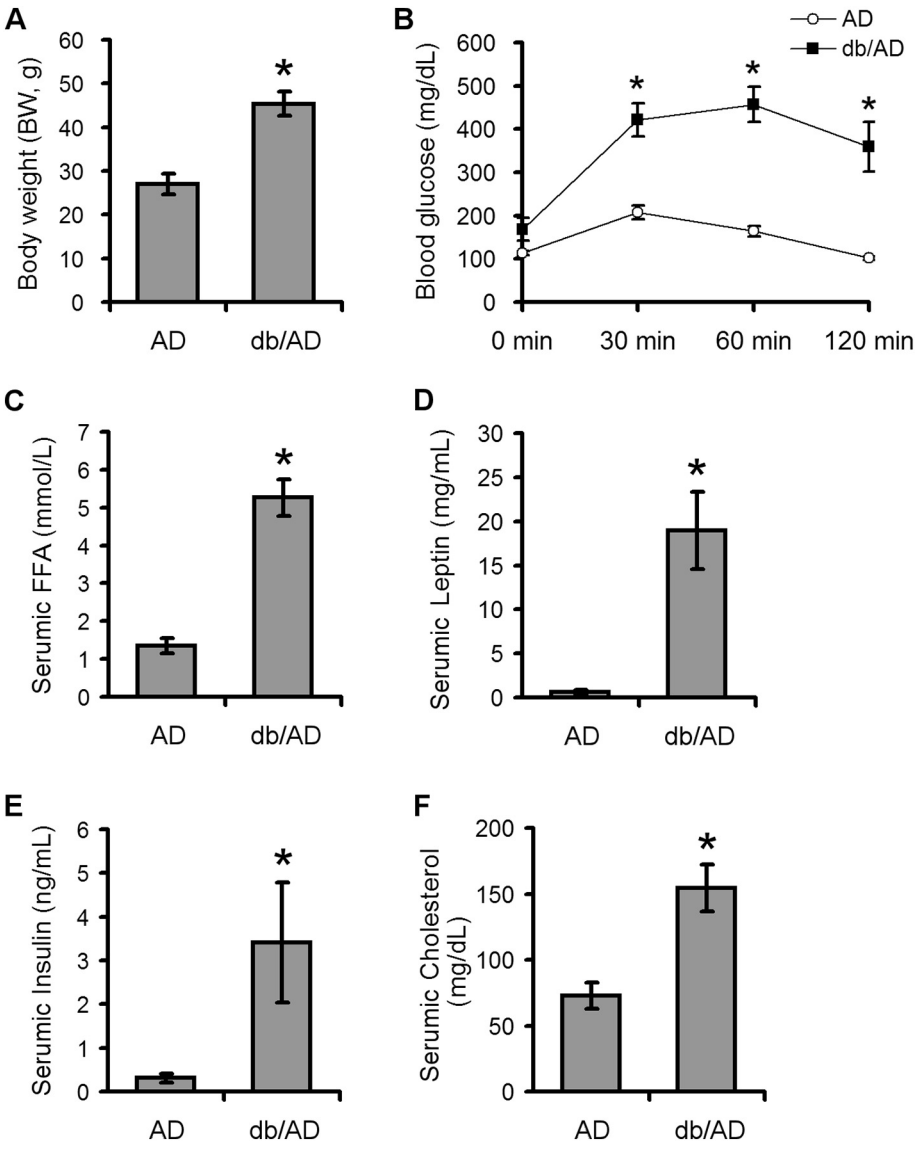

Fig. 2. Mouse GTT and serum ELISAs at 10 weeks of age. At 10 weeks of age, db/AD mice were much heavier (A) and exhibited a significant increase in glucose levels throughout the glucose tolerance test compared to the AD mice (B). The serumic concentrations of free fatty acids (C), leptin (D), insulin (E) and total cholesterol (F) were also significantly increased in db/AD mice compared with AD mice, as determined using ELISAs. (Bars represent means ± SEM; n = 8 AD mice, n = 7 db/AD mice; *$p < 0.05$ compared with AD mice).

2.10. Statistical analysis

Data are presented as the means ± standard errors of the means (SEMs) for the number of replicates indicated. Statistical analyses of two selected groups were performed using unpaired Student's *t*-test. A significant difference was defined as $p < 0.05$.

3. Results

3.1. db mutation affected weight gain and serumic metabolic parameters in the young AD mice

At 6 weeks of age, the db/AD mice already exhibited an obese phenotype, with significantly higher body weight (BW, AD: 23.59 ± 2.36 g vs. db/AD: 29.14 ± 2.79 g, $p < 0.05$), increased whole body fat content (AD: 14.45% ± 1.16% vs. db/AD: 40.24% ± 2.62%, $p < 0.05$) and lowered body muscle mass content (AD: 72.38% ± 1.65% vs. db/AD: 52.91% ± 2.02%, $p < 0.05$) as expressed as the percentage of BW (Fig. 1).

At 10 weeks of age, db/AD mice were much heavier (BW, AD: 27.04 ± 2.07 g vs. db/AD: 45.39 ± 2.90 g, $p < 0.05$) (Fig. 2A), accompanied by elevated fasting glucose levels and impaired glucose clearance compared to the AD mice (Fig. 2B). The serumic metabolic parameters of mice were also measured. The db/AD mice exhibited significantly higher serumic free fatty acid (FFA), leptin, insulin and total cholesterol levels than the AD mice ($p < 0.05$) (Fig. 2C–F).

3.2. db mutation decreased Aβ levels in the brains of young AD mice

The Aβ content was subsequently examined after serial extraction from increasingly insoluble fractions of the mouse brain, phosphate-buffered saline (PBS), 2% sodium dodecyl sulfate (SDS), and finally, 70% formic acid (FA), as described in our previous studies [4,21,23,26,27]. The amount of $A\beta_{40}$ in the SDS fraction was approximately two-fold higher than the level of $A\beta_{42}$ in the brains of each group of mice, while the solubility profiles (the proportional amounts of Aβ) in PBS and SDS fractions of the two groups appeared similar (Fig. 3). The FA fraction of $A\beta_{40}$ was not detectable due to technical

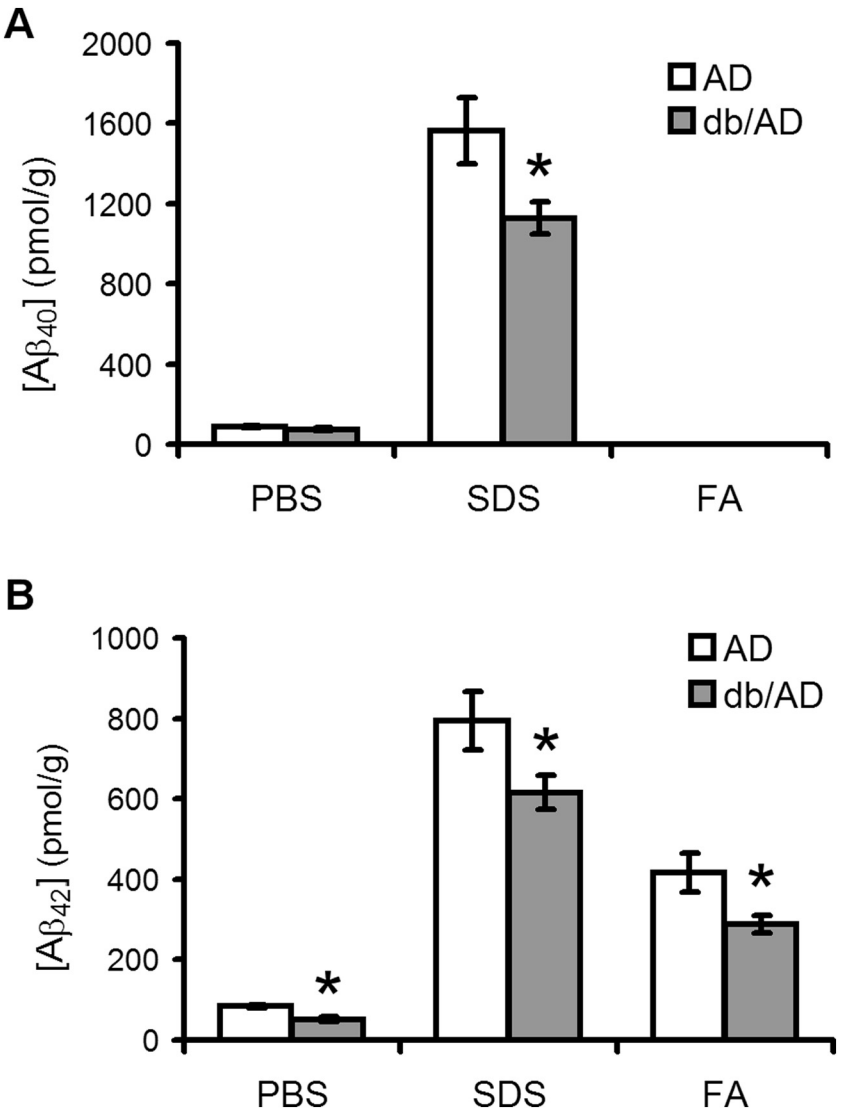

Fig. 3. db mutation decreased the Aβ levels in the brains of young AD mice at 10 weeks of age. The $A\beta_{40}$ (A) and $A\beta_{42}$ (B) levels in the PBS, SDS and FA fractions of the AD and db/AD mouse brains were measured and compared. The db mutation significantly decreased the $A\beta_{40}$ level in the SDS fraction of the mouse brain and significantly decreased the $A\beta_{42}$ levels in all 3 fractions of the mouse brain. (Values in pmol/g tissue, Bars represent means ± SEM, n = 6 mice/group. *$p < 0.05$ compared with AD mice).

limitations.

Compared to the AD mice, the db/AD mice exhibited significantly lower $A\beta_{40}$ levels in the SDS fraction of the brain ($p < 0.05$) and significantly lower $A\beta_{42}$ levels in all 3 fractions of the brain ($p < 0.05$), indicating that the db mutation decreased the Aβ content in the brains of young AD mice (Fig. 3). Although we have seen small reductions in $A\beta_{42}$ in db/AD versus AD mice in some studies, the relative magnitude of the difference appears to decrease as the animals age [4]. In fact, there are no differences in the total amounts of Aβ from 3 to 12 months of age in this mouse line ([4], and *unpublished data*). Interestingly, this suggests that this effect is likely a transient early life event that may vanish or reverse by maturity. It is also worth noting that since histologically detectable amyloid deposits are exceptionally rare at this age, these numbers almost exclusively represent biochemically soluble forms of Aβ.

3.3. db mutation did not affect β-/γ-secretase components in the brains of young AD mice

Aβ synthesis in the brains of young AD and db/AD mice was first assessed to explore the potential mechanisms underlying the decreased Aβ content observed in the db/AD mice. The protein levels of the subunits of β- and γ-secretase in the mouse cortex were compared, including betasite APP cleaving enzyme1 (BACE1), betasite APP cleaving enzyme2 (BACE2), Presenilin 1, Presenilin 2, Nicastrin and Presenilin enhancer 2 (PEN2). No significant differences were observed in the levels of the β- and γ-secretase components, suggesting that the db mutation might not affect Aβ synthesis in the brains of young AD mice via changes in the expression levels of these enzymes (Fig. 4A).

3.4. db mutation activated the mTOR signaling pathway in the young AD mice

We next analyzed the levels and activation of proteins involved in the mTOR signaling pathway in the mouse cortex, including the mTOR protein and its downstream targets: Rictor, 4EBP1, and p70-S6K (Fig. 4B). Compared to the AD mice, db/AD mice exhibited significantly elevated levels of the mTOR protein and increased phosphorylation of the 4EBP1 and p70-S6K proteins ($p < 0.05$), while the levels of the Rictor, 4EBP1 and p70-S6K proteins remained relatively unchanged

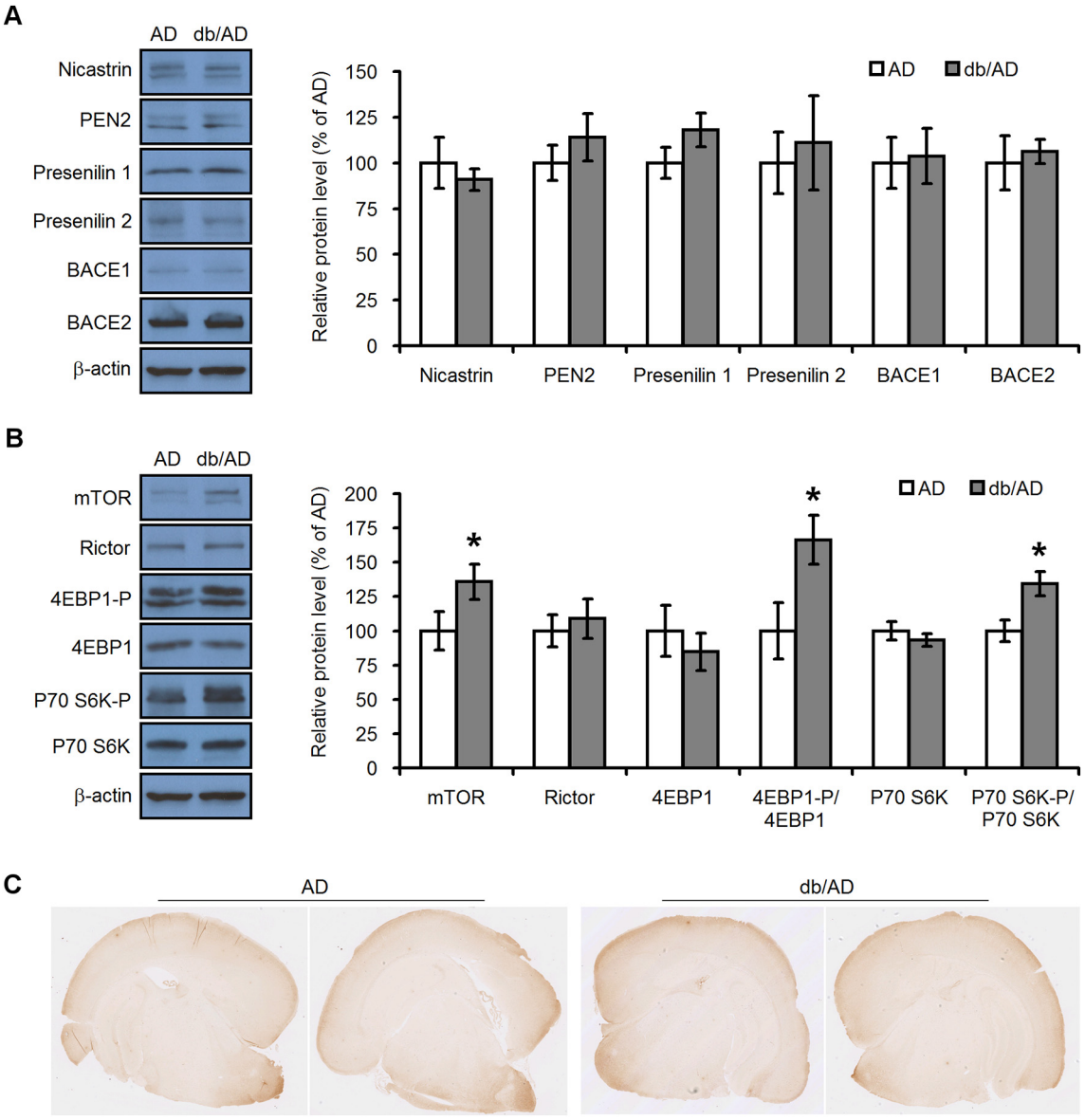

Fig. 4. Assessment of Aβ synthesis, mTOR activation and tau pathology in AD and db/AD mice at 10 weeks of age. (A) Representative western blots and relative protein levels of the β- and γ-secretase subunits in the cortex of the AD and db/AD mice, including Nicastrin, PEN2, Presenilin 1, Presenilin 2, BACE1 and BACE2. Diabetes did not significantly alter the protein levels of β- and γ-secretase subunits in the cortex of the AD mice. (B) Representative western blots and relative levels of the proteins involved in the mTOR signaling pathway. Diabetes significantly increased the level of the mTOR protein and increased the phosphorylation of the 4EBP1 and p70-S6K proteins in the cortex of young AD mice, while the levels of the Rictor, 4EBP1 and p70-S6K proteins remain relatively unchanged. β-actin was used as the loading control for all western blot analyses in the present study. (C) IHC staining for AT8 revealed a lack of significant tau pathology in the brains of both young AD and db/AD mice. (Bars represent means ± SEM, n = 6 mice/group. *$p < 0.05$ compared with AD mice).

($p > 0.05$) (Fig. 4B), suggesting an activation of the mTOR/p70-S6K/4EBP1 signaling pathway in the brains of young db/AD mice.

As the activation of mTOR and its downstream targets p70-S6K and 4EBP1 are closely associated with Aβ production and tau pathology [30,31], pathological tau deposition in the mouse brain was measured using IHC for AT8. No apparent AT8 pattern was identified in the brains of AD and db/AD mice (Fig. 4C), suggesting an absence of tau pathology in the 2 groups of mice at 10 weeks of age, or that the diabetes-induced alterations in tau phosphorylation was minimal and undetectable under our experimental conditions.

3.5. Effects of diabetes on neuroinflammation in the brains of young AD mice

The effects of diabetes on the gross inflammatory signaling in the AD mouse brain were first evaluated by measuring the levels of the IL-1β, IL-6 and TNFα mRNAs in the mouse cortex (Fig. 5A). The db/AD mice exhibited a significantly lower IL-1β mRNA level than the AD mice, while the levels of the IL-6 and TNFα mRNAs remained relatively unchanged ($p < 0.05$), suggesting a decreased inflammatory response based on the lowered IL-1β mRNA level.

Microglial and astrocyte activation were then analyzed in the brain tissues of mice. Glial fibrillary acidic protein (GFAP) and IBA1 (a calcium binding protein specifically expressed in microglia and macrophages) were used as reporters to evaluate astrocyte and microglial reactivity, respectively, in the mouse brain [25].

Diabetes did not significantly alter the total levels of the GFAP protein, but caused a slight but significant decrease in the total levels of the IBA1 protein in the brains of db/AD mice compared to the AD mice (Fig. 5B). IHC staining did not reveal significant alterations in the

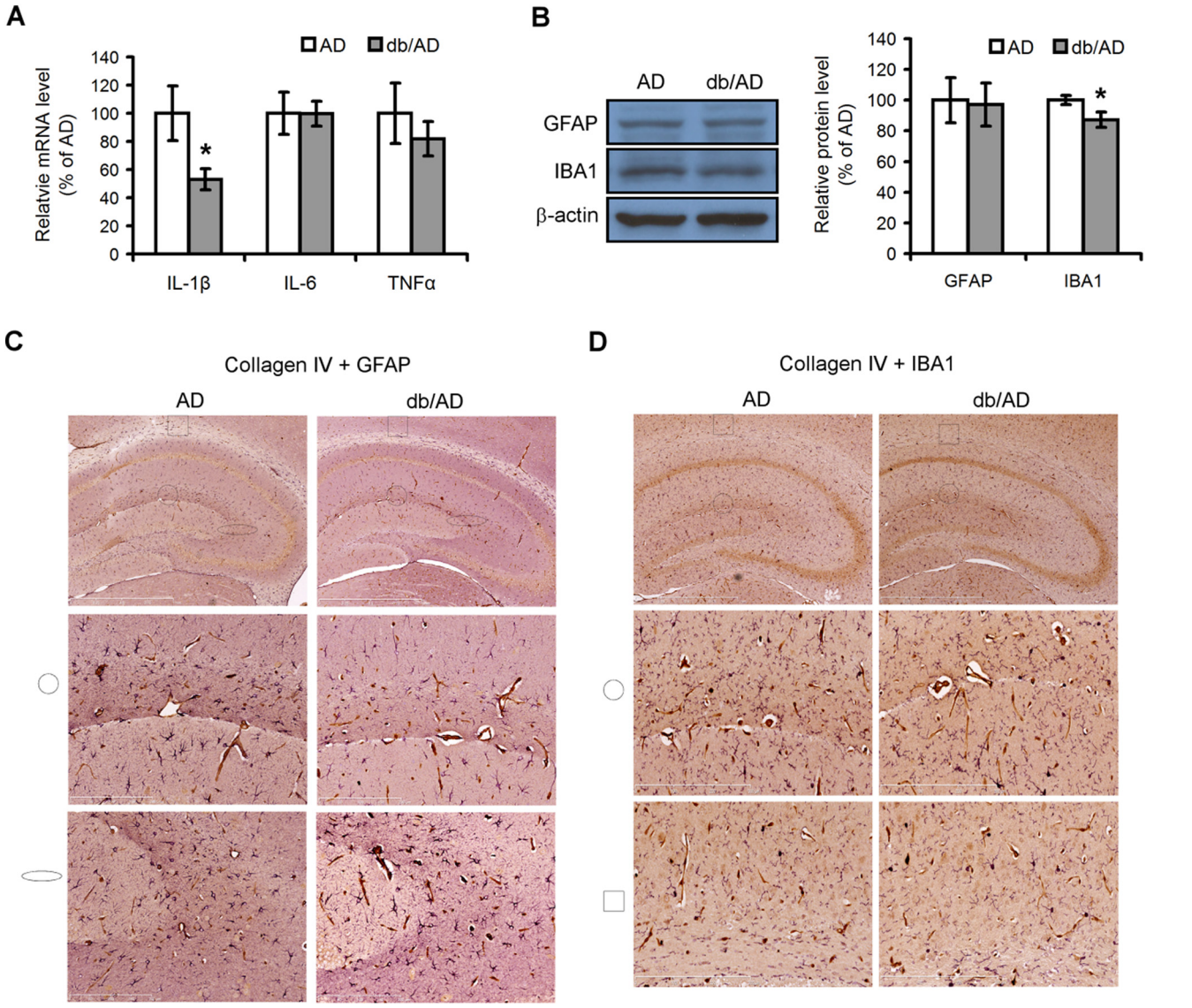

Fig. 5. Effects of db mutation on the neuroinflammatory state of young AD mice at 10 weeks of age. (A) The total inflammatory signaling pathway in mouse brain was evaluated by examining the cortical levels of the IL-1β, IL-6 and TNF mRNAs in the brains of AD and db/AD mice. The level of the IL-1β mRNA was significantly reduced in db/AD mice compared to AD mice. (B) Representative western blots and relative levels of the GFAP and IBA1 proteins in the cortex of AD and db/AD mice, with β-actin serving as the loading control. Diabetes slightly reduced the total level of the IBA1 protein in the cortex of young AD mice, while the level of the GFAP protein remained relatively unchanged. The activation of astrocytes and microglia were assessed by determining GFAP (C) and IBA1 (D) immunoreactivity (purple), respectively, in the brains of AD and db/AD mice, as well as their co-localization with cerebral blood vessels using collagen IV immunoreactivity (brown). No significant alterations in the immunoreactivity and distribution of either GFAP or IBA1 were observed in the brains of AD and db/AD mice, suggesting that diabetes did not significantly alter microglial and astrocyte activation in the young AD mice, or that the diabetes-induced alterations in microglial and astrocyte activation were minimal or undetectable under our experimental conditions. (Bars represent means ± SEM, n = 6 mice/group. $*p < 0.05$ compared with AD mice). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunoreactivity and distribution of either GFAP or IBA1 in the brains of AD and db/AD mice (Fig. 6C–D), suggesting that db mutation did not significantly alter microglial and astrocyte activation in the young AD mice, or that the diabetes-induced alterations in microglial and astrocyte activation were minimal and undetectable under our experimental conditions.

3.6. Behavioral test batteries

The impact of db mutation on the cognitive function of AD mice was evaluated using the fear conditioning test and stone T-maze test (see Supplementary data 1).

During the stone T-maze test (see Supplementary data 1), the average latency of the db/AD mice was consistently higher than that of the AD mice, without committing more errors throughout the acquisition training of 15 trials in the maze, suggesting that db mutation affected the motor function of AD mice without significantly affecting the memory and learning of the young AD mice. As the db/AD mice were much fatter than AD mice (Fig. 1), the latency observed in the db/AD mice could be simply due to an overweight-reduced motor function of db/AD mice.

During the fear conditioning test, no significant difference was observed on day 1 throughout the acclimation period or day 3 of the cued fear conditioning test, suggesting that db mutation had no significant impact on the cognitive function that is mainly dependent upon the integrity of the basolateral amygdala, and that the obesity of the young db/AD mice had no significant impact on the freezing behavior of db/AD mice compared to the young AD mice under our experimental

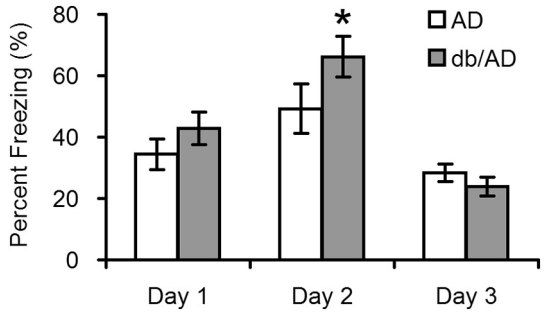

Fig. 6. Effects of db mutation on the cognitive function of young AD mice at 10 weeks of age, as assessed using the fear conditioning test. During the fear conditioning test, the average percentage of freezing behaviors throughout the acclimation period (day 1), the contextual test period of 10 min (day 2), and the average of the 5 first freezing responses to the auditory conditioning stimulus (day 3) are shown. Young db/AD mice exhibited a significantly higher percentage of freezing behaviors on day 2 of contextual fear conditioning compared to the young AD mice. (Bars represent means ± SEM, n = 6 mice/group. *$p < 0.05$ compared with AD mice).

conditions. A significantly higher percentage of freezing behaviors was recorded for the db/AD mice than the AD mice during day 2 of contextual fear conditioning ($p < 0.05$) (Fig. 6), suggesting that db mutation might improve the cognitive function of 10-week-old AD mice that is dependent on the integrity of the hippocampus.

4. Discussion

4.1. Effect of db mutation on the metabolism of young AD mice

In this study, we examined the effect of the db mutation on the metabolism, Aβ pathology, and cognitive function of AD mice at 6 and 10 weeks of age.

At 6 weeks of age, the young db/AD mice already exhibited significant obesity, with a higher body weight and body fat content, as well as a lower body muscle mass than the AD mice (Fig. 1). At 10 weeks of age, the young db/AD mice were already severely obese and exhibited a series of metabolic disturbances, including a dramatic increase in circulating leptin, insulin, FFA, and cholesterol levels, accompanied by elevated fasting glucose levels and impaired glucose clearance compared to the AD mice (Fig. 2), consistent with the previous characterizations of the db mice [18,32]. The db/AD mice develop a much higher fasting glucose level, worse glucose clearance and insulin resistance at 9 months of age [4]; thus, the metabolic disturbances observed in the 10-week-old db/AD mice could characterized as a pre-state of obesity/diabetes in the db/AD mice. We should point out that mice at 10 weeks of age are not predicted to have the development of pathology or cognitive problems, and the effects of db mutation on the young AD mice could be translated as an evaluation of the ability of a loss of metabolic control to accelerate the development of pathology in the brain.

4.2. db mutation and neuroinflammation in young AD mice

In the present study, the young db/AD mice exhibited a lower level of the pro-inflammatory IL-1β mRNA (Fig. 5A) and a slightly lower level of the IBA1 protein (Fig. 5B) in the cortex compared to their AD littermates, while significant immune-mediated activation of astrocytes and microglia was not observed between the 2 groups (Fig. 5C–D), suggesting that the db mutation potentially affects the levels of certain inflammation-related factors in the mouse cortex, but had no significant influence on the global inflammatory state of the brains of young AD mice. This result is consistent with a previous finding that diabetes does not induce neuroinflammation in the brain regions affected in AD [33]; thus, neuroinflammation is not likely to be the mechanism underlying the close connection between diabetes and AD.

4.3. db mutation activated the mTOR signaling pathway in young AD mice

The activation of the mTOR signaling pathway plays central roles in regulating a variety of biological and pathological process, including autoimmune disorders, neuroinflammation, cancer, obesity and aging [34–37]. Metabolic dysfunction, including hyperglycemia, hyperlipidemia, insulin dysfunction and diabetes, might all activate the mTOR signaling pathway, which could in turn function as a central regulator of metabolism and lipid storage [16,35,36,38–40], as well as the pathogenesis and progression of AD [30,31,37,41]. In the present study, the db mutation significantly increased the expression of mTOR protein and activation of its downstream targets S6K and 4EBP1 via phosphorylation (Fig. 4B), without promoting apparent tau pathology in the brains of young AD mice under our experimental conditions (Fig. 4C). Combined with the presence of a hyperphosphorylated tau protein caused by diabetes in both db mice and db/AD mice at 8–9 months old [4,18], the metabolic dysfunction induced by the db mutation (Fig. 2) is postulated to activate the mTOR signaling pathway without an apparent aggravation of tau pathology in the brains of young AD mice at 10 weeks old, but eventually leads to tau pathology as the mice age.

The activation of mTOR also promotes Aβ production, accumulation and senile plaque formation during the onset and progression of AD, by decreasing Aβ clearance through the inhibition of the functions of the autophagy/lysosome system and increasing Aβ generation through interactions with several key signaling pathways involved in Aβ synthesis [30,31,41,42]. In our study, the protein levels of β- and γ-secretase components in the brains of young AD and db/AD mice remained relatively constant (Fig. 4A), suggesting that the db mutation and mTOR activation did not exert significant effects on Aβ synthesis in AD mice, and the decrease in the Aβ content observed in the young db/AD mice was not likely caused by alterations in Aβ synthesis.

In this study, instead of aggravated Aβ pathology in response to mTOR activation, the db mutation conversely promoted a slight alleviation of Aβ pathology by decreasing the levels of both $A\beta_{40}$ and $A\beta_{42}$ (Fig. 3) in the brains of young db/AD mice at 10 weeks of age. Further, the mice showed a slight improvement in memory, as evidenced by the increased percentage of freezing behaviors of young db/AD mice upon re-presentation of the contextual fear conditioning stimulus (Fig. 6). These results are slightly different from those in older (3+ months) db/AD mice, in which the db mutation did not significantly impact the overall amyloid accumulation. Although we did observe a modest reduction in $A\beta_{42}$ levels in the db/AD mice as compared to the AD mice in this earlier study [4], the magnitude of this difference was much smaller than in the current study. Further, in this earlier study we observed no significant differences in the total amount of Aβ using a different assay system (for $A\beta_{1\text{-}x}$) at 3, 6, and 12 months of age. Together, these data suggest that the differences seen in the current study might only be present at a very young age in these mice, and may decrease as the mice age. Intriguingly, this also raises the possibility that there may exist a crossover point (Fig. 7) where the opposite may occur, and where the db/AD mice may have more severe Aβ pathology than the AD mice. This remains to be tested in future studies.

Importantly, AD mice are a well-established mouse model of Aβ pathology, and our previous studies have confirmed the dramatic increase in Aβ accumulation in the brains of AD mice compared to the wild-type mice or animals with a mild cognitive impairment (MCI) [4,21,23,26,43]. Because of the fast development of Aβ pathology in AD mice, the magnitude of disturbances in Aβ accumulation caused by mTOR activation could be minimal, and thus we would be unable to easily discern whether mTOR activation accelerated Aβ pathology in the young db/AD mice.

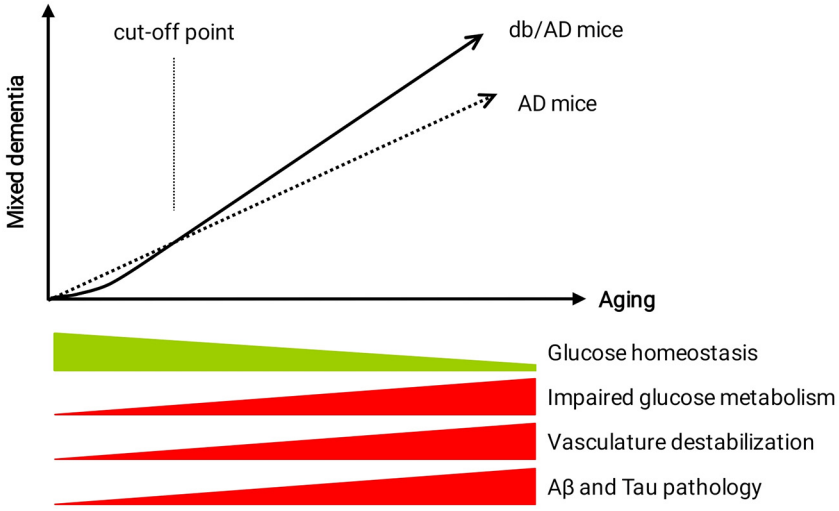

Fig. 7. Proposed model for the development of mixed dementia in db/AD mice based on the findings from the present study and our previous studies. During the early stage of life, obesity/diabetes-induced increases in the glucose content and/or metabolism in the brain may transiently improve the cognitive function of db/AD mice compared to their counterpart AD mice. With aging, the beneficial effects of bioenergetic robustness in the brains of db/AD mice would be overshadowed by the development of diabetes-associated impairments in glucose metabolism and vascular destabilization, along with the rapid development of Aβ and tau pathology and progression. The aged db/AD mice would ultimately exhibit a more severe phenotype of mixed dementia than the aged AD mice [4,18].

4.4. Potential mechanisms underlying the improvement of cognitive function in young AD mice induced by the db/db mutation

In this study, the db mutation improved the cognitive function of young AD mice, a finding that may be partially explained by the decreased levels of both soluble and insoluble Aβ in the brains of db/AD mice compared to the AD mice. Additionally, diabetes elevated circulating glucose levels in the young db/AD mice that might also contribute to the observed improvements in the cognitive function of db/AD mice compared with AD mice.

Glucose is the only essential source of energy for brain cells. In the brain, regional cerebral glucose utilization and regional cerebral blood flow are closely coordinated with blood glucose flow, while the transport of other energy sources, such as fatty acids and amino acids, remains highly limited, mainly because of the limited permeability of the blood-brain barrier [44,45]. A shortage of brain glucose, as in the case of hypoglycemia induced by the use of anti-diabetes agents during diabetes treatment, might cause cognitive disorders and impairments both in the short and long terms [46–51].

Chronic obesity and diabetes, involving long-term impaired glucose control, insulin resistance and chronic inflammation, are invariant risk factors for pathogenesis and development of AD [18,52,53]. In contrast, a transient increased glucose level and/or metabolism in the brains of flies or mice may translate into enhanced synaptic activity and cognitive function, reduces the risk of developing AD and/or delays the onset of clinical manifestations, and ultimately these protective effects could be achieved even after the neuropathological process had begun, suggesting the therapeutic potential of a sudden increase in glucose uptake in the brain in the context of AD [54–57]. Nevertheless, increases in glucose uptake or glucose transport could also been observed in the brain of patients with AD and AD mice in different stages of the development of diabetes [58,59].

Our previous study on db/AD mice of adult and middle age [4] indicates that the db mutation-induced diabetes and/or obesity lead to a vascular dementia through destabilization of the vasculature in the older db/AD mice that also exhibit the AD-caused Aβ pathology, resulting in a mixed dementia in the older db/AD mice [4]. Thus the phenotype changes observed in the db/AD mice could also occur in other models of diabetes/obesity that could lead to an alteration in vascular function and aging including mice exposed to high calorie or/and high cholesterol diets.

In both patients with AD and AD mice, a prominent feature of AD progression is a substantial reduction in glucose metabolism that precedes the onset of clinical symptoms, and worsens with disease progression [60,61]. Thus, in the present study, the slight improvement in cognitive function observed in the young db/AD mice might also be partially explained by the transiently improved glucose homeostasis that relieved glucose hypometabolism in the brains of db/AD mice by increasing the glucose uptake and/or transport into the brain cells of AD mice (Fig. 7) before it is predominated by the development of insulin resistance that reduces glucose uptake and attenuates the beneficial effects of glucose on the cognitive function of AD mice [4,62]. Simultaneously, the development of Aβ and tau pathology and progression of obesity/diabetes-associated vascular destabilization in the db/AD mice would also offset the beneficial effects of the elevated brain glucose level on the cognitive function of mice, leading to a mixed dementia phenotype in the older db/AD mice [4] (Fig. 7).

In conclusion, in this study, we examined the impact of a relatively short timeframe of hyperglycemia (db/db) on multiple aspects of brain homeostasis in a mouse model of AD pathology, at an age prior to the known establishment of AD pathological features. Surprisingly, we observed that db/AD mice had improved cognitive function and reduced inflammation. These data suggest that the impact of glucose control on brain homeostasis could be dependent on the duration of glucose dysfunction, the severity of glucose dysfunction, and the presence of AD pathological features (β-amyloid/tau pathology). In this model, an acute loss of glucose control in the absence of AD pathology is something the brain is able to withstand, in contrast to a chronic or severe loss of glucose control (Fig. 7). Taken together, our study suggests that glucose regulation in maintaining a glucose homeostasis while simultaneously be preventive against hyperglycemia-associated metabolic disorders could be a strategy for prevention and treatment of neurodegenerative diseases like AD, and the db/AD mice at young age could represent a unique mouse model to explore the early interactions between loss of glucose control and development of cognition dysfunction and pathology.

Authors contributions

LZ, S-OF, TLB, DMN, KK, KD, AJB, MPM and JNK acquired the data and contributed to the data analysis and interpretation. LZ drafted the manuscript with contributions from all other authors. LZ, MPM and JNK contributed to the conception and design of the study, and take responsibility for the final content of the manuscript.

Declaration of conflicts of interest

None.

Transparency document

The Transparency document associated this article can be found, in online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dummy.2019.01.002.

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