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Dietary Quercetin Ameliorates Memory Impairment in a Murine Model of Alzheimer's Disease with Obesity and Diabetes, Suppressing ATF4 Expression

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Abstract

Background: While type 2 diabetes is a known risk factor for Alzheimer's disease (AD), the underlying mechanism of this relationship remains unclear. In a previous study, we demonstrated that brain expression of activating transcription factor 4 (ATF4) is increased in aged (12-month-old) amyloid- β precursor protein (APP) 23 mice (APP) and in the young (3-month-old) offspring of APP mice crossed with obese and diabetic *db/db* mice (APP;*db/db*). On this premise, we examined the relationship between ATF4 expression and memory in APP;*db/db* mice.

Methods and Findings: We demonstrate that ATF4 expression at 6-9 months of age was higher in the brains of APP;*db/db* mice than that in the brains of non-obese APP mice. Both APP and APP;*db/db* mice learned to discriminate contextual and auditory cues in contextual and auditory fear conditioning. However, novel object recognition (NOR) memory was impaired in APP;*db/db* mice, but was unaffected in APP mice. Five weeks of dietary supplementation with quercetin, which is a polyhydroxylated flavonoid, ameliorated NOR memory deficits in APP;*db/db* mice in association with suppressed ATF4 expression in the brain.

Conclusion: These results indicate that dietary quercetin suppresses the obesity-induced ATF4 expression in the brain. Therefore, control of the integrated stress response (ISR) with biologically active compounds that reduce ATF4, such as quercetin, may be valuable for the treatment of memory impairments in early-stage AD, and particularly in cases with comorbid obesity and/or diabetes.

Keywords: ATF4; Quercetin; Novel object recognition; Alzheimer's disease

Abbreviations: ATF4: Activating Transcription Factor 4; APP: Amyloid- β Precursor Protein; CREB: Cyclic Adenosine Monophosphate Response Element Binding Protein;

eIF2 α : Eukaryotic Translation Initiation Factor 2 α ; ISR: Integrated Stress Response

Introduction

Activating transcription factor 4 (ATF4) is translated at low levels in the presence of the eukaryotic translation initiation factor 2 α (eIF2 α)-GTP-tRNA^{Met} ternary complex [1]. The eIF2 α protein can be phosphorylated by four mammalian eIF2 α kinases, namely, protein kinase RNA (PKR)-like ER-localized eIF2 α kinase (PERK), general control non-derepressible 2 (GCN2), PKR, and heme-regulated inhibitor [2]. In contrast, in the presence of the catalytic subunit of protein phosphatase 1 (PP1c)-binding protein (PPP1R15A), which is also known as growth arrest and DNA damage-inducible gene 34 (GADD34) or MyD116, PP1c dephosphorylates eIF2 α to reduce ATF4 expression [3]. ATF4 plays several critical roles in cellular physiology. For example, ATF4 expression is induced as a component of the integrated stress response (ISR) that regulates cellular amino acid supply, protects against oxidative stress [4], and supports normal postsynaptic function [5,6]. The ISR is implicated in several disease states [7] such as obesity and diabetes [8].

Type 2 diabetes is a risk factor for Alzheimer's disease (AD) [9] and substantially increases the risk of cognitive decline in individuals with an apolipoprotein E (ApoE) ϵ 4 allele [10]. AD is a neurodegenerative disorder that involves the deposition of pathological amyloid β (A β) fragments and neurofibrillary tangles in the brain, presumably leading to impaired memory and cognitive function [11]. The pathological A β fragment, A β ₁₋₄₂, is generated by γ -secretase complex cleavage of amyloid precursor protein (APP), which includes presenilins 1 and 2 (PS1 and PS2), nicastrin, Aph-1, and Pen-2 [12]. Previously, we showed that endoplasmic reticulum (ER) stress [13] and autophagy impairment [14] increased A β production via γ -secretase activation, which was mediated by increased binding of ATF4 to the promoter region of *PS1* to increase *PS1* gene expression [15]. Moreover, it is reported that memory deficits and A β deposition are exacerbated in high fat diet-induced murine models of AD with obesity [16], and in the offspring of AD mice crossed with diabetic *ob/ob* mice [17,18]. Taken together, these findings suggest that ER stress, and

specifically the ISR, may play a role in AD-related memory deficits [19,20]. In support of this hypothesis, ISR inhibitor (ISRIB) has been shown to regulate eIF2B guanine nucleotide exchange factor (GEF), reduce ATF4 expression *in vitro*, and promote memory function in wild-type mice *in vivo* [21,22].

Currently, it is unknown whether ATF4 is involved in obesity-induced memory impairment; however, ATF4 expression is increased in the brains of an aged APP23 AD mouse model [23] and AD patients [24]. Moreover, phosphorylated PERK is observed in AD patient neurons [25]. Furthermore, the cognitive impairment that is related to obesity and/or diabetes may be mediated by interleukin (IL) 1 β [26] or deficits in glucocorticoid-mediated hippocampal neurogenesis [27]. ATF4 expression is induced by IL1 β with IL6 in pancreatic islets [28] and by insulin in the presence of glucocorticoid in mouse L cells [29], therefore increased ATF4 as a consequence of obesity and/or diabetes may be involved in related cognitive impairments.

In the present study, we evaluated memory impairments in early-stage AD model obese mice, and evaluated the ability of dietary quercetin to ameliorate these impairments in an ATF4-dependent manner.

Methods

Animals

APP23 mice, which express a human APP₇₅₁ cDNA with a Swedish double mutation on a C57BL/6 genetic background [30], were kindly provided by Dr. M. Staufenbiel (Novartis Pharma, Ltd.; Basel, Switzerland). Obese and diabetic *db/db* (*Lepr^{db/db}*) [31,32] mice on a C57BL/6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). To generate obese AD model mice (APP;*db/db*), hemizygous APP23 mice with *Lepr^{db/+}* were crossed with *Lepr^{db/+}* mice. All mice were individually housed in a temperature- and light-controlled room (24°C; 12-hour light/dark cycle) and provided with water and 4 g/day of AIN93G diet (Table 1, Oriental Yeast Co., Ltd.; Tokyo, Japan). AIN93G diet containing 0.5% quercetin was fed at 7 months of age. Blood glucose measurements were performed using the Freestyle Freedom Lite apparatus (Nipro; Osaka, Japan). All animal studies were approved by the Gifu University Graduate School of Medicine Animal Care and Use Committee and conducted according to the Guidelines for Experiments on Animals provided by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Table 1 Ingredients of AIN93G (Oriental Yeast Co., Ltd; Tokyo, Japan) diet.

Ingredient (g/100 g diet)	AIN-93G
Casein	20
L-cystine	0.3
Vitamin mixture ¹	1
Mineral mixture ²	3.5

Corn Starch	39.486
α -Corn Starch	13.2
Soybean oil	7
Choline bitartrate	0.25
Sucrose	10
Cellulose	5
t-Butylhydroquinone	0.0014
¹ AIN-93 Vitamin mixture (mg/kg diet): Vitamin A 800 (400,000U); Vitamin B12 2.5; Vitamin D3 250 (100,000U); Vitamin E 7,500; Vitamin K 75; Thiamin HCl 600; Riboflavin 600; Pyridoxine-HCl 700; Nicotinic acid 3,000; Calcium pantothenate 1,600; Folic acid 200; D-Biotin 20; Sucrose 974.655	
² AIN-93 Mineral Mixture (mg/kg diet): Calcium carbonate 357,000; Potassium phosphate Monobasic 196,000; Potassium citrate monohydrate 70,780; Sodium chloride 74,000; Potassium sulfate 46,600 Magnesium oxide 24,000; Ferric citrate 6,060; Zinc carbonate 1,650; Manganous carbonate 630; Copper (II) Carbonate monohydrate 324; Potassium iodate 10; Sodium selenate 10.25; Ammonium molybdate tetrahydrate 7.95; Sodium metasilicate Nonahydrate 1,450; Chromium (III) Potassium Sulfate dodecahydrate 275; Lithium chloride 17.4; Boric acid 81.5; Sodium fluoride 63.5; Nickel (II) Carbonate basic tetrahydrate 30.58; Ammonium vanadate 6.6; Sucrose 221,003.2	

Behavioral procedures

A Freeze-Frame system (Coulbourn Instruments; Whitehall, PA) and Actimetrics Freeze-Frame software (Coulbourn Instruments) were used for contextual and fear conditioning, as described previously [23]. Briefly, mice (n=7, three female and four male mice per group) were habituated for 15 minutes per day for 3 days. The experiments were performed in chamber A, where foot shocks were delivered through the floor, and chamber B, which had a non-shock floor and bright aluminum sidewalls and where no shocks were delivered. Mice were trained in chamber A and chamber B after habituation every day. Each training trial lasted for 420 seconds and paired three auditory cues (2,800 Hz, 85 dB, 30 seconds) with a co-terminating electrical foot shock (0.6 mA for 2 seconds) at the 210-second, 270-second, and 330-second time points. One minute after the last training trial, mice were returned to their home cages. Contextual fear conditioning was assayed 2 and 26 hours after training by monitoring freezing behavior in chamber A for three minutes. Auditory fear conditioning was assayed in chamber B 1.5 hours after the contextual fear conditioning test carried out 26 hours after training. The auditory fear conditioning test involved the presentation of a pre-conditioning stimulus (tone -) for 120 seconds followed by the presentation of the paired auditory cue (tone +) for 120 seconds.

Novel object recognition (NOR) testing was performed as previously described by Ennaceur et al. [33] using the SMART v3.0 tracking system (Panlab, Spain) with video (HDC-HS350; Panasonic; Osaka, Japan). Briefly, mice were habituated for 10 minutes in a white plastic chamber (30 × 30 × 16 cm). On the next day, the mice were exposed to two identical objects (11.5-cm tall 5-cm in diameter filled with beads) placed in proximity to walls with different patterns for 5 minutes per day for 2 days. After a retention interval, one of the objects was replaced with a new object (a 6.5 cm-tall glass pyramid with a

49 cm² base) in the same position (novel), and the animal's behavior was observed. Exploration of familiar and novel objects was defined as directing the nose within 2 cm of a given object, as previously described. The behavior of the mouse was monitored using video recording and an automated tracking system (SMART v3.0 software). Objects were cleaned with 70% ethanol and 1% acetic acid between trials.

Western blot

Western blot was performed as described previously [23]. Mouse brain tissues (hippocampus, amygdala, cerebral cortex) were frozen by dry ice and homogenized in buffer A (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1% Nonidet P-40) containing protease and phosphatase inhibitors. After centrifugation at 13,100 x g, supernatants of hippocampus, amygdala and cerebral cortex were used for western blot. The antibodies used for western blot were as follows: anti- α -tubulin (Sigma-Aldrich Co., LLC), anti-ATF4 (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA), anti-phosphorylated-cyclic adenosine monophosphate response element binding protein (CREB) (Ser133) and anti-CREB (Cell Signaling Technology; Beverly, CA), and horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (heavy and light chain) antibodies (SouthernBiotech; Birmingham, AL). Quercetin was used for diet supplementation (Extrasynthese; Genay, France; and Sigma-Aldrich Co., LLC). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Kanto Chemical Co., Inc., (Tokyo, Japan), and Sigma-Aldrich Co., LLC.

Immunohistochemistry

Immunohistochemistry was performed as previously described [34] and analyzed using an inverted microscope (BZ-9000; KEYENCE, Osaka, Japan). Briefly, APP mice (9 months) and APP;*db/db* mice (8-9 months) were anesthetized using isoflurane inhalation solution (Pfizer, Groton, CT, USA) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1-M phosphate buffer (PB). Mouse brains were post-fixed for 2 h in the same 4% paraformaldehyde fixative, and then cryoprotected in 15% sucrose overnight in 0.1-M PB. Sections (14-30 μ m) were cut and mounted onto slides with anti-Iba1 (1:1,000 dilution, Wako Pure Chemical Industries, Ltd. Osaka, Japan) in PBS containing 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and 0.1% Triton X-100 for 12 h at 4°C. Alexa Fluor 488-conjugated F(ab')₂ anti-rabbit IgG

(H + L) (Life Technologies Corporation, Carlsbad, CA) was used to visualize primary antibody signal.

Statistical analysis

Statistical significance ($p < 0.05$) was determined using Student's t tests or one-way repeated measures analyses of variance followed by Dunnett *post hoc* tests when necessary. Data are presented as the mean \pm standard error of the mean of 3 independent experiments.

Results

NOR memory is impaired in early-stage APP23 mice with obesity and diabetes

To examine the roles of obesity and diabetes in memory impairment in early-stage AD model mice, we crossed hemizygous APP23 mice expressing a human APP₇₅₁ cDNA with leptin receptor-mutated obese *db/db* mice. In APP23 mice, the earliest detectable A β deposition occurs at approximately 6 months of age and region-specific neuronal loss occurs at approximately 14–18 months of age [30]. In our study, A β deposits were observed in animals that were older than 11 months of age, but not in animals 6-9 months of age. Body weights and blood glucose levels of double-transgenic APP;*db/db* mice were significantly increased relative to those of APP mice (**Figure 1a**). Because ATF4 expression is significantly increased in the brains of APP;*db/db* mice relative to APP mice at 4-6 months of age [23], we examined the effects of ATF4 on memory impairment in early-stage AD model mice 8-11 months of age. We used two well-established memory tasks, namely, fear conditioning and NOR. For the fear conditioning task, we trained mice to associate a 2-second foot shock with the offset of an auditory stimulus (**Figure 1b**). APP and APP;*db/db* mice showed significant increases in contextual fear conditioning (**Figure 1c**) and auditory fear conditioning (**Figure 1d**) 1 day after training, although the difference in % freezing in APP;*db/db* mice after the tone presentation was smaller than that in APP mice. Next, we used the NOR task (**Figure 1e**) to evaluate episodic-like memory [35]. Exploration time for the novel object was significantly increased in the early-stage AD model mice (**Figure 1f**). In contrast, APP;*db/db* mice failed to show a novelty preference after the 3-minute interval. This is indicative of a failure to establish short-term memory (STM) (**Figure 1f**). This result suggests that obesity and diabetes result in the impairment of episodic-like memory in early-stage APP mice.

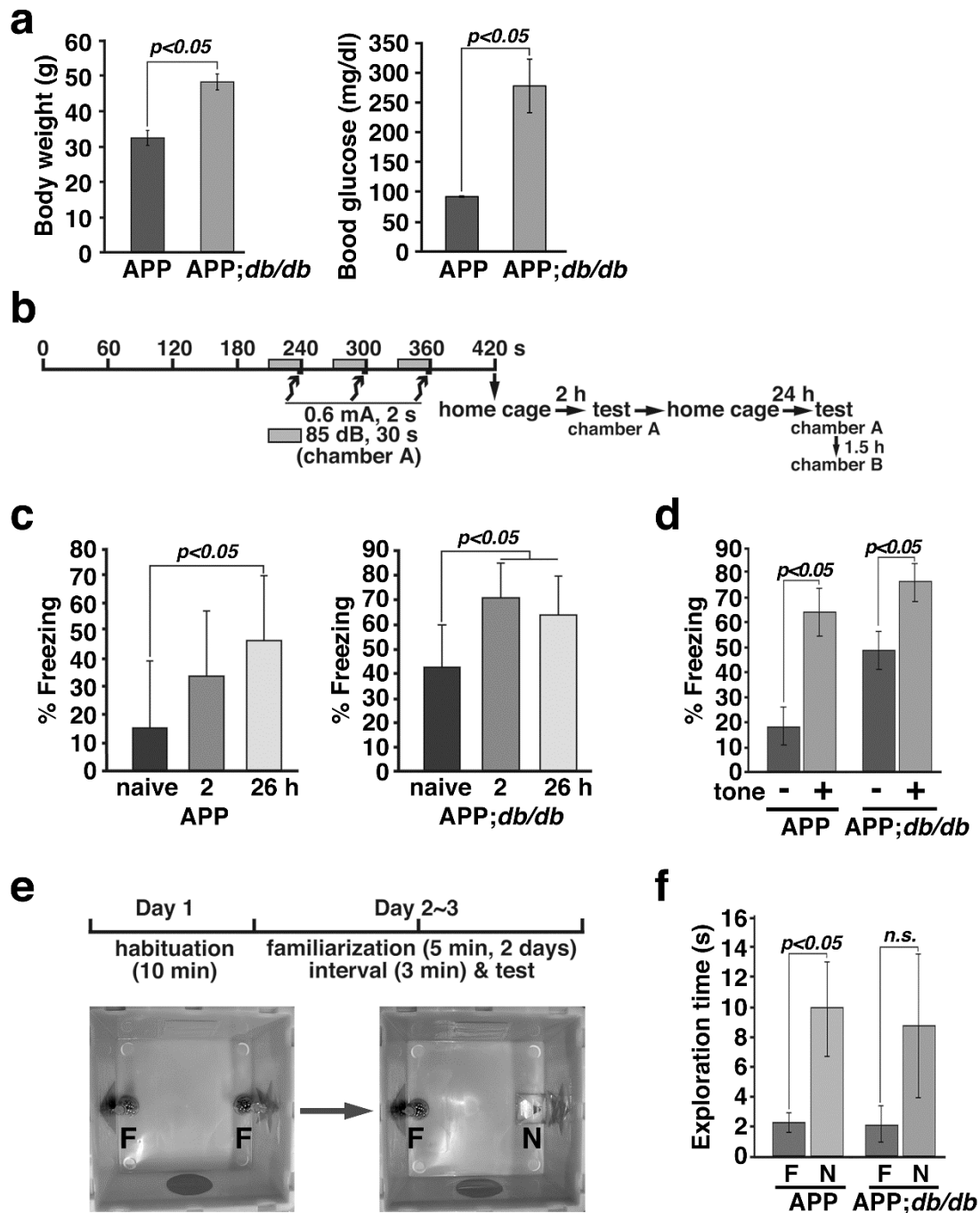


Figure 1 NOR memory is impaired in early-stage APP23 mice with obesity and diabetes. **(a)** Body weight (left panel) and blood glucose levels (right panel) in APP and APP;*db/db* mice are shown. **(b)** Behavioral schedule for fear conditioning. **(c)** Contextual fear conditioning was assessed in chamber A based on the observation of freezing behavior for 180 seconds before training (naive) and 2 and 26 hours after training. One-way analyses of variance with repeated measures followed by Dunnett *post hoc* tests were used for statistical comparisons. **(d)** Freezing in response to the paired tone was assessed in chamber B 27.5 hours after training for 120 seconds before the tone presentation (-) and for 120 seconds during tone presentation (+). Panels: APP mice (left); APP;*db/db* mice (right). **(e)** Behavioral schedule for novel object recognition (upper panel). Top-down view of the white plastic chamber and object placements (lower panels). F: familiar object; N: novel object. **(f)** Novel object recognition memory was assessed by comparing exploration of the novel and familiar objects for each group ($n=7$). Panels: APP mice (left); APP;*db/db* mice (right). n.s., not significant.

Quercetin improves episodic-like memory in early-stage APP23 mice with obesity and diabetes

Recently, Roy and colleagues have shown that memory recall is impaired in a mouse model of early-stage AD, and that engram cell stimulation rescues this impairment [36]. We have also demonstrated that memory recall is improved by dietary quercetin supplementation in a mouse model of AD and in patients with early-stage AD [37]. We thus examined whether obesity and diabetes-related impairments in short-term episodic-like memory in early-stage APP mice are improved by quercetin supplementation. Dietary supplementation with 0.5% quercetin (in AIN93G) for 5 weeks had no effects on body weight (**Figure 2a**) or blood glucose (**Figure 2b**) in APP;*db/db* mice. However, in the NOR task, APP;*db/db* mice showed an increased preference for the novel object relative to the familiar one (**Figures 2c and 2d**). Since quercetin has anti-inflammatory effects [38] and microglia mediates synapse loss in AD [39], we examined the morphology of microglia by immunostaining using Iba1 antibody according to previously report by Hong et al. [39]. Immunohistochemical analyses indicated that morphology of brain Iba1-positive cells after quercetin supplementation in APP;*db/db* mice were similarly observed to morphology without quercetin (**Figure 2e**). Next, we examined the effects of quercetin on ATF4 expression in the brain. ATF4 expression levels in the brain were significantly decreased in APP;*db/db* mice after quercetin supplementation (**Figure 2f**) to near wild type levels (data not shown). Since quercetin may enhance phosphorylation of CREB in primary mouse cortical neurons in culture [40] and in mouse brain [41], we measured the levels of phosphorylated CREB in the brains of APP;*db/db* mice. We found that phosphorylated CREB was not increased after quercetin supplementation (**Figure 2g**).

Discussion

Although type 2 diabetes is a significant risk factor for Alzheimer's disease, the underlying mechanisms for this association remain unclear. Here we report that obesity and diabetes enhance ATF4 expression in the brains of early-stage AD model mice and impair short-term episodic-like memory. Moreover, both increased ATF4 expression and STM impairments were improved by dietary quercetin supplementation.

Memory impairment in the NOR task, which is a task that requires no external motivation, reward, or punishment, was exacerbated in APP;*db/db* mice [35]. Interestingly, NOR memory impairment is ameliorated in 18-week-old *db/db* mice after treadmill training. This effect is mediated by the inhibition of IL1 β -mediated neuroinflammation [26] and is observed in ~11-week-old *db/db* mice after the normalization of corticosterone levels, which are associated with decreases in fasting glucose levels and insulin concentrations [27]. NOR memory deficits were improved in APP;*db/db* mice after quercetin supplementation. This effect was correlated with a decrease in ATF4 expression in the brain. Since IL1 β and insulin induce ATF4 expression in *db/db* pancreatic islets [28] and in

mouse L cells [29], respectively, quercetin may reduce ATF4 expression by controlling the levels of IL1 β and insulin. In *Aplysia*, micro-RNA (miRNA) 124 and PIWI-interacting RNA-F, which are small noncoding RNAs, regulate serotonin-induced neuronal plasticity through CREB1 translation by reducing the levels of miRNA 124 and suppressing CREB2 (also called ATF4) transcription via the methylation of the promoter for CREB2, respectively [42]. Although we have shown that quercetin suppresses ATF4 translation by reducing eIF2 α phosphorylation [23], transcriptional regulation of ATF4 should be assessed in future experiments. The suppression of ATF4 expression may improve synaptic plasticity [43] through CREB-dependent transcription [44] pathways, such as those involving brain-derived neurotrophic factor (BDNF) [45]. CREB is involved in the allocation [46] and recall [47] of fear memory, and the activation of subsets of neurons in the amygdala [46,47]. In our experiment, the expression of phosphorylated CREB was not increased in mouse brains after quercetin supplementation, suggesting that quercetin may not affect the expression of phosphorylated CREB. Several studies have suggested that the ISR is important for the regulation of synaptic plasticity and memory formation [48]. Reduced eIF2 α phosphorylation improves memory and neurodegeneration in mice; eIF2 α phosphorylation and neuronal loss are increased in mice with prion disease in a manner that is prevented by growth arrest and DNA damaged-inducible gene (GADD)34 overexpression [49]. In addition, NOR impairments related to prion-infected mice are prevented via reduced eIF2 α phosphorylation and ATF4 expression by oral PERK inhibitor treatment after inoculation [50]. Additionally, ISRIB, which stimulates eIF2B and reduces ATF4 expression [22], improves memory in 10-week-old wild-type mice [21] and prevents neurodegeneration in mice with prion disease [51]. Consistent with the above findings, we have shown that quercetin induces GADD34 expression and decreases ATF4 expression in the brain [23]. Taken together, these findings and our results suggest that ATF4 may play a role in memory impairment in AD with obesity and diabetes. Therefore, control of the ISR with biologically active compounds that reduce ATF4, such as quercetin, may be valuable for the treatment of memory impairments in early-stage AD, and particularly in cases with comorbid obesity and/or diabetes.

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Competing Interests

The authors declare no competing interests with regard to this study or the preparation of the manuscript.

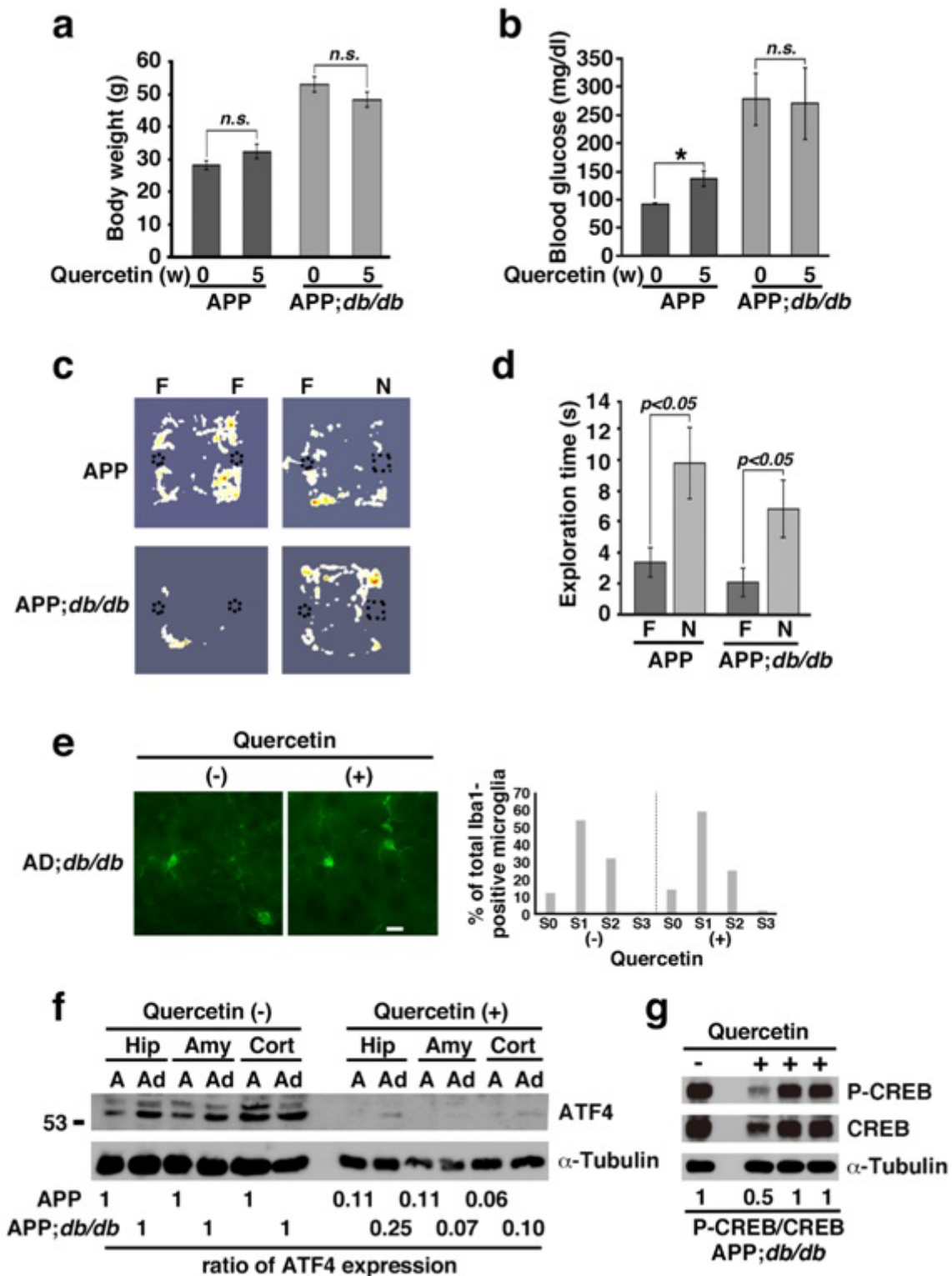


Figure 2 Quercetin improves episodic-like memory in early-stage APP23 mice with obesity and diabetes. **(a)** Body weight and **(b)** blood glucose levels were not significantly changed after 5-week dietary quercetin supplementation in both groups. *, $p < 0.05$. n.s., not significant. W: week, 0: before quercetin, 5: 5-week dietary quercetin supplementation. **(c)** Representative heat maps showing exploration times for familiar (F: black dotted circle) and novel (N: black dotted square) objects. **(d)** Novel object recognition memory was assessed by comparing the exploration of the novel vs. the familiar object in each group. Student's t tests were used for statistical comparisons. Data represent the mean \pm standard error of the mean (APP mice, $n=5$; APP;*db/db* mice, $n=6$). **(e)** Iba1 immunostaining in the brains of APP;*db/db* mice. Scale bar: 10 μ m. Images are representative of two independent experiments (left). Percentage of microglia with morphology score in total Iba1-positive microglia (right). S0: thin and long processes with many branches; S1: thick processes and branches; S2: thick and short processes with few branches; S3: round microglia and no processes. **(f)** ATF4 expression levels in the hippocampus (Hip), amygdala (Amy), and cerebral cortex (Cort) in APP and APP;*db/db* mice after 5-week dietary quercetin supplementation. ATF4 expression was normalized to α -tubulin expression. Images are representative of three independent experiments. **(g)** Phosphorylated CREB was not increased in the brains of APP;*db/db* mice after 5-week dietary quercetin supplementation.

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