GADD34 suppresses eIF2α phosphorylation and improve cognitive function in Alzheimer's disease model mice

Biochemical and Biophysical Research Communications 654 (2023) 112–119

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

GADD34 suppresses eIF2α phosphorylation and improves cognitive function in Alzheimer's disease-model mice

Miki Hayakawa-Ogura, Tana, Toshiyuki Nakagawa, Masanori Itoh*

Department of Neurobiology, Gifu University Graduate School of Medicine, Gifu, 501-1194, Japan

ARTICLE INFO

Article history:
Received 31 January 2023
Accepted 26 February 2023
Available online 28 February 2023

Keywords:
GADD34
eIF2α
ATF4
BACE1
Alzheimer's disease
Memory

ABSTRACT

Alzheimer's disease (AD) causes neurodegeneration, leading to cognitive impairment and memory loss. Our previous studies have demonstrated that the induction of growth arrest and DNA damage-inducible gene 34 (*GADD34*) by quercetin can affect eukaryotic translation initiation factor 2α (eIF2α) phosphorylation-activated transcription factor 4 (ATF4) signaling. However, the relationship between GADD34 expression and cognitive function has not been clarified. In this study, we determined the direct effect of GADD34 on memory. To achieve this, truncated *GADD34* (*GADD34.5*) was injected into the mouse brain to suppress eIF2α phosphorylation and evaluate memory. The injection of *GADD34.5* into the hippocampus in AD-model mice did not improve novel object recognition but improved novel object location. The injection of *GADD34.5* into the amygdala also resulted in the maintenance of contextual fear memory based on the fear condition test. These results suggest that GADD34 is effective in improving memory for spatial cognition and contextual fear conditioning in AD by inhibiting eIF2α phosphorylation. In summary, GADD34 suppresses eIF2α phosphorylation in the brain and prevents memory loss. As quercetin feeding increases GADD34 expression, it might be used in preventative applications for AD.

1. Introduction

Alzheimer's disease (AD) is characterized by amyloid plaque deposition and neurofibrillary tangles, which typically cause memory loss and cognitive decline [1]. Dementia cases, including AD, are estimated to increase to 131.5 million by 2050 [2]. However, no cure has been established to date.

Aging-induced impairment of autophagy leads to amino acid starvation and activates GCN2, an amino acid sensor. GCN2 induces eukaryotic translation initiation factor 2α (eIF2α) phosphorylation [3]. This phosphorylation suppresses general protein synthesis but promotes the translation of some genes, such as activating transcription factor 4 (*ATF4*) [4]. Decreased expression of GCN2 leads to the reduced phosphorylation of eIF2α, which in turn results in reduced ATF4 expression; together, these changes enhance CREB-dependent transcription, synaptic plasticity, and learning [5].

In contrast, we previously revealed that ATF4 increases the expression of presenilin 1 (PS1), which is a core component of γ-secretase [6], to promote amyloid-β (Aβ) production [7]. Endoplasmic reticulum (ER) stress induced by diabetes and obesity activates PERK, which phosphorylates eIF2α and increases PS1 expression. Therefore, we surmised that the eIF2α-ATF4-PS1 axis might be a core signaling pathway that induces AD and that the regulation of ATF4 expression in the brain is important for the prevention of AD.

Growth arrest and DNA damage-inducible gene 34 (GADD34) forms a structural complex with the catalytic subunit of protein phosphatase 1 (PP1c) [8], which has been shown to promote the dephosphorylation of eIF2α and rescue cells from false protein folding stress [9]. We showed that quercetin, a polyphenol, induces GADD34 expression and suppresses ATF4 expression, leading to decreased Aβ production *in vitro* [10]. Quercetin administration has also been shown to increase GADD34 expression in the brains of AD-model (APP23) mice and delay memory loss in fear conditioning tests in the early stages [11].

However, the molecular mechanism underlying the effect of

Abbreviations: AD, Alzheimer's disease; GADD34, growth arrest and DNA damaged-inducible gene 34; eIF2α, eukaryotic translation initiation factor 2α; ATF4, activating transcription factor 4; PS1, presenilin 1; HEK293, human embryonic kidney 293; ER, endoplasmic reticulum; CREB, cyclic AMP response element binding protein; Aβ, amyloid-β; PP1c, protein phosphatase 1c; PCR, polymerase chain reaction; GFP, green fluorescent protein; DG, dentate gyrus; NOR, novel object recognition; NOL, novel object location; UPR, unfolded protein response; ISR, integrated stress response; LTP, long-term potentiation.

* Corresponding author.
E-mail address: m_itoh@gifu-u.ac.jp (M. Itoh).

https://doi.org/10.1016/j.bbrc.2023.02.077
0006-291X/

quercetin on brain function has not been elucidated. In this study, we investigated the direct effect of GADD34 on signal transduction in the brain and in memory. To this end, we directly injected truncated *GADD34.5* into the mouse brain for the first time and assessed memory functions in APP23 mice. Our results indicated that increased truncated *GADD34* expression in the brain improved memory, suggesting that quercetin supplementation might increase *GADD34* expression in the brain to prevent AD.

2. Materials and methods

2.1. Materials

We used the following chemicals in this study: NaCl, HCl, NaOH, $CaCl_2$, sodium deoxycholate, NP-40, polyoxyethylene (20) sorbitan monolaurate, β-glycerophosphate, Na_3VO_4, sodium dodecyl sulfate (SDS), agar powder, ampicillin sodium, kanamycin sulfate, skim milk powder, Dulbecco's modified Eagle's medium (DMEM), Mg^{2+}- and Ca^{2+}-free Hanks' Balanced Salt Solution (HBSS), HEPES, and EDTA・2Na from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); aprotinin, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), and forskolin from Sigma-Aldrich Co. (Burlington, MA, USA); 0.05% trypsin-EDTA, penicillin/streptomycin, MEM non-essential amino acids, L-glutamine, sodium pyruvate, and UltraPure Tris from Thermo Fisher Scientific (Waltham, MA, USA); and Bacto tryptone and Bacto yeast extract from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

2.2. Subcloning

Gamma 34.5 (*ICR34.5*) is known as a *GADD34* homologous gene, encoded by a virus (herpes simplex virus-1: HSV-1) [12]. We used amino acids from the 379–626 region of human GADD34, which corresponds to full-length γ34.5, and named it GADD34.5. We amplified the cDNA encoding that region via polymerase chain reaction (PCR) using KOD plus (Toyobo Co, Ltd, Osaka, Japan) and the following primers: forward, 5′-CTCAAGCTTactcctgctacaggtgtcttctt-3′, and reverse, 5′-CTCAGATCTttagggggctaaaggtggttc-3′; the underlined and lowercase text indicate the enzyme site and coding region, respectively. This was then subcloned into the *Hin*dIII and *Bgl*II sites of pFLAG-CMV2 (Sigma-Aldrich Co.), fusing the FLAG tag to the N-terminus of the inserted gene. Subsequently, the *GADD34.5* gene together with the FLAG-tag gene was amplified through PCR using the following primers: forward, 5′-CTCGGAT**CCAAC**atggactacaagacgatgac-3′, and reverse, 5′-CTCGAATTCttagggggctaaaggtgg-3′; the underlined, lowercase, and bold text indicate the enzyme site, coding region, and Kozak sequence, respectively. This was then subcloned into the *Eco*RI and *Bam*HI sites of the FUGW vector (Addgene, Watertown, MA, USA, #14883 [13]; the GFP gene was removed). The Kozak sequence (CCAAC; same as the sequence indicated in bold for the forward primer for the second PCR described previously) was inserted upstream of the start codon. Plasmids were purified using the Plasmid midi kit (Qiagen, Düsseldorf, Germany).

2.3. Cell culture

Human embryonic kidney (HEK) 293T cells were grown in complete DMEM supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and penicillin/streptomycin. To produce the lentivirus, 293FT cells (Thermo Fisher Scientific) were used. Cells were maintained at 37°C in an atmosphere containing 5% CO_2.

2.4. Western blotting

Western blotting was performed as previously described [7]. Mouse tissues and cultured cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, and 5% deoxycholate), containing protease inhibitors (2 mg/mL aprotinin, 2 mg/mL leupeptin, 1 mg/mL pepstatin A, and 100 μM PMSF) and phosphatase inhibitors (20 μM β-glycerophosphate and 2 μM Na_3VO_4) [11]. After SDS polyacrylamide gel electrophoresis, proteins were transferred onto an Immobilon-P PVDF Membrane (MilliporeSigma, Burlington, MA, USA) and blocked with 5% skim milk or 5% BSA (for phosphorylated proteins) solution solved in TBST (20 mM Tris-HCl [pH 7.6], 138 mM NaCl, and 0.05% polyoxyethylene (20) sorbitan monolaurate). The following antibodies were used: anti-CREB2 (ATF4) antibody (#sc-200, 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho eIF2α (Ser51) (1:1000 dilution) and anti-eIF2α (1:1000 dilution) antibodies (Cell Signaling Technology, Beverly, MA, USA), anti-alpha tubulin (1:10,000 dilution) and anti-FLAG (1:5000 dilution) antibodies (Sigma-Aldrich Co.), and HRP-conjugated anti-mouse and anti-rabbit IgG (H + L) antibodies (1:3000 dilution; Southern Biotech, Birmingham, AL, USA). Signals were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) and detected with an Image Quant LAS-4000 mini (GE Healthcare, Wauwatosa, WI, USA). Antibodies were normally diluted in 5% skim milk/TBST and diluted in CanGet Signal (Toyobo) for phosphorylated proteins.

2.5. Lentiviral expression

The 293FT cells were grown in complete DMEM supplemented with 10% FBS, 0.1 mM MEM non-essential amino acids, 6 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. The cells were seeded in 10 cm dishes (4×10^6 cells per dish) and transfected with 20 μg of *GADD34.5* gene-inserted FUGW, 15 μg of pxPAX2 (Addgene #12260), and 6 μg of pMD2.G (Addgene #12259) for each dish using the calcium phosphate-DNA co-precipitation method. Briefly, plasmids were suspended in 250 mM $CaCl_2$ solution and dropped into 2 × HEPES-buffered saline (pH 6.95). Cells were cultured in medium containing 10 μM forskolin. The culture medium containing the virus was collected and concentrated (Himac CP 60E from Hitachi, Tokyo, Japan) at 100,000×*g* for 2 h at 4°C for 5 d sequentially. Finally, all viral suspensions were centrifuged at 100,000×*g* for 2 h at 4°C, and the precipitated virus was suspended in Mg^{2+}-and Ca^{2+}-free HBSS. The viral suspension was aliquoted and stored at −80°C.

2.6. Surgery

Viruses were injected into the dentate gyrus (DG) region of the hippocampus. Briefly, anesthesia solution, a mixture of ketamine hydrochloride (50 mg/mL, Daiichi Sankyo, Tokyo, Japan) and xylazine hydrochloride (20 mg/mL, Bayer HealthCare, Leverkusen, Germany), at a ratio of 4:1, was administered at 3 mL/kg of body weight (112.5 mg/kg ketamine hydrochloride and 15 mg/kg xylazine hydrochloride) via subcutaneous injection before surgery. The mouse was placed in a fixed frame (stereotaxic instrument for mice: SR-5M [Narishige Co, Ltd, Tokyo, Japan]; Stereotaxic Micromanipulator: SM-15 [Narishige Co, Ltd.]). The skull was exposed, the lambda and bregma were set to 0 on the vertical plane, and a small hole was made in the skull using a microgrinder HD20A-SET (Urawa Corporation Co, Ltd, Saitama, Japan). We injected the target bilaterally into the DG (−2.0 mm anteroposterior, ±1.3 mm mediolateral, −1.9 mm dorsoventral) [14] or amygdala (−1.8 mm anteroposterior, ±3.4 mm mediolateral, −1.9 mm dorsoventral)

[15]. One microliter of virus solution was injected using a syringe 701 N (Hamilton Company, Reno, NV, USA) and a motorized microinjector (IMS-10; Narishige Co, Ltd), for 5 min, and the needle was kept for 10 min on the left and right sides. The holes in the skull were plugged with dental resin cement (UNIFAST III, GC Corporation, Tokyo, Japan). After injection, the scalp skin was sutured with a needle using suture (A11-50B1; Natsume Seisakusho Co, Ltd, Tokyo, Japan), and the mice were warmed on a heating mat (KN-475; Natsume Seisakusho Co, Ltd.) and returned to their homes.

2.7. Novel object recognition (NOR)

The experiments were conducted in a 30 × 45 cm rectangular box. All mice were transferred to the behavioral room and acclimated for 30 min before the training session. Mice were habituated for 15 min in an empty box for 3 d. For training, two identical objects (cylindrical glass bottle; diameter × height: 5.4 cm × 13.4 cm) were placed in defined locations. One hour and 24 h after training, we placed a novel object (plastic cube, 10 cm width, height, and depth) on one side of the trained object, and the mouse explored these objects for 10 min (Fig. 2a). All movements of each mouse were recorded using video (Panasonic Corporation, Osaka, Japan) and a computerized video-tracking system (SMART v2.5, Panlab, Barcelona, Spain). Object exploration was defined as the time when the nose or forepaws of the mouse touched a novel object. The time spent exploring the object was manually analyzed in a blinded manner. For each mouse, the box and objects were cleaned with 1% acetic acid and 70% ethanol.

2.8. Novel object location (NOL)

The experiments were performed in the same box as the NOR. On the first day, the mice were habituated in an empty box for 15 min. From the second to the fourth day, one object different from the NOR (pyramidal glass bottle; diameter × height: 5 × 11.5 cm) was placed in the defined place and explored for 15 min as training. Three minutes after the final training, the object was placed in a symmetric position, and the mice explored the object for 5 min for the test (Fig. 2d). The recording method and procedure were the same as those used for the NOR.

2.9. Contextual and auditory fear conditioning test

A FreezeFrame system (Coulbourn Instruments, Whitehall, PA, USA) was used for contextual and fear conditioning, and Actimetrics FreezeFrame software (Coulbourn Instruments) was used for monitoring. Fear conditioning tests were performed as previously described [11]. Two chambers were used. The first (chamber A) contained a shocking floor with clear plastic walls, and the second (chamber B) contained a non-shock floor and clear plastic only in the front wall, with the other walls being made of bright aluminum. Mice were habituated for 15 min per chamber per day for 3 days. Following this habituation period, the mice were conditioned in chamber A, which involved an initial 2 min acclimatization (naïve), followed by the delivery of an auditory cue (2800 Hz, 85 dB for 30 s) and an electrical foot shock simultaneously delivered with the end of the auditory cue (0.35 mA for 1 s). One minute after the foot shock, the mouse was returned to its home cage. The contextual fear conditioning test was assayed in chamber A twice by studying the frequency of freezing during exposure to the training environment for 5 min, first 24 h after training and second after 1 month. Four hours after each of these tests, auditory fear conditioning tests were conducted. This involved a 2 min acclimatization to chamber B, followed by a 3 min auditory cue of the same volume and frequency (2800 Hz, 85 dB). As in the contextual fear conditioning test, behavior was studied as the frequency of freezing.

2.10. Mice

C57BL/6J female mice (older than 6 months) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). APP23 mice, which express human *APP751* complementary DNA with a Swedish double mutation in the C57BL/6 genetic background [16], were kindly provided by Dr. M. Staufenbiel (Novartis Pharma Ltd.; Basel, Switzerland). The mice were housed in a temperature- and light-controlled room (24°C; 12h light–dark cycle). All animal studies were approved by the Gifu University Graduate School of Medicine Animal Care and Use Committee under the guidelines for experiments on animals provided by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

2.11. Statistical analysis

Statistical analyses were performed using Kaleida Graph software (Synergy Software, Reading, PA, USA). Statistical significance ($p < 0.05$) was determined by performing paired or unpaired two-tailed student's *t*-test or two-way repeated measures ANOVA, followed by a Bonferroni post-hoc test. Data are presented as the mean ± standard error.

3. Results

3.1. Truncated GADD34 (GADD34.5) suppresses the phosphorylation of eIF2α and expression of ATF4

To reveal the direct effect of GADD34 on brain function, we created a GADD34-expressing virus and infected the mouse brain. First, we tried to use the lentivirus to express full-length *GADD34* but could not insert it in our plasmid possibly because it was too long (data not shown). Therefore, a lentivirus was created using a part of the human *GADD34* gene. Full-length GADD34 has four and a half repeats of approximately 34 amino acids in length, which encompasses three predicted PEST sequences and a C-terminal region of 60–80 amino acids that shows sequence homology to the HSV-1 neurovirulence factor ICP34.5 (γ34.5) [12]. The γ34.5 protein is a GADD34 homologue that binds to PP1 and dephosphorylates eIF2α [17]. We compared amino acid sequences between human GADD34 and γ34.5 and then truncated the human *GADD34* gene from amino acids 379 to 626 (because it is the γ34.5 homologous region in human *GADD34*) and named it GADD34.5 (Fig. 1a). We fused the FLAG tag to the N-terminus of GADD34.5 and inserted this *FLAG-GADD34.5* gene into the FUGW vector after removing the EGFP-encoding gene.

To test the effect of FLAG-GADD34.5, HEK293T cells were transfected with the plasmid using the calcium phosphate-DNA co-precipitation method. Forty-eight hours later, the lysate was prepared, and eIF2α phosphorylation and ATF4 protein were detected via western blotting. eIF2α phosphorylation and ATF4 expression were decreased by GADD34.5, confirming the functional similarity between GADD34.5 and full-length GADD34 in terms of eIF2α dephosphorylation (Fig. 1b).

3.2. GADD34.5 enhances spatial memory in the AD mouse model

We then examined whether the decreased expression of ATF4 mediated by GADD34.5 might affect memory. NOR and NOL are non-aversive learning paradigms that measure spontaneous exploratory behavior. We used the NOR and NOL tasks to test memory impairment. We injected the virus into the DG of 5–6-

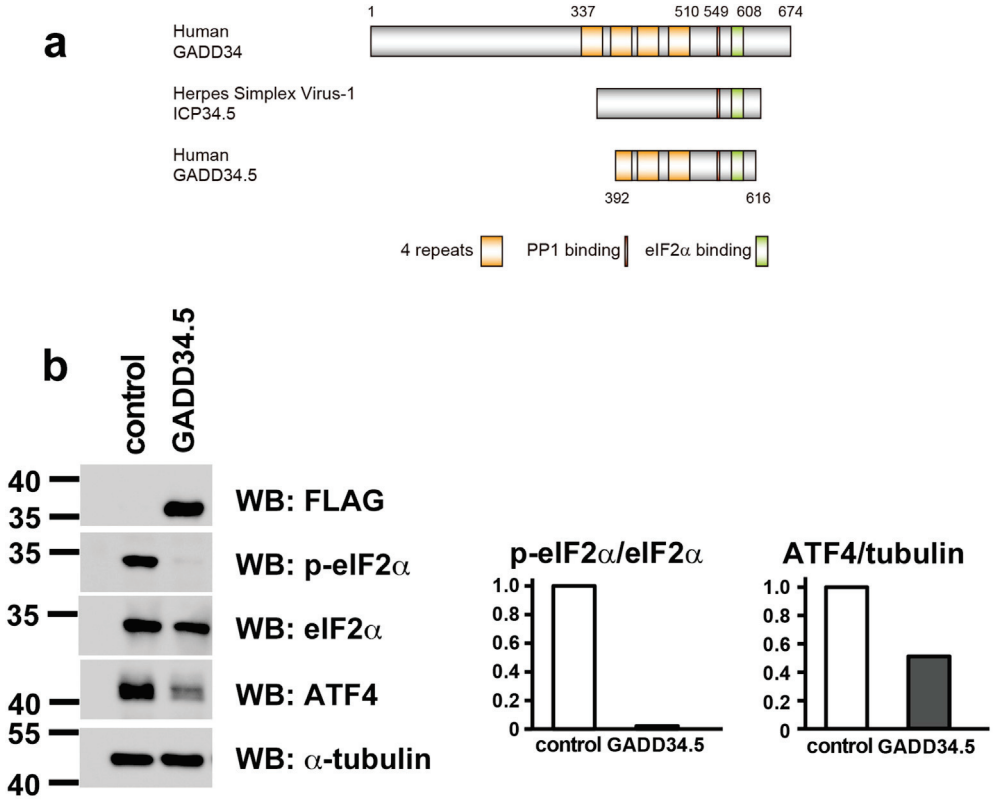

Fig. 1. Truncated *GADD34* (GADD34.5) suppresses the phosphorylation of eIF2α and expression of ATF4 in cells. (a) Schematic of GADD34.5. (b) GADD34.5 transfection in HEK293T cells was confirmed through western blotting using an anti-FLAG antibody. The suppression of eIF2α phosphorylation and ATF4 expression was also evaluated via western blotting. Tubulin was used as an internal control for FLAG-GADD34.5 and ATF4.

month-old APP23 mice and performed NOR and NOL at 4 and 7–8 months after injection, respectively. There was no significant difference in the exploration time in the NOR test between the GADD34.5-infected APP23 mice and control (vector-infected) mice (Fig. 2b). However, when NOL was tested 24 h after 3 days of training, GADD34.5-infected APP23 mice exhibited a significantly increased exploration time of the novel object compared to that with control mice (Fig. 2e). We examined proteins downstream of GADD34 in the virus-infected mice and observed that eIF2α phosphorylation was significantly decreased in the brains of GADD34.5-infected APP23 mice (Fig. 2g). ATF4 levels were also reduced but not significantly. In conclusion, these results indicate that GADD34.5 inhibits eIF2α phosphorylation and enhances spatial memory in AD-model mice.

3.3. *GADD34.5 injected into the amygdala improves contextual fear memory*

We investigated whether GADD34 expression in the amygdala affects fear conditioning memory. GADD34.5 was injected into the amygdala of 5–8-month-old APP23 mice, and an auditory fear conditioning test was performed 6 months later. In the contextual fear test, both control and GADD34.5-infected groups showed a significant increase in the percentage of freezing behavior at 24 h compared with that in naïve mice. The percentage of freezing behavior in control mice at 1 month was decreased compared to that at 24 h and was not significantly different from that of naïve mice. However, the significant increase in the freezing behavior of GADD34.5-infected APP23 mice persisted even after 1 month (Fig. 3a). Two-way repeated measures of ANOVA using all infection factors and time factors showed a difference in the time factor ($F(2,15) = 5.93$, $p = 0.013$) but not in the infection factor ($F(1,15) = 0.186$, $p = 0.672$) and interaction ($F(2,15) = 1.58$, $p = 0.238$). The time factor was further analyzed via one-way ANOVA followed by a Bonferroni post-hoc test in naïve vs 24 h ($F(2,10) = 8.78$, $p = 0.006$ for control, $F(2,14) = 2.79$, $p = 0.001$ for GADD34.5), 24 h vs 1 month ($F(2,10) = 8.78$, $p = 0.130$ for control, $F(2,14) = 2.79$, $p = 1.000$ for GADD34.5), and naive vs 1 month ($F(2,10) = 8.78$, $p = 0.273$ for control, $F(2,14) = 2.79$, $p = 0.002$ for GADD34.5)) groups. In contrast, the auditory fear condition test revealed no difference between the two groups of mice (Fig. 3b). Two-way repeated measures ANOVA showed the following results: $F(1,10) = 7.865$, $p = 0.019$ for all infection and time factors, $F(1,10) = 1.093$, $p = 0.320$ for the infection factor, and $F(1,10) = 0.146$, $p = 0.711$ for the interaction. eIF2α phosphorylation and the expression of ATF4 were similarly decreased in the amygdala and the DG (Fig. 3c). A paired *t*-test showed no significant difference between 24 h and 1 month later (control: $p = 0.0539$, GADD34.5: $p = 0.406$). This suggests that GADD34.5 might contribute to improvements in contextual fear memory in the amygdala.

4. Discussion

Previously, we revealed that ER stress increases PS1 expression and that quercetin inhibits this by inducing GADD34 [10,11]. Herein, we investigated the direct effect of GADD34 on memory. The unfolded protein response (UPR) is activated in the brains of AD patients, and eIF2α, a UPR activation marker, is increased in patients with AD [18]. In prion-infected mice, GADD34 expression reduces eIF2α phosphorylation and ATF4 expression, improves synaptic function, and prevents neurodegeneration [19]. *GADD34*, a target

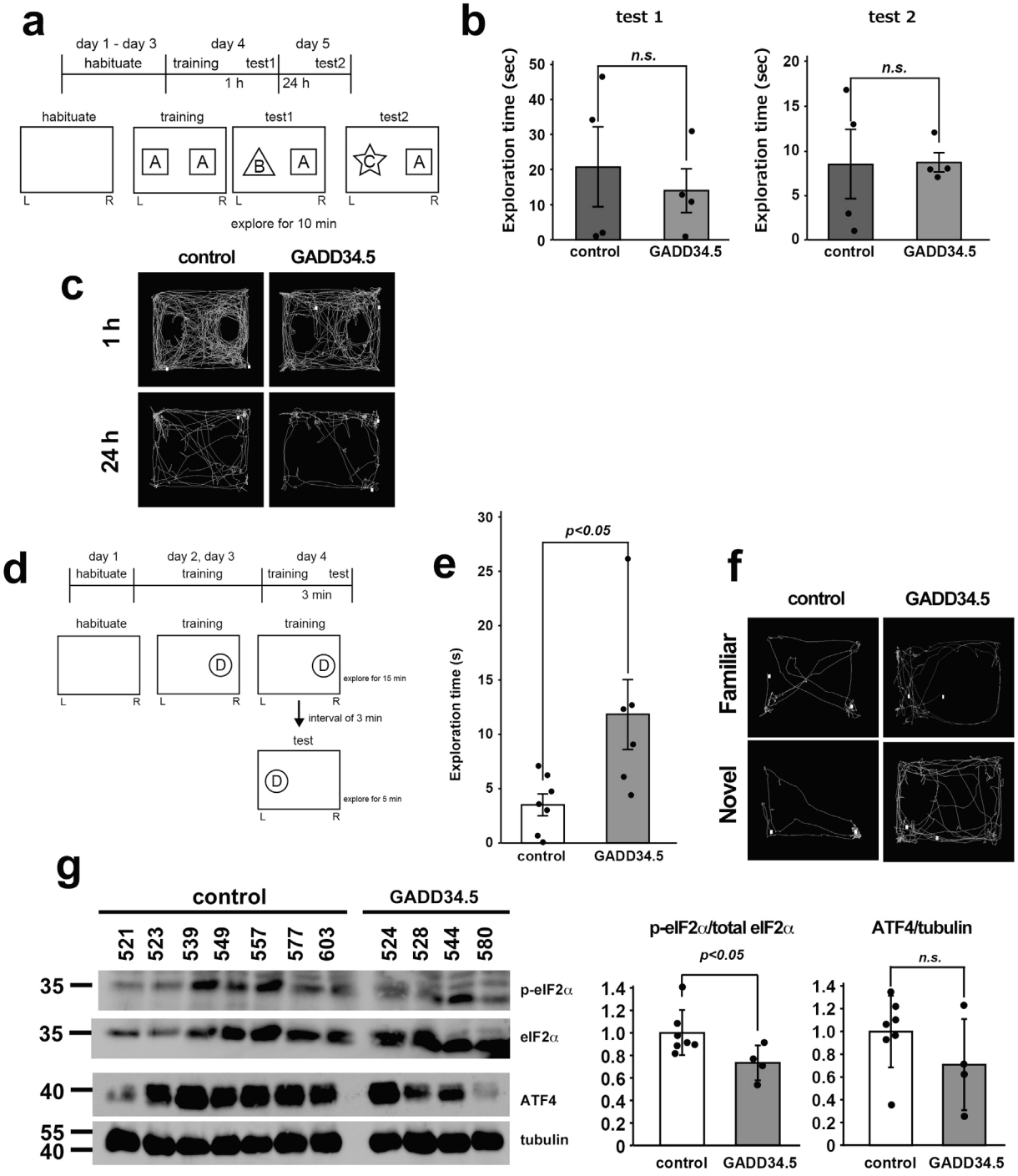

Fig. 2. GADD34.5-infected APP23 mice show improved spatial cognitive function. (a, d) Schematic of the novel object location (NOL) (a) and novel object recognition (NOR) (d) tests during training and test sessions. (b) GADD34.5-infected APP23 mice (n = 4) exhibited a similar exploration time for the novel object compared to that with the control object (n = 4). (c) Mouse trajectory of NOR detection during testing, analyzed using SMART 2.5. (e) GADD34.5-infected APP23 mice (n = 6) had significantly longer novel location object search times than control mice (n = 7). (f) Mouse trajectory of NOL detection during final training and testing, analyzed using SMART 2.5. (g) Levels of eIF2α phosphorylation and ATF4 expression in the dentate gyrus (DG) of GADD34.5-infected APP23 mice compared with those in control mice based on western blotting.

gene of ATF4 [11], interacts with PP1c to reduce the levels of eIF2α phosphorylation, thereby negatively regulating the eIF2α–ATF4 pathway [8]. eIF2α phosphorylation reduces general protein synthesis but allows for the translation of some mRNAs, including *ATF4*. The small-molecule integrated stress response (ISR) inhibitor ISRIB blocks the downstream signaling of eIF2α phosphorylated kinase and enhances rodent spatial and fear-related learning memory [16]. Quercetin induces GADD34, regulates phosphorylated eIF2α and ATF4 signaling, and suppresses PS1 expression and Aβ production [11].

The NOR and NOL tests are non-rewarded and etiologically relevant. In the NOR test, no significant difference was observed between the GADD34.5-injected and control mice (Fig. 2b). In contrast, the NOL test results were significantly improved by GADD34.5 injection into the dorsal hippocampus, which is implicated in spatial memory (Fig. 2e). Hippocampal function is

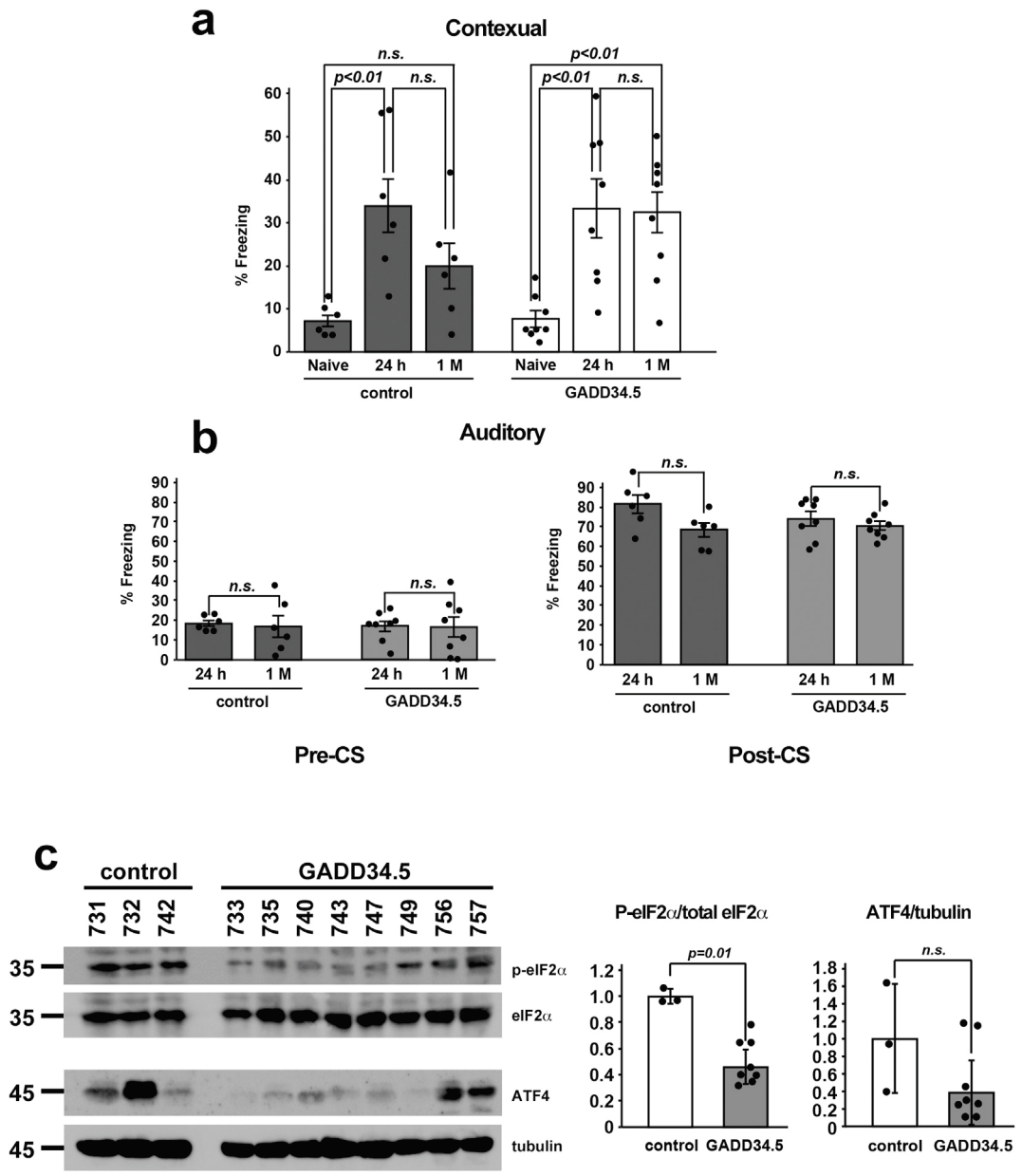

Fig. 3. GADD34.5 injected into the amygdala results in the maintenance of contextual fear memory. (a) Contextual fear conditioning was assessed in chamber A based on freezing episodes that occurred within a 3 min period before training (naïve) and 24 h and 1 month after training using GADD34.5-infected APP23 (n = 8) or control (n = 6) mice. The data showed a significant difference in the average between naïve mice and those 1 month after training for GADD34.5-infected APP23 mice. (b) Auditory fear conditioning was assessed in chamber B based on freezing episodes that occurred between 24 h and 1 month after training for a 3 min period without (pre-CS) and with (post-CS) sound. Two-way repeated measures ANOVA, including all infection and time factors, showed a difference in the time factor; however, no difference in the infection factor and interaction was observed. A paired *t*-test based on these results showed no significant difference between 24 h and 1 month later. The same mice as in Fig. 3a were used. (c) Levels of eIF2α phosphorylation and ATF4 expression in the amygdala of GADD34.5-infected APP23 mice were compared with those in control mice by performing western blotting.

reportedly important for the memory of an object position, particularly spatial memory involving the dorsal hippocampus [20]. However, object recognition memory formation does not require the hippocampus [21] and depends on several different regions [22]. These results could explain our finding that the injection of GADD34.5 into the dorsal DG specifically improved NOL.

Recently, we used aged wild-type mice and showed that the rate of freezing behavior increased in quercetin diet-fed mice in the fear conditioning test. In addition, GADD34 expression in the brain increased immediately after auditory fear conditioning in quercetin-fed mice [11]. The lateral nucleus of the amygdala is an important component of the nervous system and is involved in memory formation during fear conditioning [23–25]. In this study, mice injected with GADD34.5 into the amygdala tended to retain memory in a contextual test 1 month after fear conditioning, but control mice did not (Fig. 3a). *eIF2α*$^{+/S51A}$ mice, which express phosphorylation site-dead eIF2α mutants, show enhanced contextual and auditory fear conditioning [26], indicating that unphosphorylated eIF2α is important for fear memory. Our results suggested that GADD34 suppresses eIF2α phosphorylation to maintain memory under contextual fear conditions. Herein, auditory fear memory did not differ between GADD34.5-injected and control mice (Fig. 3b). Some previous studies also revealed that auditory fear memory tends to be maintained, even if contextual

fear memory is affected [27,28]. Auditory fear memory might be strong even in control mice, and we could not show the difference.

To date, some studies have revealed the involvement of eIF2α phosphorylation and ATF4 expression in brain functions. For example, the deficiency of PERK, an eIF2α kinase, reduces ATF4 expression and rescues synaptic plasticity and spatial memory in AD-model mice [29]. In mice, ATF4 impairs synaptic plasticity and memory formation [30]. In aplysia, ATF4 inhibits long-term potentiation (LTP) [31].

However, few studies have also indicated that ATF4 is important for memory formation. Pasini et al. reported that specific inhibition of the expression of ATF4 in the mouse hippocampus results in worse results in the Morris water maze test and inhibits LTP in the mouse brain [32]. Liu et al. revealed that ATF4 knockdown in cultured hippocampal and cortical neurons reduces spine density and dendritic PSD-95 and GluR1 and that ATF4 influences synaptic morphology via actin filament remodeling [33]. In contrast, eIF2α phosphorylation is positively associated with increased BACE1 and Aβ in humans and AD-model mice [34]. Arctigenin, extracted from *Fructus arctii*, inhibits Aβ production and improves memory impairment by suppressing BACE1 translation, including the eIF2α-mediated pathway [35].

Previously, we reported that the Aβ production pathway (eIF2α phosphorylation and ATF4 expression) is increased in aged or APP23 mouse brains [11]. In this study, we focused on this pathway, which was inhibited by introducing GADD34.5 into the DG or amygdala (Figs. 2g and 3c). However, a BACE1 reduction due to the suppression of eIF2α phosphorylation, mediated by GADD34, might contribute to learning ability. In both cases, GADD34 is considered to directly promote eIF2α dephosphorylation and prevent Aβ production. However, because ATF4 has contradictory effects on synaptic plasticity, the morphology and properties of synapses should be accurately examined to determine the effect of ATF4 on memory formation.

In this experiment, we used the AD model mice to examine memory. Since eIF2α phosphorylation and ATF4 expression are increased in patients with Alzheimer's disease [36,37], our strategy of increasing GADD34 expression in the brain to prevent memory loss might be applicable to humans as well. As quercetin feeding increased GADD34 expression, quercetin might be a potential compound used to prevent AD.

5. Conclusion

In summary, our results suggest that GADD34 suppresses eIF2α phosphorylation in the brain and prevents memory loss.

Author contributions

T.N. contributed to the experimental design. M.H–O, Tana, T.N, and M. I performed the experiments. M.H–O, M. I, and T. N. analyzed the data and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Dr. M. Staufenbiel (Novartis Pharma Ltd, Basel, Switzerland) for providing APP23 mice.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.02.077.

References

[1] M.A. DeTure, D.W. Dickson, The neuropathological diagnosis of Alzheimer's disease, Mol. Neurodegener. 14 (2019) 32, https://doi.org/10.1186/s13024-019-0333-5.
[2] M. Prince, A. Wimo, M. Guerchet, G.C. Ali, Y.T. Wu, M. Prina, Alzheimer's Disease International, World Alzheimer Report 2015 - the Global Impact of Dementia: an Analysis of Prevalence, Incidence, Cost and Trends, Alzheimer's Disease International, London, 2015, p. 84.
[3] G.S. Hotamisligil, Endoplasmic reticulum stress and the inflammatory basis of metabolic disease, Cell 140 (2010) 900–917, https://doi.org/10.1016/j.cell.2010.02.034.
[4] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell Biol. 8 (2007) 519–529, https://doi.org/10.1038/nrm2199.
[5] M. Costa-Mattioli, D. Gobert, H. Harding, B. Herdy, M. Azzi, M. Bruno, M. Bidinosti, C. Ben Mamou, E. Marcinkiewicz, M. Yoshida, H. Imataka, A.C. Cuello, N. Seidah, W. Sossin, J.C. Lacaille, D. Ron, K. Nader, N. Sonenberg, Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2, Nature 436 (2005) 1166–1173, https://doi.org/10.1038/nature03897.
[6] K. Ohta, A. Mizuno, M. Ueda, S. Li, Y. Suzuki, Y. Hida, Y. Hayakawa-Yano, M. Itoh, E. Ohta, M. Kobori, T. Nakagawa, Autophagy impairment stimulates PS1 expression and gamma-secretase activity, Autophagy 6 (2010) 345–352, https://doi.org/10.4161/auto.6.3.11228.
[7] T. Mitsuda, Y. Hayakawa, M. Itoh, K. Ohta, T. Nakagawa, ATF4 regulates gamma-secretase activity during amino acid imbalance, Biochem. Biophys. Res. Commun. 352 (2007) 722–727, https://doi.org/10.1016/j.bbrc.2006.11.075.
[8] I. Novoa, H. Zeng, H.P. Harding, D. Ron, Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha, J. Cell Biol. 153 (2001) 1011–1022, https://doi.org/10.1083/jcb.153.5.1011.
[9] P. Tsaytler, H.P. Harding, D. Ron, A. Bertolotti, Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis, Science 332 (2011) 91–94, https://doi.org/10.1126/science.1201396.
[10] K. Ohta, A. Mizuno, S. Li, M. Itoh, M. Ueda, E. Ohta, Y. Hida, M.X. Wang, M. Furoi, Y. Tsuzuki, M. Sobajima, Y. Bohmoto, T. Fukushima, M. Kobori, T. Inuzuka, T. Nakagawa, Endoplasmic reticulum stress enhances gamma-secretase activity, Biochem. Biophys. Res. Commun. 416 (2011) 362–366, https://doi.org/10.1016/j.bbrc.2011.11.042.
[11] M. Hayakawa, M. Itoh, K. Ohta, S. Li, M. Ueda, M.X. Wang, E. Nishida, S. Islam, C. Suzuki, K. Ohzawa, M. Kobori, T. Inuzuka, T. Nakagawa, Quercetin reduces eIF2alpha phosphorylation by GADD34 induction, Neurobiol. Aging 36 (2015) 2509–2518, https://doi.org/10.1016/j.neurobiolaging.2015.05.006.
[12] J.H. Connor, D.C. Weiser, S. Li, J.M. Hallenbeck, S. Shenolikar, Growth arrest and DNA damage-inducible protein GADD34 assembles a novel signaling complex containing protein phosphatase 1 and inhibitor 1, Mol. Cell Biol. 21 (2001) 6841–6850, https://doi.org/10.1128/MCB.21.20.6841-6850.2001.
[13] C. Lois, E.J. Hong, S. Pease, E.J. Brown, D. Baltimore, Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors, Science 295 (2002) 868–872, https://doi.org/10.1126/science.1067081.
[14] D.S. Roy, A. Arons, T.I. Mitchell, M. Pignatelli, T.J. Ryan, S. Tonegawa, Memory retrieval by activating engram cells in mouse models of early Alzheimer's disease, Nature 531 (2016) 508–512, https://doi.org/10.1038/nature17172.
[15] H. Li, M.A. Penzo, H. Taniguchi, C.D. Kopec, Z.J. Huang, B. Li, Experience-dependent modification of a central amygdala fear circuit, Nat. Neurosci. 16 (2013) 332–339, https://doi.org/10.1038/nn.3322.
[16] M.E. Calhoun, K.H. Wiederhold, D. Abramowski, A.L. Phinney, A. Probst, C. Sturchler-Pierrat, M. Staufenbiel, B. Sommer, M. Jucker, Neuron loss in APP transgenic mice, Nature 395 (1998) 755–756, https://doi.org/10.1038/27351.
[17] B. He, M. Gross, B. Roizman, The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 843–848, https://doi.org/10.1073/pnas.94.3.843.
[18] J.J. Hoozemans, R. Veerhuis, E.S. Van Haastert, J.M. Rozemuller, F. Baas, P. Eikelenboom, W. Scheper, The unfolded protein response is activated in Alzheimer's disease, Acta Neuropathol. 110 (2005) 165–172, https://doi.org/10.1007/s00401-005-1038-0.
[19] J.A. Moreno, H. Radford, D. Peretti, J.R. Steinert, N. Verity, M.G. Martin, M. Halliday, J. Morgan, D. Dinsdale, C.A. Ortori, D.A. Barrett, P. Tsaytler, A. Bertolotti, A.E. Willis, M. Bushell, G.R. Mallucci, Sustained translational repression by eIF2alpha-P mediates prion neurodegeneration, Nature 485 (2012) 507–511, https://doi.org/10.1038/nature11058.
[20] M.S. Fanselow, H.W. Dong, Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65 (2010) 7–19, https://doi.org/10.1016/j.neuron.2009.11.031.

[21] A.M. Oliveira, J.D. Hawk, T. Abel, R. Havekes, Post-training reversible inactivation of the hippocampus enhances novel object recognition memory, Learn, MEM (Miner. Electrolyte Metab.) 17 (2010) 155–160, https://doi.org/10.1101/lm.1625310.

[22] J.K. Denninger, B.M. Smith, E.D. Kirby, Novel object recognition and object location behavioral testing in mice on a budget, JoVE 141 (2018), https://doi.org/10.3791/58593, 10.3791/58593.

[23] S.M. Rodrigues, G.E. Schafe, J.E. LeDoux, Molecular mechanisms underlying emotional learning and memory in the lateral amygdala, Neuron 44 (2004) 75–91, https://doi.org/10.1016/j.neuron.2004.09.014.

[24] J. Hitchcock, M. Davis, Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm, Behav. Neurosci. 100 (1986) 11–22, https://doi.org/10.1037/0735-7044.100.1.11.

[25] J. Muller, K.P. Corodimas, Z. Fridel, J.E. LeDoux, Functional inactivation of the lateral and basal nuclei of the amygdala by muscimol infusion prevents fear conditioning to an explicit conditioned stimulus and to contextual stimuli, Behav. Neurosci. 111 (1997) 683–691, https://doi.org/10.1037/0735-7044.111.4.683.

[26] M. Costa-Mattioli, D. Gobert, E. Stern, K. Gamache, R. Colina, C. Cuello, W. Sossin, R. Kaufman, J. Pelletier, K. Rosenblum, K. Krnjević, J.C. Lacaille, K. Nader, N. Sonenberg, EIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory, Cell 129 (2007) 195–206, https://doi.org/10.1016/j.cell.2007.01.050.

[27] G.E. Schafe, N.V. Nadel, G.M. Sullivan, A. Harris, J.E. LeDoux, Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase, Learn, MEM (Miner. Electrolyte Metab.) 6 (1999) 97–110, https://doi.org/10.1101/lm.6.2.97.

[28] C.R. Pugh, K. Kumagawa, M. Fleshner, L.R. Watkins, S.F. Maier, J.W. Rudy, Selective effects of peripheral lipopolysaccharide administration on contextual and auditory-cue fear conditioning, Brain Behav. Immun. 12 (1998) 212–229, https://doi.org/10.1006/brbi.1998.0524.

[29] T. Ma, M.A. Trinh, A.J. Wexler, C. Bourbon, E. Gatti, P. Pierre, D.R. Cavener, E. Klann, Suppression of eIF2alpha kinases alleviates Alzheimer's disease-related plasticity and memory deficits, Nat. Neurosci. 16 (2013) 1299–1305, https://doi.org/10.1038/nn.3486.

[30] Z. Jiang, J.E. Belforte, Y. Lu, Y. Yabe, J. Pickel, C.B. Smith, H.S. Je, B. Lu, K. Nakazawa, EIF2alpha phosphorylation-dependent translation in CA1 pyramidal cells impairs hippocampal memory consolidation without affecting general translation, J. Neurosci. 30 (2010) 2582–2594, https://doi.org/10.1523/JNEUROSCI.3971-09.2010.

[31] D. Bartsch, M. Ghirardi, P.A. Skehel, K.A. Karl, S.P. Herder, M. Chen, C.H. Bailey, E.R. Kandel, Aplysia CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change, Cell 83 (1995) 979–992, https://doi.org/10.1016/0092-8674(95)90213-9.

[32] S. Pasini, C. Corona, J. Liu, L.A. Greene, M.L. Shelanski, Specific downregulation of hippocampal ATF4 reveals a necessary role in synaptic plasticity and memory, Cell Rep. 11 (2015) 183–191, https://doi.org/10.1016/j.celrep.2015.03.025.

[33] J. Liu, S. Pasini, M.L. Shelanski, L.A. Greene, Activating transcription factor 4 (ATF4) modulates post-synaptic development and dendritic spine morphology, Front. Cell. Neurosci. 8 (2014) 177, https://doi.org/10.3389/fncel.2014.00177.

[34] T. O'Connor, K.R. Sadleir, E. Maus, R.A. Velliquette, J. Zhao, S.L. Cole, W.A. Eimer, B. Hitt, L.A. Bembinster, S. Lammich, S.F. Lichtenthaler, S.S. Hébert, B. De Strooper, C. Haass, D.A. Bennett, R. Vassar, Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis, Neuron 60 (2008) 988–1009, https://doi.org/10.1016/j.neuron.2008.10.047.

[35] Z. Zhu, J. Yan, W. Jiang, X.G. Yao, J. Chen, L. Chen, C. Li, L. Hu, H. Jiang, X. Shen, Arctigenin effectively ameliorates memory impairment in Alzheimer's disease model mice targeting both beta-amyloid production and clearance, J. Neurosci. 33 (2013) 13138–13149, https://doi.org/10.1523/JNEUROSCI.4790-12.2013.

[36] R.C. Chang, A.K. Wong, H.K. Ng, J. Hugon, Phosphorylation of eukaryotic initiation factor-2alpha (eIF2alpha) is associated with neuronal degeneration in Alzheimer's disease, Neuroreport 13 (2002) 2429–2432, https://doi.org/10.1097/00001756-200212200-00011.

[37] J. Lewerenz, P. Maher, Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression, J. Biol. Chem. 284 (2009) 1106–1115, https://doi.org/10.1074/jbc.M807325200.