Soybean Meal Extract Preserves Memory Ability By Increasing Presynaptic Function and Modulating Gut Microbiota in Rats

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Research Article

Keywords: soybean meal extract, memory function, synaptic proteins, hippocampus, gut microbiota

Posted Date: October 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-973062/v1

Version of Record: A version of this preprint was published at Molecular Neurobiology on January 10th, 2022. See the published version at https://doi.org/10.1007/s12035-021-02669-3.

Abstract

Age-related degenerative brain diseases frequently manifest as memory deficits. Dietary interventions or nutraceuticals may provide efficacious treatments through prevention and cure. Soybean meal, a byproduct of soy oil refining, has health benefits, but its effect on memory function is unknown. Therefore, we evaluated the effect of the oral administration of soybean meal extract (SME) for 2 weeks on memory function using the Morris water maze (MWM) test in healthy rats and investigated the possible underlying mechanisms. First, analysis of the composition revealed that SME is rich in isoflavones; SME did not exhibit hepatotoxicity or renal toxicity at the different doses tested. The MWM results revealed that the escape latency and movement distance of rats were significantly shorter in the SME group than in the control group, indicating that SME can help in memory preservation. In addition, SME increased the levels of presynaptic proteins such as synaptophysin, synaptobrevin, synaptotagmin, syntaxin, synapsin I, and 25-kDa synaptosome-associated protein as well as protein kinases and their phosphorylated expression, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)] in the hippocampal nerve terminals (synaptosomes). Transmission electron microscopy also indicated that SME increased the number of synaptic vesicles in hippocampal synaptosomes. Furthermore, SME rats exhibited increased gut microbiota diversity and altered microbiota composition compared with control rats. Therefore, our data suggest that SME can increase presynaptic function and modulate gut microbiota, thus aiding in memory preservation in rats.

Introduction

With the aging of society worldwide, the incidence of degenerative brain diseases, including Parkinson disease, Alzheimer disease (AD), Huntington disease, amyotrophic lateral sclerosis, and multiple sclerosis, is increasing [1]. Memory impairment is one of the main symptoms of age-related degenerative brain diseases [2, 3]. Because no effective therapies exist for memory impairment, preventive approaches, such as dietary interventions or nutraceuticals, have been receiving increasing attention [4–7]. Consumption of foods rich in polyphenols is associated with improved cognitive performance and a reduced risk of cognitive impairment in humans [8–10].

Soybean contains numerous nutritional and biofunctional compounds, especially isoflavones [11]. It has estrogenic, hypocholesterolemic, anticancer, antiosteoporotic, antioxidative, anti-inflammatory, and neuroprotective effects [11–18]. Soybean and its extract have been reported to improve cognitive deficits by reducing β amyloid (1-42) formation, increasing neurogenesis, and suppressing neuroinflammation in several animal models [19–21].

In the present study, we focused on the soybean meal, which is a byproduct of soybean oil extraction and is widely used in feed and food industries [22]. It contains functional bioactive compounds similar to soybeans, such as phenolics and isoflavones [23]. Similar to soybean, soybean meal has diverse biological effects, including antioxidative, anti-inflammatory, anticancer, and antiphotodamage effects

[24–27]. However, no study has focused on the role of soybean meal in memory function. Here, we (i) determined whether the oral administration of soybean meal extract (SME) for 2 weeks affected memory in healthy rats using the Morris water maze (MWM) test, (ii) elucidated the mechanisms of action of SME and (iii) determined whether SME produced any side effects in rats. In addition, the feces of rats were collected to analyze gut microbiota, which is associated with memory function [28].

Materials And Methods

Materials

Soybean meal was purchased from the local market. Anti-synaptophysin (#36406S), anti-synapsin I (#5297S), anti-β-actin (#3700S), anti-extracellular signal-regulated kinases 1 and 2 (ERK1/2, #9102S), anti-phospho-protein kinase C (PKC, #9371S), and anti-phospho-ERK1/2 (#4370S) were purchased from Cell Signaling (MA, USA). Anti-synaptotagmin (#ab13259), anti-synaptobrevin (#ab18013), anti-syntaxin (#ab188583), anti-synaptosomal-associated protein 25 kDa (SNAP 25, #ab41455), anti-PKC (#ab23511), anti-Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII, #ab92332), and anti-phospho-CaMKII (#ab171095) were purchased from Abcam (Cambridge, UK). Anti-phospho-synapsin I site-4,5 (Ser 62, 67) (#GTX82591), anti-phospho-synapsin I site-3 (Ser 603) (#GTX82589), and anti-horseradish peroxidase-conjugated secondary antibodies (#GTX213110-01, #GTX213111-01) were purchased from Gentex (MI, USA). Other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of SME

The extract was prepared as described in previous studies [26, 27]. Briefly, 50 g of soybean meal was dissolved in 150 mL of ethanol/water (1:1 v/v) by continuously stirring for 2 h at 4°C. Then, the supernatants were obtained by centrifugation (6000 rpm for 20 min at 25 °C) and lyophilized to obtain a powder. The extract powder was then stored at -20 °C for further use in the biological assays.

LC–MS/MS analysis

Isoflavone and phenolic acid of SME were separated on an Agilent Eclipse plus C18 column (100 mm × 4.6 mm, 3.5 μm) at a flow rate of 0.35 $mLmin^{-1}$ by using an Agilent 1200 series binary pump. Gradient elution was performed with mobile phase A (5% methanol with 0.1% formic acid) and mobile phase B (methanol with 0.1% formic acid). The initial condition was 50:50 mobile phase A/mobile phase B (*v/v*) for 2.5 min, after which is was changed to 80% mobile phase B in 0.1 min that was maintained for 2 min. Finally, the solvent composition was quickly reverted to the initial conditions and equilibrated for 11 min. Mass spectrometry was operated in multiple ion-monitoring mode (MRM) and negative polarity at −4200 V by using API 3000 (MDS SCIEX, Applied Biosystems, Ontario, Canada).

Experimental animals

The International Guidelines for Care and Use of Laboratory Animals were followed for all experiments, and the experimental protocol was approved by the Animal Care Committee of Fu Jen Catholic University (approval number: A11018). Thirty male Sprague-Dawley rats (Taiwan BioLASCO) weighing 160–200 g were used. They were housed in plastic cages and were fed on pellets with free access to tap water. Room temperature was controlled at 22 ± 2°C with a 12-h light:12-h dark cycle. After 3 days of training with MWM, rats were divided into SME and tap water (control) groups; they were orally administered SME solution or an equal volume of 0.9% normal saline, respectively, daily for 2 consecutive weeks. After 2 weeks, the behavioral test was conducted 30 min by using the MWM video analysis system. Next, the rat's body weight was measured, and stool samples were collected and immediately stored at -80°C for gut microbiota analysis. Finally, the rats were deeply anesthetized and killed, and the hippocampus was collected to prepare synaptosomes for glutamate release assay, transmission electron microscopy (TEM), and Western blotting. In addition, the liver and kidney were collected from the rats after sacrifice for hematoxylin-eosin (H&E) staining.

MWM test

The MWM test was conducted to evaluate the performance of spatial learning and memory, as described by previous study [29]. A circular pool with a diameter of 55 cm and height of 25 cm was filled with opacified water (20 cm depth) at 25 ± 1°C. The pool was divided into four quadrants, and the platform was placed at the center of one fixed quadrant for all trials. Training (days 1—4) was conducted four times a day, and the escape latency time for each rat to go to the platform was measured for 120 s. Rats reaching the platform were allowed to be remain there for 15 s. Rats that failed to locate the platform were guided to the platform and allowed to stay for 30 s. The latency period of the failed rats was recorded within 120 s. The swimming path from the entry to the hidden platform, escape latency, and movement distance in the coverage zone were recorded using a video-tracking system (Version 1.17, SINGA Technology Corporation, Taipei, Taiwan).

H&E staining

Liver or kidney tissues were fixed in 4% PFA, dehydrated with graded alcohol, and embedded in paraffin wax. A series of paraffin sections (5 μm) were cut using a Leica rotation microtome and stained with H&E, and images were captured under a microscope with 400x magnification. Histological changes in the liver and kidney sections were determined in terms of cytoplasmic color fading, vacuolization, nuclear condensation, nuclear fragmentation, nuclear fading, and erythrocyte stasis [30].

TEM

Rat hippocampal synaptosomes from each group were placed in an electron microscope fixative solution for 1 day. Samples were then postfixed in 1% osmium tetraoxide for 2 h, followed by gradient ethanol dehydration, soaking, and embedding in pure epoxy resin. Samples were cut into 70-nm-thick sections and stained with uranium and lead. Finally, sections were observed under a TEM (JEM-1400, JEOL, Japan).

Western blotting analysis

Western blotting was performed as described by previous reported [29]. Briefly, hippocampal synaptosomes were homogenized and the concentrations of proteins were determined using Bradford's method, with bovine serum albumin (BSA) as a standard. Equal protein amounts (20 mg) were subjected to sodium dodecylsulfate polyacrylamide (SDS-PAGE) gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST) for 1h at room temperature and incubated overnight at 4°C with primary antibodies. The antibodies used were anti-synaptophysin (1:200000), anti-synaptotagmin (1:1000), anti-synaptobrevin (1:800), anti-syntaxin (1:10000), anti-synapsin I (1:50000), anti-SNAP 25 (1:50000), anti-PKC (1:700), anti-phospho-PKC (1: 2000), anti-CaMKII (1:10000), anti-phospho-CaMKII (1:2000), anti-ERK1/2 (1:5000), anti-phospho-ERK1/2 (1:2000), anti-phospho-synapsin I site-4, 5 (1:2000), anti-phospho-synapsin I site-3 (1:2000), and anti-b-actin (1:1000). Next, the membrane was washed with TBST three times and incubated with a secondary horseradish peroxidase-conjugated antibody (1:5000) at room temperature for 1 h. Protein bands were visualized using a chemiluminescence reagent (Amersham, Buckinghamshire, UK). The intensity of the protein bands was analyzed using ImageJ software (Synoptics, Cambridge, UK).

Gut microbiota

Gut microbiota analysis was conducted by the Biotools Microbiome Research Center (Taipei, Taiwan). Briefly, DNA was extracted from fecal samples using the QIAamp PowerFecal DNA kit (Qiagen, CA, USA). The 16s rDNA amplicon sequencing of the V4 hypervariable region was performed with an Illumina HiSeq (paired-end 250 bp). Primers was designed to target the V4 region of the 16S rDNA (position 319 of the bacterial 16s rRNA gene to position 806). Each reaction was denatured at 95°C for 3 min followed by 25 cycles of (95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec), followed by a final extension at 72°C for 5 min [31]. Reactions each contained a unique sequence index to enable pooling. Pools were purified with the AMPure XP beads and sequenced on an Illumina HiSeq platform. The 16S rDNA data were analyzed with the open-source bioinformatics pipeline Quantitative Insights into Microbial Ecology (QIIME). The sequences were grouped into operational taxonomic units (OTUs) by UCLUST at a minimum of 97% sequence similarity. Representative sequences from each OTU were aligned using the PyNAST software (v.1.2). Taxonomy was assigned using the Silva database (v.132).

Statistical analysis

Statistical analysis was done using the SPSS.16.0 software. The data were expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was run followed by Tukey post hoc comparisons test. The criterion for the statistical significance was $p < 0.05$.

Results

Isoflavones and phenolic acid content in SME

Figure 1 indicates that isoflavone content is higher than the phenolic acid content. The major compounds in SME are glucoside and malonyl types of isoflavone (Table 1). The content of different functional groups can be ranked as glucoside >malonyl>> acetyl >aglycone, and the basic structure of isoflavone as daidzein>genistein>>glycitein. Unlike isoflavones, the phenolic acid content is very low; among the phenoic acids, p-coumaric acid content is significantly the highest (Table 1).

Table 1
Isoflavone and phenolic acid content in SME (concentration)

Analyte	ng/mL
Protocatechuic acid	15.82 ± 6.21
p-Coumaric acid	389.32 ± 137.09
Gallic acid	14.13 ± 2.89
Caffeic acid	2.99 ± 0.90
Ferulic acid	146.33 ± 22.32
Catechin	3.93 ± 0.37
Chlorogenic acid	14.21± 4.73
Rutin	5.88 ± 0.86
Daidizein	1727.50 ± 1007.22
Genistein	1373.25 ± 869.15
Glycitein	663.29 ± 391.40
Daidizin	18676.91 ± 2631.96
Genistin	18546.38 ± 1967.54
Glycitin	2699.11 ± 233.28
Acetyldaidzin	3395.27 ± 402.31
Acetylgenistin	4145.27 ± 571.26
Acetylglycitin	432.30 ± 61.52
Malonyldaidzin	15302.70 ± 3146.35
Malonylgenistin	19047.02 ± 3474.71
Malonylglycitin	2883.97 ± 840.35
Data are presented as mean ± SEM (n = 3).	

Body weight and hepatic and renal toxicity

The experimental design is presented in Fig. 2A. SME was orally administered to rats once daily for 14 days. The effects of SME on the body weights and hepatic and renal toxicity were investigated at the end of the experimental period, and the results are shown in Fig. 2B and C. Compared with the control group, the body weight of rats in the SME group was not significantly different [$F(2, 27) = 0.03$, $p = 0.9$]. In addition, no obvious morphological changes were observed in the liver and kidney between the two groups (Fig. 2C), indicating that chronic administration of SME does not cause liver and kidney damage.

Memory retention in rats administered SME

To determine the effect of SME on spatial learning and memory, we analyzed the rats performance in the MWM test (Fig. 3A). In the MWM test, rats in the SME group had a shorter time for finding the platform than the control group [$F(2, 21) = 54.9$, $p < 0.001$, Fig. 3B]. Similarly, the movement distance of rats in the SME group was significantly shorter than that of the control group [$F(2, 21) = 77.9$, $p < 0.001$; Fig. 3C]. No significant difference was observed between rats administered SME 50 mg/kg and SME 100 mg/kg groups ($p = 0.9$). These results suggest that the memory retention of the SME group were superior to that of the control group.

Increased synaptic protein expression in the hippocampal synaptosomes of rats administered SME

Synaptic function, often measured in terms of presynaptic protein levels, indicates cognitive brain reserve [32]. To confirm that synaptic function was involved in the effects of SME, the levels of presynaptic proteins including synaptophysin, synaptotagmin, synaptobrevin, syntaxin, synapsin-1, and SNAP-25 in the hippocampal synaptosomes were detected. As shown in the Fig. 4, the administration of SME led to significant increases in the levels of synaptophysin by 44% [$F(2, 12) = 22.9$, $p < 0.001$], synaptotagmin by 42% [$F(2, 12) = 8.4$, $p < 0.01$], synaptobrevin by 81% [$F(2, 12) = 21.2$, $p < 0.001$], syntaxin by 54% [$F(2, 12) = 11.9$, $p < 0.01$], SNAP-25 by 67% [$F(2, 12) = 62.4$, $p < 0.001$], and synapsin I by 53% [$F(2, 12) = 20.5$, $p < 0.001$] in the hippocampal synaptosomes compared with those in the control group ($p < 0.001$, n = 5; Fig. 4). No significant difference was observed between the effects of SME 50 mg/kg and SME 100 mg/kg groups ($p > 0.05$).

Increased protein kinase expression and phosphorylation in the hippocampal synaptosomes of rats administered SME

Because synaptic vesicle-related proteins are regulated by extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [33], we

detected the levels of ERK1/2, PKC, and CaMKII, and their phosphorylated expression (Fig. 5A). The expression of PKC [F (2, 12) = 5.9, $p < 0.01$], CaMKII[F (2, 12) = 10.4, $p < 0.01$], and ERK1/2 [F (2, 12) = 28.3, $p < 0.001$] and their phosphorylated levels in the hippocampal synaptosomes were higher in the SME group than in the control group [p-PKC, F (2, 12) = 6.3, $p < 0.01$; p-CaMKII, F (2, 12) = 38.9, $p < 0.01$; p-ERK1/2, F (2, 12) = 28.3, $p < 0.001$; Fig. 5B]. In addition, a significant increase in the phosphorylation of synapsin I at ERK1/2-specific sites 4 and 5 and CaMKII-specific sites 3 was observed in the SME group compared with the control group [p-synapsin I site 4,5, F (2, 12) = 19.1, $p < 0.001$; p-synapsin I site 3, F (2, 12) = 7.1, $p < 0.01$; Fig. 5C]. No significant difference was observed between rats administered SME 50 mg/kg and SME 100 mg/kg ($p > 0.05$).

Increased synaptic vesicles in the hippocampal synaptosomes of rats administered SME

We observed the changes in synaptic vesicles in the hippocampal synaptosomes of rats under a transmission electron microscope (Fig. 6A). The number of synaptic vesicles in the hippocampal synaptosomes of the SME group was higher in the SME group than in the control group [F (2, 6) = 45.3, $p < 0.001$; Fig. 6B]. No significant difference was observed between rats administered SME 50 mg/kg and SME 100 mg/kg ($p = 0.9$).

Changes in gut microbiota in rats administered SME

In addition, increasing evidence has demonstrated that the gut microbiota is associated with memory function [28]. To explore gut microbiota composition and alterations, we used the next-generation sequencing technology to measure the bacterial community in fecal samples of rats. Fig. 7 presents the microbiota profiles of rats treated with SME. Alpha-diversity was estimated based on observed OTUs, and the results indicated that the observed species in the gut microbiota of the SME 100 mg/kg group was significantly increased compared with that in the control group [F (2, 6) = 6.6, $p < 0.05$; Fig. 7A]. These results were confirmed using rarefaction curves analysis (Fig. 7B), indicating that the SME treatments increased the species richness and diversity compared with the control group. At the phylum level, the relative abundance of three main phyla—Bacteroidates, Firmicutes, and Proteobacteria—was approximately 97% (Fig. 7C). The relative abundance of Firmicutes in the SME 100 mg/kg group was significantly lower than that in the control group [F (2, 11) = 4.9, $p < 0.05$; Fig. 7D], whereas that of Bacteroidates was not significantly different between the two groups [F (2, 12) = 0.3, $p > 0.05$; Fig. 7D]. The Firmicutes to Bacteroidates(F/B) ratio in the SME 100 mg/kg group was reduced compared with that in the control group [F (2, 9) = 4.3, $p < 0.05$; Fig. 7D, inset]. By contrast, the relative abundance of Proteobacteria and Actinobacteria was higher in the SME group than in the control group [Proteobacteria, F (2, 12) = 4.3, $p < 0.05$; Actinobacteria, F (2, 7) = 5.3, $p < 0.05$; Fig. 7D]. At the genus level, Bacteroides, Lactobacillus, and other unclassified bacterial strains were the main bacteria in the control and SME groups (Fig. 7E). The relative abundance of Lactobacillus, Prevotellaceae_UGG_001, Romboutsia,

Turicibacter, and Parabacteroides decreased in the SME group, whereas that of Akkermansia, Prevotellaceae_NK3B31_group, Parasutterella, and other unclassified bacterial strains was nonsignificantly higher increased in the SME group. However, most of these differences were not statistically significant ($p > 0.05$; Fig. 7F), except the between-group differences in the relative abundance of other unclassified bacteria [$F(2, 9) = 4.8$, $p < 0.05$; Fig. 7F].

Discussion

Considerable research attention has been directed to factors, particularly natural products, that can enhance the memory of older adults or prevent the development of cognitive deficits. Soybean meal is a cheap, readily available source of bioactive health-promoting compounds [24–27]. We investigated the effect of SME on memory function in rats through the MWM test and evaluated its possible underlying mechanisms.

The MWM test is the most widely accepted model for evaluating hippocampal-dependent spatial learning and memory in rodents [34–35]. In the MWM test, a lower score in the escape latency is used as an index of enhanced spatial learning and memory [36]. In the present experiments, orally administered 50 or 100 mg/kg/day of SME for 14 days led to significantly decreased escape latencies compared with that in the control group. The shorter escape latencies suggest that the oral administration of SME plays a significant role in memory retention.

Memory formation and storage are tightly linked to synaptic plasticity [37, 38], which is regulated by numerous neurochemical alterations, including changes in neurotransmitter release [39, 40]. In synaptic terminals, neurotransmitter release can be regulated by multiple synaptic proteins, including SNAP-25, synaptobrevin, synapsin I, and syntaxin, which are involved in vesicle docking, priming, and triggering fast neurotransmitter exocytosis [41, 42]. The phosphorylation of these proteins by various protein kinases, such as ERK1/2, PKC, and CaMKII [43–45], increases the availability of vesicles in the active zone and, thus, increases neurotransmitter release [33, 46, 47], contributing to synaptic plasticity and memory formation and retention in the hippocampus [48–50]. In the present study, in the hippocampal nerve terminals of the SME group, (i) the protein levels of presynaptic proteins (synaptophysin, synaptotagmin, synaptobrevin, synapsin-1, and SNAP-25) and protein kinases (ERK1/2, PKC, CaMKII) were higher; (ii) the phosphorylation of these protein kinases was higher; (iii) the phosphorylation of synapsin I at ERK1/2-specific sites 4 and 5 and CMKII-specific sites 3 was higher; and (iv) number of synaptic vesicles was higher than the corresponding values of the control group. These findings imply that the increases in the levels of synaptic proteins and vesicles in the hippocampal nerve terminals may have contributed to increased neurotransmitter release and memory retention in rats in the MWM tasks. This speculation is supported by evidence showing that high levels of presynaptic proteins, including synaptophysin, SNAP-25, syntaxin, and synaptobrevin, are associated with higher cognitive performance and lower risk of dementia in older adults [51–53]. Nevertheless, how SME-induced increases in synaptic proteins and vesicles in the hippocampal nerve terminals lead to memory preservation requires further research.

Several studies have indicated that the memory ability is associated with alterations in gut microbiota, usually in microbiota richness and diversity [54, 55]. In the present study, SME rats exhibited an increase in the alpha-diversity of gut microbiota. Alpha-diversity reflects the richness, evenness, and diversity of species of gut microorganisms. Thus, SME may help maintain better health flora by increasing the richness and diversity of gut microbiota in rats. With respect to the gut microbiota composition, phyla Bacteroidetes and Firmicutes are the predominant divisions in the gut flora [56]. In our study, the number of phyla Bacteroidetes was not significantly changed, but that of Firmicutes was decreased in the SME rats, thereby decreasing the F/B ratio. This was ac companied by an increase in the phylum Actinobacteria. Previous preclinical and clinical studies have demonstrated a decrease in the microbiota diversity and Actinobacteria content and a increase in the F/B ratio in AD and aging; thus, these alterations are related to cognitive decline [55, 57–63]. In our study, SME increased microbiota diversity and decreased the F/B ratio, leading to functional profile changes in the microbial community, which may have contributed to the improved memory retention. Further research is required to determine how intestinal flora alterations by SME affect memory function.

High concentrations of isoflavones and low concentrations of phenolic acids were detected in SME rats in the present study, which is consistent with previous findings [24, 26, 27]. A higher intake of polyphenols, including isoflavones and phenolic acids, has been linked to higher cognitive function in both animals and humans [64–68]. In the current study, polyphenols might have been played a role in memory retention in SME rats. Polyphenols exert a direct action on the brain by crossing the blood-brain barrier (BBB), and they also affect brain function by modifying the gut microbiota composition and functions [69, 70]. Short-chain fatty acids and some metabolites produced by the gut microbiota can penetrate the BBB and affect neuronal function [71]. However, the interactions and relationships between polyphenols and gut microbiota and between gut microbiota and memory function are complex and warrant further research. In addition, SME is water-solubility, and our results revealed that it did not produce significant changes in the morphology of the liver and kidney in rats; this implies that SME administration may be safe. Thus, it has potential for use as a functional food ingredient.

Conclusion

Our study is the first to demonstrate that SME helps in memory preservation in rats. This beneficial effect might be due to the enhancement of presynaptic integrity and the modulation of microbiota composition, as summarized in the schematic (Fig. 8). Our findings imply that soybean meal has potential as a food ingredient or supplement for preventing memory impairment. Future studies should investigate the effects of soybean meal or SME on human cognitive function.

Declarations

Acknowledgements : Not applicable.

Author contributions: KCH, HLL and TYH performed the experiments. CFH, HLL and TYH analyzed the results and edited fgures. SJW wrote preliminary draft of the manuscript. SJW and CFH designed experiments and revised the manuscript. All authors approved fnal version of the manuscript.

Funding: This research was funded by grant MOST 109-2320-B-030-009 from the Ministry of Science and Technology and Taiwan, and grant TYAFGH-A-110004 from the Taoyuan Armed Forces General Hospital in Taiwan

Data Availability: The data that support the fndings of this study are available from the corresponding author upon reasonable request.

Ethics Approval: Animal studies were approved by the Animal Care Committee of Fu Jen Catholic University.

Consent to Participate: Not applicable.

Consent for Publication: Not applicable

Conflicts of interest The authors declare no competing interests.

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Figures

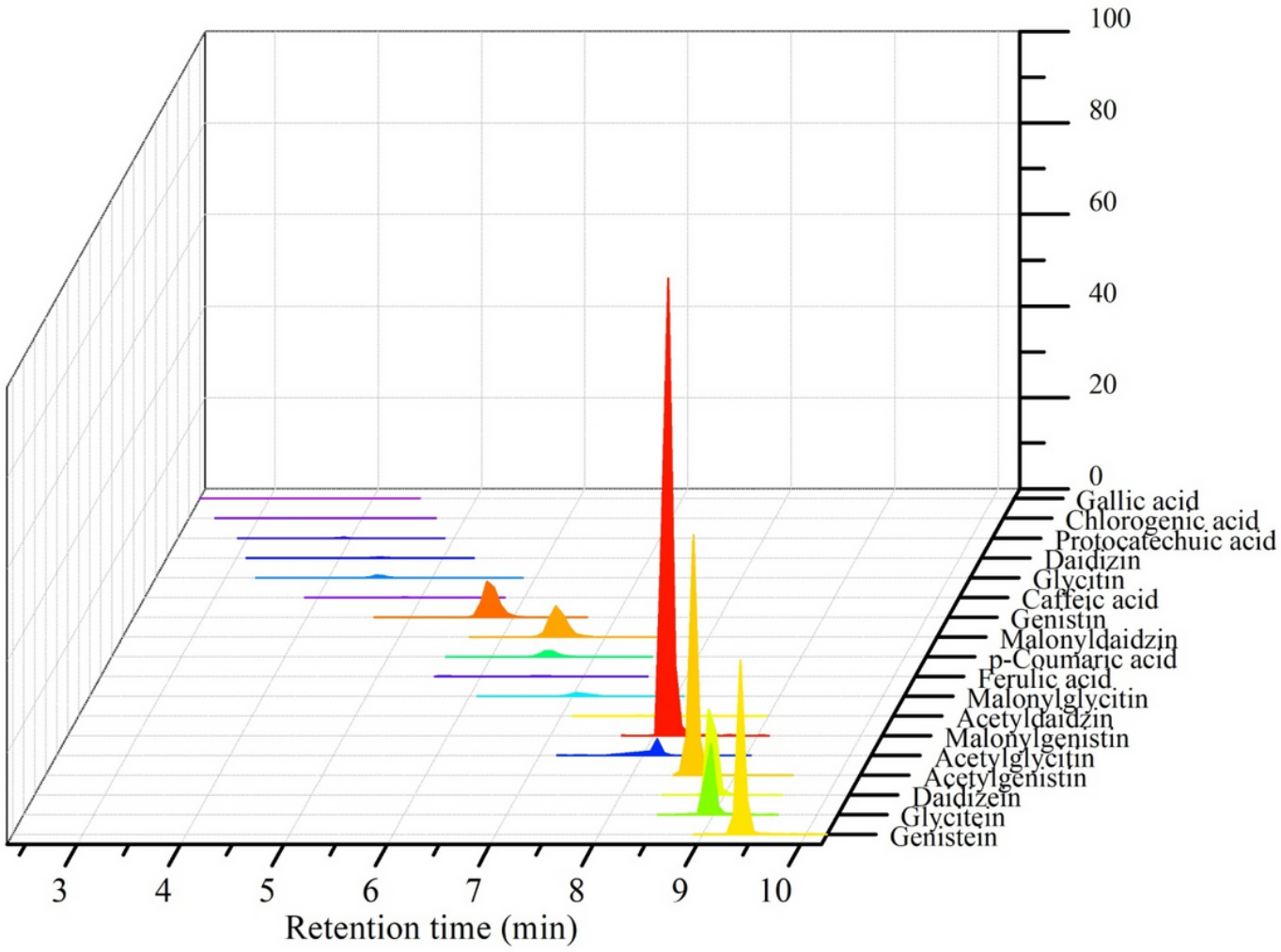

Figure 1

Chromatogram of 20 fold dilution SME. Graphe is expressed in relative intensity

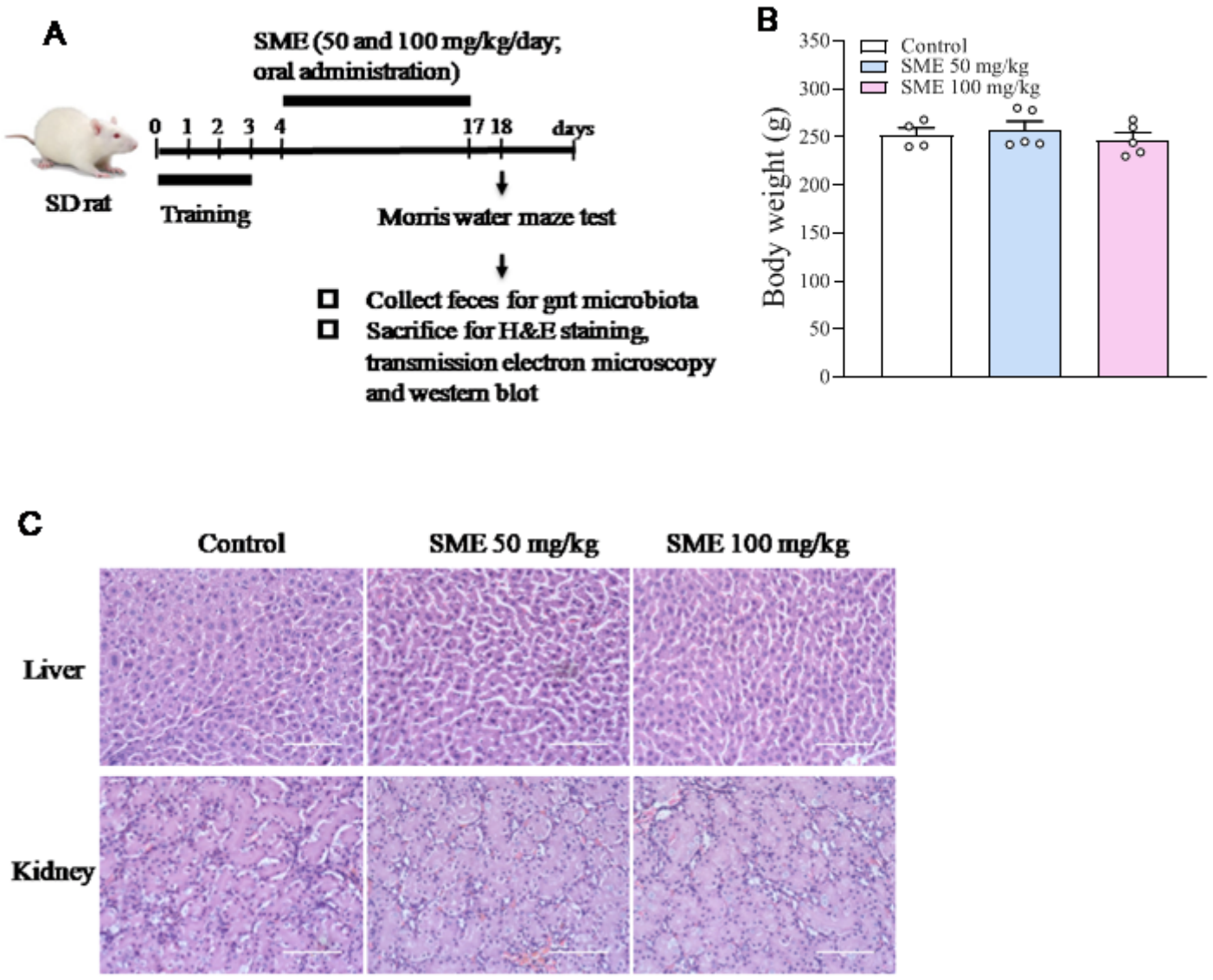

Figure 2

Effects of orally administration of SME on body weight, liver, and kidney in rats. (A) Timeline of experimental studies. The body weight (B) and hepatic and renal toxicity (C) were assayed at the end of the experimental period. Liver or kidney morphology was examined by H&E staining (400 ×). Scale bars = 10 μm. Data are presented as mean ± SEM (n = 10 per group).

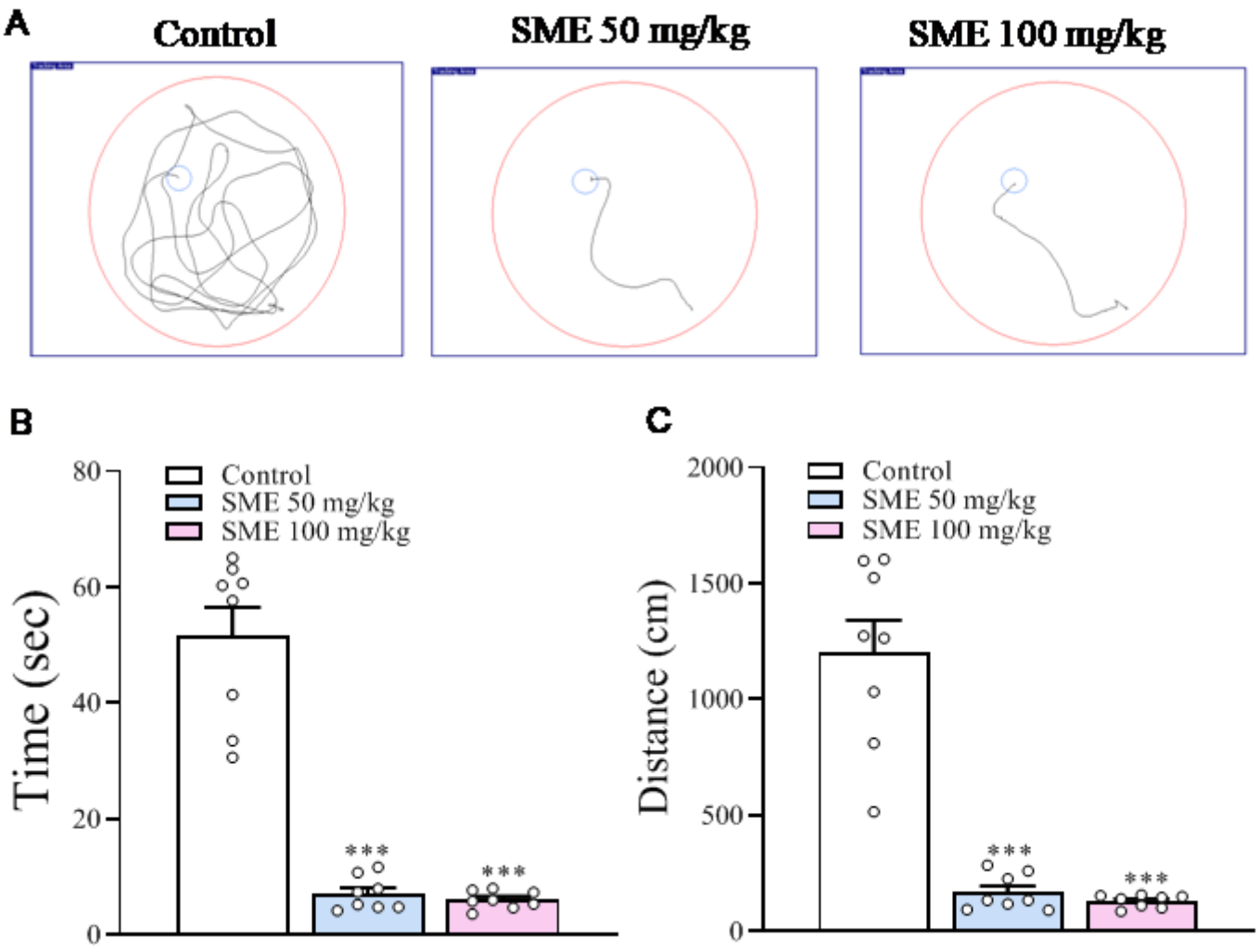

Figure 3

Effects of orally administration of SME on learning and memory of rats in a MWM. (A) MWM trajectories. (B) Time latency and (C) distance traveled to reach the platform. Data are presented as mean ± SEM (n = 8 per group). ***p < 0.001 compared with the control group.

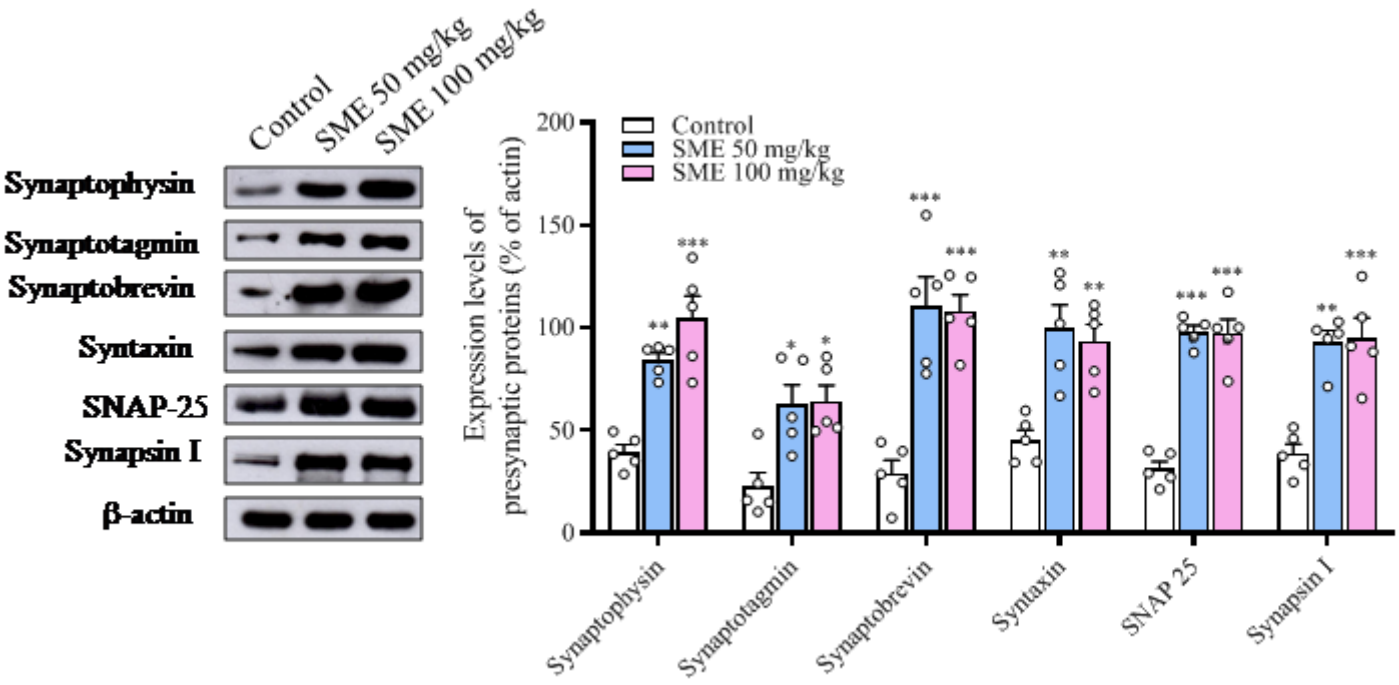

Figure 4

Effects of orally administration of SME on the expression levels of synaptic proteins in the hippocampal synaptosomes of rats. Western blot showing the expression levels of synaptophysin, synaptotagmin, synaptobrevin, syntaxin, synapsin-1, and SNAP-25 in the hippocampal synaptosomes for each group. Relative protein levels were quantified. Data are presented as mean ± SEM (n = 5 per group). *p < 0.01, **p < 0.01, ***p < 0.001 compared with the control group

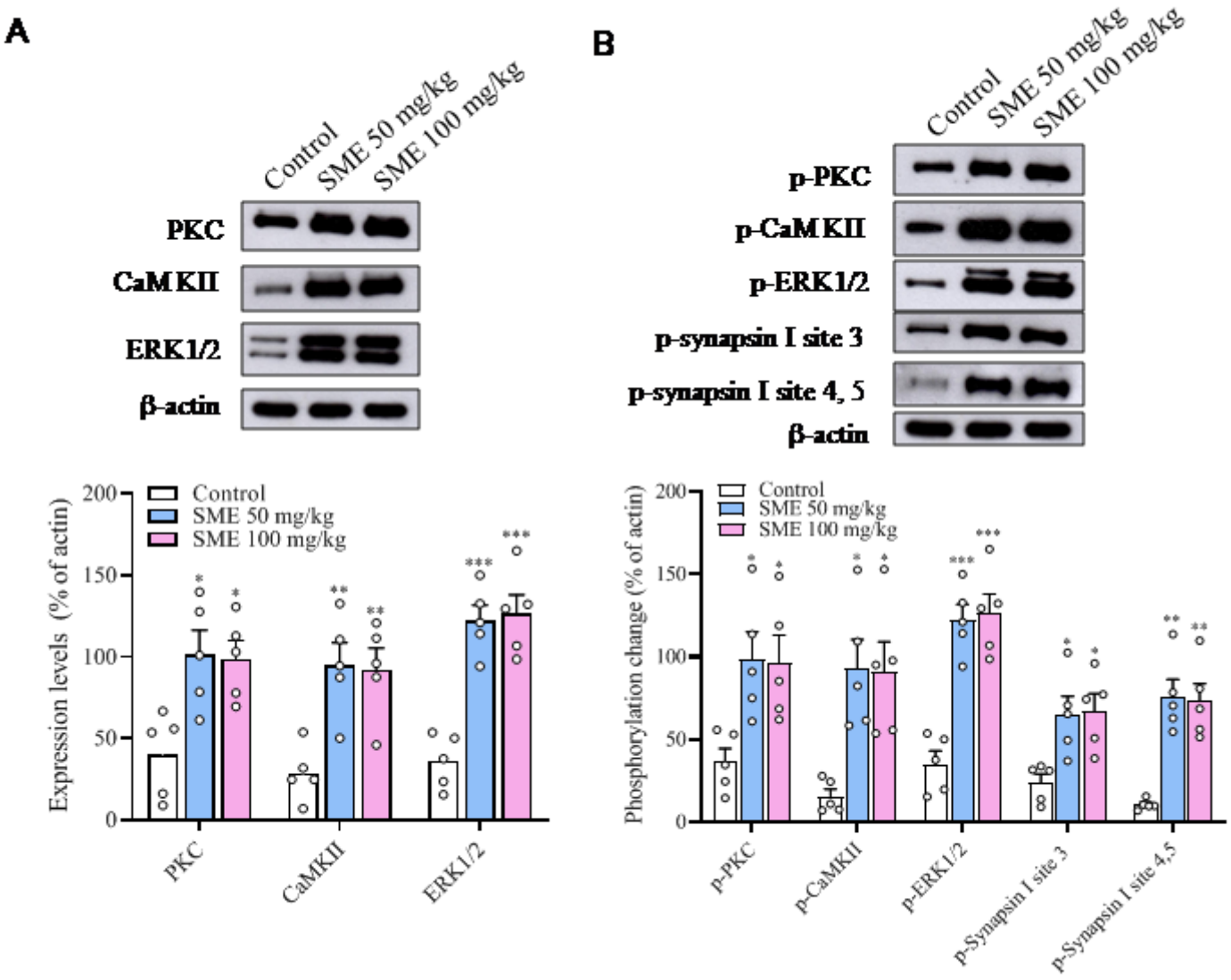

Figure 5

Effects of orally administration of SME on the levels of PKC, CaMKII, and ERK1/2 and their phosphorylated expression in the hippocampal synaptosomes of rats. (A) Western blot showing the protein expression of PKC, CaMKII, and ERK1/2 in the hippocampal synaptosomes for each group. (B) Western blot showing the phosphorylation levels of PKC, CaMKII, ERK1/2, and synapsin I site 3 and sites 4, 5 in the hippocampal synaptosomes for each group. Relative protein levels were quantified. Data are presented as mean ± SEM (n = 5 per group). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group

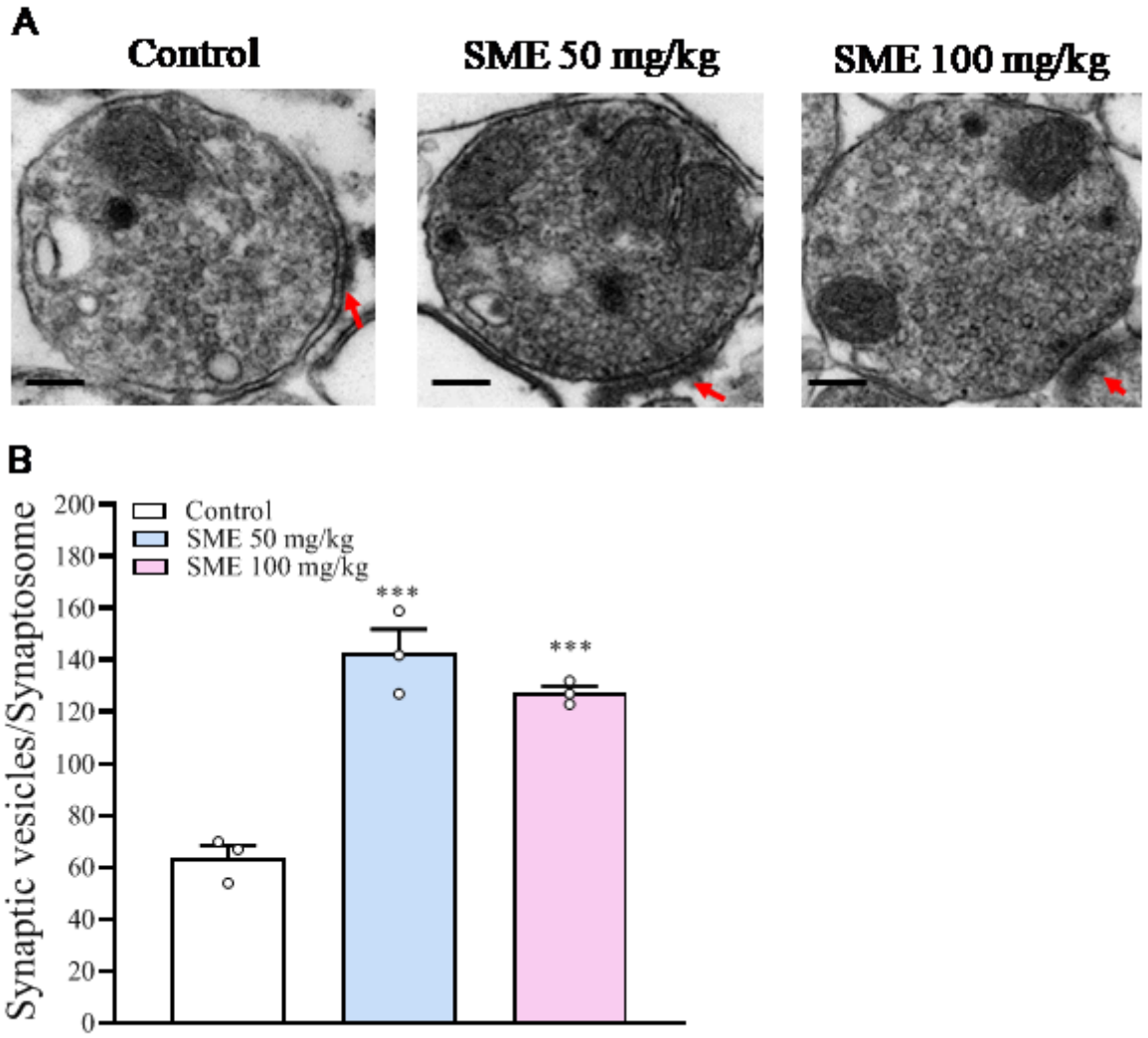

Figure 6

(A) Transmission electron micrographs of hippocampal synaptosomes. Each synaptosome contains mitochondrias, numerous synaptic vesicles, and a synaptic junction with postsynaptic density (arrowhead). Scale bar, 200 nm. (B) The number of synaptic vesicles in hippocampal synaptosomnes was counted. Data are presented as mean ± SEM (n = 3 per group). ***p < 0.001 compared with the control group.

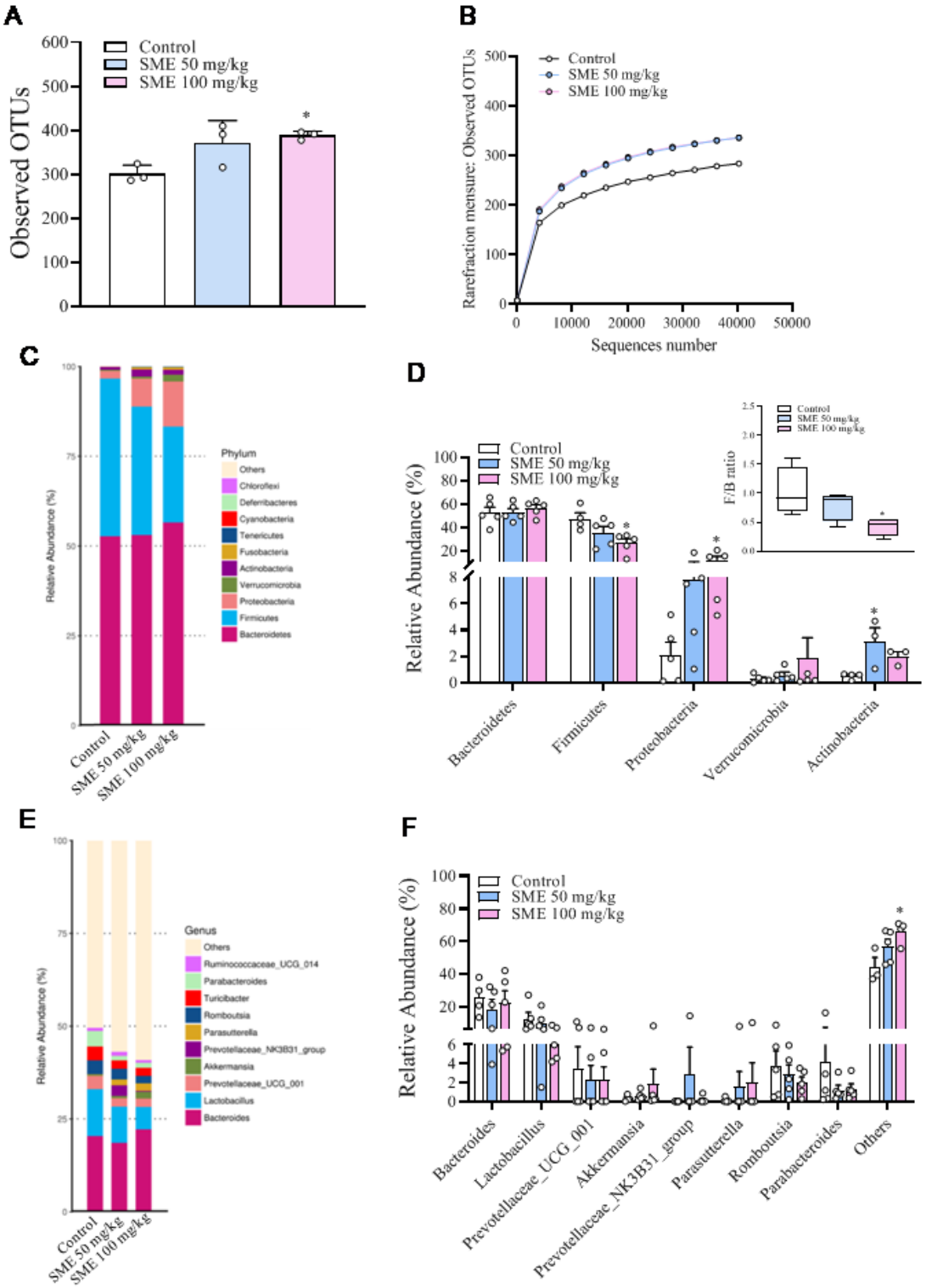

Figure 7

Effects of orally administration of SME on the gut microbiota in rats. (A) Alpha diversity. (B) Rarefaction curves. Relative abundance at the phylum (C) and genus (E) level.. Relative abundance of major bacterial OTUs in phylum (D) and genus (F) level. The ratio of Firmicutes to Bacteroidates (inset D). Data are presented as mean ± SEM (n = 3-5 per group). *p < 0.05 compared with the control group

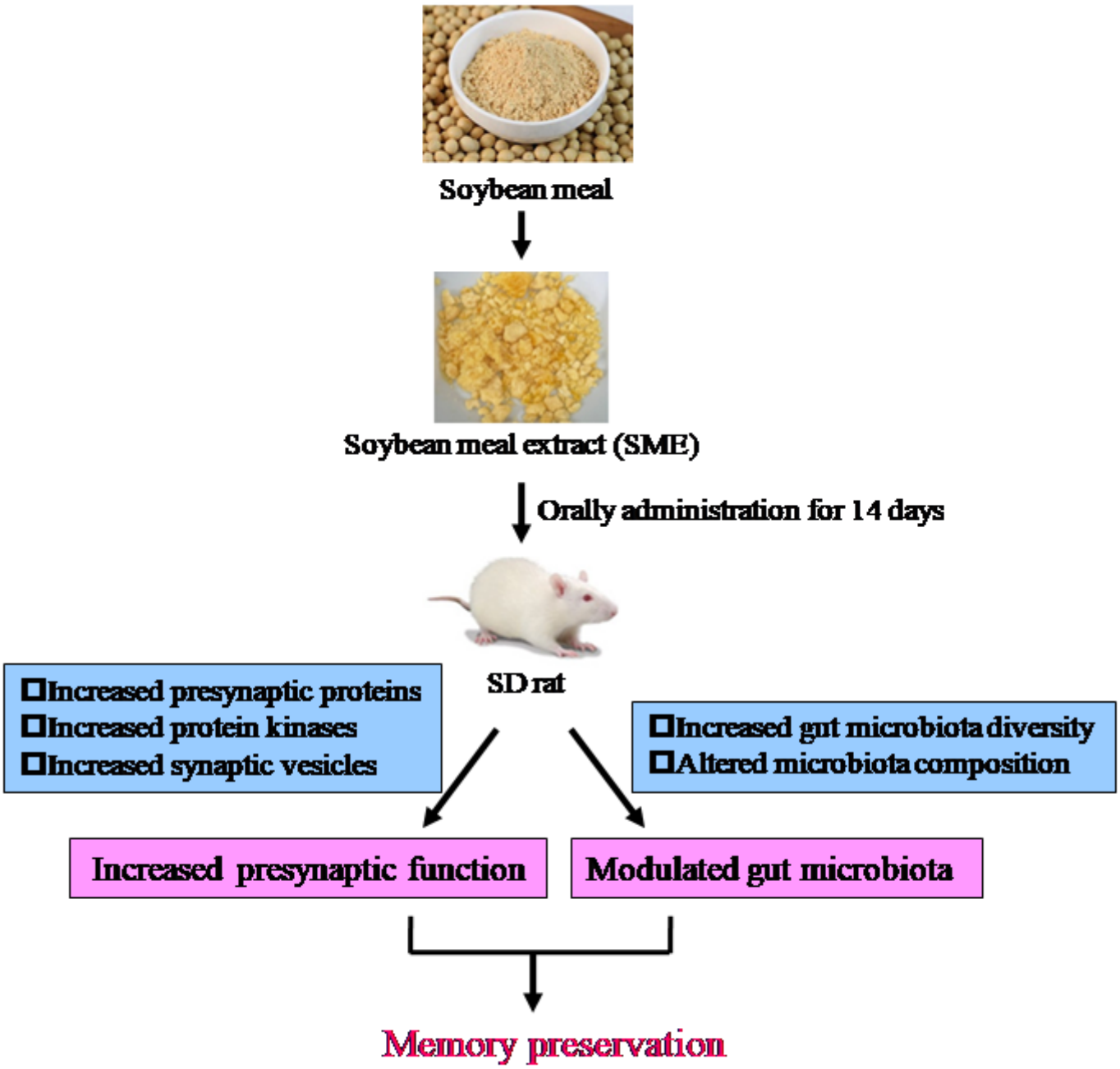

Figure 8

Suggested graphical representation of the possible mechanisms of memory retention by SME in rats. The effect of oral administration of SME on memory might result from the increased expression of synaptic proteins, phosphorylation of protein kinases, and number of synaptic vesicles in the hippocampal nerve terminals, thus enhancing neurotransmitter release and synaptic plasticity in the hippocampus of these rats. Further, the preservation of memory in the SME rats might be related to its modulatory effects on gut microbiota composition