**Doctoral Dissertation**

**(Shinshu University)**

# Neuroprotective and cognitive decline-suppression activities of fermented beverage-derived biomaterials

# （発酵飲料由来機能性素材の神経保護および認知低下抑制作用）

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**Table of Contents**

## ABSTRACT

The potential preventive effects of *Lactobacillus* strains isolated from rice wine lees (sake kasu) and fermented rice bioactive peptide on age-related cognitive decline were investigated in this doctoral dissertation.

Chapter 1 describes the introduction, background literature, and objectives. Aging is a risk for the deterioration of brain function and its main symptom is cognitive deficit. Many factors contribute to the onset of age-related cognitive decline. Oxidative stress is implicated to the pathology of age-related neurodegenerative disease. Aging is associated with the reduction of neurotrophins such as brain-derived neurotrophic factor (BDNF), which regulates not only neuronal development and survival but also long-term potentiation and synaptic plasticity. The dietary intervention has been proposed as an effective therapeutic approach to delay or prevent brain aging and memory impairments. Fermented foods and their by-products may exert potent effects on the brain and cognitive health promotion.

Rice is one of the main cereal crops and staple food for most of the world's population. In some Asian countries, rice is also utilized in the preparation of fermented food products such as Amazake (traditional fermented rice beverage in Japan) and rice wine (sake). In rice wine production, a large amount of residue called rice wine lees containing beneficial microorganisms is generated. However, the potential of rice wine lees as a viable source of probiotics with cognitive-enhancing effects is not yet investigated. The fermentation process allows the production of bioactive peptides from food protein with the aid of microbe's proteases. Several health benefits of Amazake have been reported; however, its neuroprotective effects have not yet been explored.

Chapter 2 deals with the evaluation of preventive effects of long-term dietary supplementation with *Lactobacillus* strains isolated from sake lees on cognitive decline in a mouse model of aging. Fourteen-week-old female senescence-accelerated mouse prone 8 (SAMP8) mice were fed for 43 weeks a standard diet containing 0.1% (w/w) *Lactobacillus casei* subsp. *casei* 327 (L. 327) or *Lactobacillus paracasei* K71 (L. K71). Results showed that age-related memory impairment was reduced in aged SAMP8 mice that were fed an L. K71-supplemented diet. L.K71 group had better cognitive performance compared with the control and L. 327 groups in Barnes maze and passive avoidance tests. L. K71 long-term administration resulted in increased BDNF protein expression and cAMP response element-binding (CREB) protein phosphorylation in the hippocampus. The levels of serotonin, which stimulates BDNF expression, were also elevated in the serum and brain tissue of L. K71-fed mice.

In Chapter 3, the effects of Amazake fermented rice peptides (FRPs) against scopolamine-induced memory impairment in mice were investigated. Mice were pretreated with FRPs (25 and 100 mg/kg body weight) via intraperitoneal injection for 7 days, followed by intraperitoneal injection of scopolamine. FRP pretreatment improved scopolamine-induced cognitive impairment in passive avoidance test. Compared with controls, the scopolamine-treated mice showed significantly decreased acetylcholine levels and increased acetylcholine-esterase activity in the hippocampus, which was reversed by FRP pretreatment. Scopolamine treatment significantly increased malondialdehyde (MDA) level and decreased superoxide dismutase (SOD) activity but these changes were suppressed by FRP pretreatment. Western blot analysis revealed that FRP treatment significantly attenuated the scopolamine-induced suppression of the protein expressions of BDNF and induced the phosphorylation of CREB protein and extracellular signal-regulated kinase (ERK) in the hippocampus. Among

the fractions separated by size-exclusion chromatography, the non-glycosylated peptide fraction of FRP suppressed H2O2-induced neuronal damage in SK-N-SH cells by upregulating BDNF expressions. Results indicated that FRP prevented memory impairment and that the underlying mechanism might involve regulation of the cholinergic systems and ERK/CREB/BDNF signaling pathway.

Lastly, Chapter 4 provides a summary and conclusion of the studies. Our findings suggested that long-term administration of a diet supplemented with *Lactobacillus paracasei* K71 isolated from rice wine lees and intraperitoneal administration of Amazake fermented rice peptide could alleviate age-related cognitive decline in mice models of aging by regulating signaling pathways associated to neuroprotection. Taken together, fermented rice beverage-derived biomaterials such as Amazake fermented rice peptides and *Lactobacillus paracasei* K71 isolated from sake lees might have beneficial effects in preventing age-related cognitive dysfunction and dementia among the elderly.

# CHAPTER 1

# INTRODUCTION

## 1. Background

Aging is an inevitable biological process which constitutes the progressive decline of mental and physical capacities of an individual. Memory impairment is one of the prominent pathological hallmarks of aging. Neurodegenerative diseases (e.g., Alzheimer's disease) are characterized by a progressive loss of cognitive abilities, which affects learning and memory function in daily activity. Alzheimer's disease (AD) results from the deposition of amyloid plaques, tau protein aggregation, cerebral oxidative stress, neuroinflammation and cholinergic dysfunction accompanied by psychological and pathophysiological complications such as anxiety, depression, concentration problems, and motor disturbances.[1] With an increasing number of aging populations, neurodegenerative diseases are recognized as major health concerns globally. Dementia is also responsible for the increased risk factor for illness and death among older people. Moreover, it is costly, in terms of the financial, personal and societal burdens.[2]

Dietary interventions have been identified as a potential means to delay or prevent the progression of human diseases including neurodegenerative diseases. A growing body of evidence indicates the potential of fermented foods as functional foods for the brain and cognitive health promotion. Fermented foods and beverages have traditionally been used all over the world for centuries and are one of the oldest and cheapest forms of food preservation. Functional fermented beverages have attracted attention due to the reported health benefits of bioactive compounds present in these drinks. Fermentation enhances the sensory quality and nutritional value of foods. The process also increases the number of beneficial microorganisms which are now termed as "probiotics" that helps in maintaining a healthy gut

flora. These microorganisms aid in the breakdown of complex macronutrients to simple products with desired properties and they also secrete many metabolites during the process.[3,4] Moreover, fermentation has also been proven as one of the strategies to produce bioactive peptides from food protein with the aid of microbes proteases.[5]

Rice is the staple crop for more than half of the world's population. However, in some Asian countries, white rice is also used in various food products and fermented beverage preparation such as rice wine (*sake*) and Amazake. Amazake, a traditional beverage made from fermented nonwaxy rice, is regarded as one of the nutritious fermented beverages in Japan. Steamed rice mixed with rice-*koji* (*Aspergillus* spp.) and water is heated to 55°C to 60°C, and enzymes breakdown the rice into simpler compounds, such as glucose, amino acids, and peptides.[6] The health benefits of Amazake include suppression of liver cirrhosis and anti-obesity and anti-hypertensive effects;[7,8] however, the neuroprotective effects of fermented-rice beverages, and particularly the associated bioactive peptides, have not yet been explored. Fermented products are a well-documented source of bioactive peptides. They can be generated by the proteolytic systems of starter during the manufacture of fermented foods. Microbial fermentation provides a natural technology applicable for the enrichment of fermented foods in bioactive peptides from animal or plant origin.[3] Consequently, the neuroprotective effects of bioactive peptides derived from fermented rice beverages have not yet been studied. Therefore, investigation of the potential neuroprotective roles of fermented rice peptides is timely and desirable.

Accumulating evidences showed that food-derived peptides have beneficial roles in neuroprotection and age-related cognitive decline. Oral administration of marine collagen peptide isolated from Chum Salmon (*Oncorhynchus keta*) skin by enzymatic hydrolysis enhanced brain-derived neurotrophic factors (BDNF) and PSD95 expressions in the

hippocampus of aged female C57BL/6J mice.[9] Chai et al. (2016) reported that peptides isolated from lantern fish (*Benthosema pterotum*) reduced $H_2O_2$-induced apoptotic cell death in human neuroblastoma SH-SY5Y cells and attenuated memory and learning deficiency of D-galactose-induced neurodegenerative/aging ICR mice via activation of intracellular antioxidant defense system and enhancement of BDNF expression. Soy peptide comprising of di- and tri-peptides suppressed cognitive decline by increasing the mRNA and protein expressions of BDNF and neurotrophin-3 (NT-3) levels in the brain of aged SAMP8 mice as well as the phosphorylated CREB protein level.[11] It has been demonstrated that antioxidant peptides could protect neuronal cells from oxidative damage through the induction of genes for antioxidant enzymes. Peptides extracted from defatted walnut meal, a main byproduct of walnut oil production, effectively suppressed $H_2O_2$-induced apoptosis in PC12 neuronal cells.[12]

Fermented rice products and by-products are viable source of beneficial microorganisms. In rice wine production, a large amount of residue called rice wine lees (*sake kasu*) is generated and often discarded as industrial waste after the juice or wine is extracted. The full potential of rice wine lees as a vaiable source of probiotics is not yet fully investigated. Therefore, studying the physiological effects of rice wine lees probiotics and bioactive compounds and utilizing it for functional foods or supplement development would be an attractive value-adding and recycling strategy for this waste product.

There is much compelling evidence documenting the probiotics' potential health effect of gut microbiota on brain function. Recently, there is growing interest in the potential beneficial effects of probiotics on behavior, mood, and mental health. Probiotics are commensal bacteria offering potential health benefit to the host when provided in adequate

amount. Among these bacteria, certain *Lactobacillus* and *Bifidobacterium* families have been shown to improve gut health, as well as mood disorders and stress-induced alterations.[13] Several studies have shown that ingestion of some probiotics could not only rescue stress-related disorders but also improve cognitive performance. Certain Bifidobacterial strains (*B. longum* 1714*, B. breve* 1205) are able to induce some positive effects on cognition in fear-related cognitive tasks, by decreasing anxiety in mice.[13] Administration of *Lactobacillus helveticus* NS8 in rat also improved chronic restraint stress-induced behavioral (anxiety and depression) and cognitive dysfunction.[14] Distrutti et al. (2014) showed that age-related deficit in long-term potentiation was markedly attenuated in rats that received VSL#3 which is a mixture of eight different strains of bacteria. Considering the modulation effects of some probiotics in cognition, we hypothesized that long-term consumption of some *Lactobacillus* species isolated from rice wine lees could also prevent age-related cognitive decline such as spatial learning and memory.

## 2. Review of Literature

### 2.1 Amazake as functional rice-based fermented beverage

Amazake is a traditional fermented rice beverage in Japan.[16] The name Amazake was derived from the two Japanese words "Ama" and "zake" means sweet and sake/wine, respectively. Although the preparations of Amazake is almost similar to that of sake (rice wine), this drink contains no alcohol because the fermentation condition is usually kept at higher temperature which prevents the proliferation of yeasts which is responsible for alcohol production.[6] Preparation of Amazake is very simple. Cooked rice is added with water and starter culture containing *Aspergillus oryzae* which produces enzymes that converts the starch to simple sugars such as maltose and glucose. Rice slurry is allowed to ferment and sweeten

at 55-60 $^{o}$C for 15-18 h.[4] It has a sweet and malty flavor and contains about 20% glucose. Its thick consistency is similar to yoghurt but the texture and thickness may vary. Aside from white rice Amazake, other variants such as brown rice, germinated brown rice or combination of the three are also available. Amazake with live microorganism (lactic acid bacteria) is recently developed and available in Japanese market. Amazake is being consumed as a snack, dessert, baby food, and salad dressing and is available in supermarkets, convenience stores, vendo machines, tea-houses, and even at shrines and temples during special occasions. Amazake is highly nutritious and is consumed for its claimed health benefits. Previous studies have shown that Amazake offers various health-promoting benefits including antioxidant, antiobesity, antihypertesnion and suppression liver cirrhosis.[6,16,17]

2.2 Effects of fermented rice products on brain function

Accumulating evidences indicate the potential of fermented foods as functional foods for the brain and cognitive health promotion. Among the cereal family, rice is one of the most popular ingredients used in the preparation of cereal-based fermented foods. They have been widely consumed in various fermented forms in Asian countries. Fermented foods contain many bioactive compounds that are absent in unfermented foods. Red mold rice (RMR), is fermented rice which acquired its color after being cultivated with the mold *Monascus purpureus*.[18] It has been used as fermenting agent or ingredient to improve the color and flavor of foods. The neuroprotective effect of RMR has been investigated and the ethanol extract of RMR showed protective activity in vitro and in vivo experiments.[19] RMR extract exhibited strong protection against β-Amyloid (Aβ) 40-induced neurotoxicity in PC-12. It rescued cell viability as well as repressed inflammatory responses and oxidative stress. In AD rats infused with Aβ40 into the cerebral ventricle, administration of RMR extract potently reversed the

cognitive dysfunction in the memory task and brain damage in the biochemical assay. Furthermore, RMR prevented Aβ fibrils formation and decreased Aβ40 accumulation in the hippocampus.[19] Treatment of RMR extract suppressed cholesterol-raised β- secretase activity and increased the neuroprotective soluble amyloid precursor protein (APP) R-fragment secretion in vitro and in vivo.[19,20] Other studies revealed that RMR administration has a protective effect in a Zn-deficiency model[21] and Parkinson's disease model.[22] The administration of RMR significantly ameliorated behavioral dysfunction and improved the activity of antioxidant enzymes including glutathione reductase, glutathione peroxidase, SOD, and catalase, which all lead to markedly reduced reactive oxygen species (ROS) production.[21] The antioxidant-containing *M. purpureus* NTU 568-fermented rice extract ameliorated 6-hydrodopamine (6-OHDA)-induced neurotoxicity in SH-SY5Y and the rat model of Parkinson's disease via oxidative and anti-inflammatory mechanisms, suggesting its potential therapeutic value for PD treatment. [22] The antioxidant-containing *M. purpureus* NTU 568-fermented rice extract ameliorated 6-OHDA-induced neurotoxicity in SH-SY5Y and the rat model of Parkinson's disease via oxidative and anti-inflammatory mechanisms.[22] The aliphatic hydroxamates from lovastatin, a secondary metabolite from monascus-fermented red mold rice also displayed a neurocytoprotective effects in 6-OHDA-treated nerve growth factor (NGF)-differentiated PC12 cells.[23] Rice vinegar is another fermented rice product popularly produced in many Asian countries such as China, Japan, Korea, and Vietnam.[24] Japanese vinegar (Kurozu) feeding suppresses cognitive dysfunction and brain amyloid accumulation in senescence-accelerated P8 mice.[25] Concentrated Kurozu increased mRNA expression of heat shock 70 kDa protein 1A (HSPA1A), a protein that stabilizes proteins against misfolding and aggregation, although the result was ambiguous in mice primary neurons. The expression of

HSPA1A may be associated with the decreased accumulation of aggregated proteins in the brain.[25]

2.3 Rice bioactive peptides

Bioactive peptides, as products of fermentation, chemical and enzymatic hydrolysis of diverse food proteins, are commonly composed of two to less than 100 amino acid residues.[26] Several bioactive peptides exhibit antioxidant, anti-obesity, anti-angiogenic, and anti-hypertensive activities.[27] At present, there are no reported studies on the potential health-benefits of peptides derived from rice wine lees despite its high protein content. However, several antioxidant peptides derived from rice grain and bran have been reported. Antioxidant peptides, Phe-Arg-Asp-Glu-His-Lys-Lys (959.5 Da), produced from enzymatic hydrolysis of defatted rice endosperm protein inhibited lipid peroxidation in a linoleic acid emulsion and enhanced the viability of t-BHP induced cytotoxicity of human embryonic lung fibroblasts (MRC-5) and mouse macrophage (RAW264.7) cells.[28] Using trypsin and pepsin-trypsin enzymatic system, bioactive peptides ranging from 800-2100 Da have been isolated from rice bran and displayed antioxidant activity in various *in vitro* assays.[29] A novel pentapeptide (Glu-Gln-Arg-Pro-Arg, 685.378 Da) was also isolated from rice bran and possessed cancer growth inhibitory properties on colon, breast, lung and liver cancer cells.[30] Antioxidant activity is one of the most studied and crucial biological roles of bioactive peptides. Antioxidant activity of bioactive peptides can be associated with their radical scavenging, inhibition of lipid peroxidation and metal ion chelation properties. It has been proposed that structure and amino acid sequence can affect the peptide antioxidative properties.[31]

2.4 Effects of food-derived peptides and fermented rice products on brain function

Recent studies have shown that food-derived peptides have beneficial roles in neuroprotection and age-related cognitive decline. Oral administration of marine collagen peptide isolated from Chum Salmon (*Oncorhynchus keta*) skin by enzymatic hydrolysis enhanced BDNF and PSD 95 expressions in the hippocampus of aged female C57BL/6J mice.[9] Chai et al. (2016) reported that peptides isolated from lantern fish (*Benthosema pterotum*) reduced $H_2O_2$-induced apoptotic cell death in human neuroblastoma SH-SY5Y cells and attenuated memory and learning deficiency of D-galactose-induced neurodegenerative/aging ICR mice via activation of intracellular antioxidant defense system and enhancement of BDNF expression. Soy peptide comprising of di- and tri-peptides suppressed cognitive decline by increasing the mRNA and protein expressions of BDNF and NT-3 levels in the brain of aged SAMP8 mice as well as the phosphorylated CREB protein level.[32] It has been demonstrated that antioxidant peptides could protect neuronal cells from oxidative damage through the induction of genes for antioxidant enzymes. Peptides (WSREEQEREE and ADIYTEEAGR) extracted from defatted walnut meal, a main byproduct of walnut oil production, effectively suppressed $H_2O_2$-induced apoptosis in PC12 neuronal cells.[12] Consequently, the neuroprotective effects of bioactive peptides from rice and rice-based products have not yet been studied. Therefore, investigation of the potential neuroprotective roles of rice-derived peptides is necessary.

2.5 Serotonergic system and cognition

The role of the serotonergic system in cognition is modulated by the activity and function of serotonin receptors (5HTR) classified into seven groups, which differ in structure, action, and localization. Many 5HTRs are located in the regions linked to various cognitive

processes. The serotonergic system influences behavior and cognitive function and serotonin receptors in various parts of the brain such as cortex, amygdala, and hippocampus play a key role in learning and memory.[33] Preclinical studies using animal and human studies have shown that alteration of serotonergic activity influence cognitive performance. Reduced serotonergic neurotransmission negatively influences cognitive functions and that normalization of serotonergic activity may have beneficial effects, suggesting that serotonin and its receptors represent important pharmacological targets for cognition enhancement and restoration of impaired cognitive performance.[34] Serotonin receptors have been widely studied and their involvement in cognition and memory have been reported. Converging evidence suggests that the administration of 5-$HT_{2A/2C}$ or 5-$HT_4$ receptor agonists or 5-$HT_{1A}$ or $5HT_3$ and 5-$HT_{1B}$ receptor antagonists attenuated memory impairment and promotes learning in situations involving a high cognitive demand. On the other hand, opposite effects on memory and learning was observed for receptor antagonists for 5-$HT_{2A/2C}$ and 5-$HT_4$, or agonists for 5-$HT_{1A}$ or 5-$HT_3$ and 5-$HT_{1B}$.[35]

Depletion of serotonin levels by manipulating tryptophan levels has been performed to investigate the role of serotonin in learning and memory.[34] Musumeci et al.[36] showed that chronic administration of a high-tryptophan diet increased the brain serotonin level and prevented the reduction of BDNF protein expression in the aged rat hippocampus and frontal cortex. It is observed that the detrimental effects of acute tryptophan depletion on working memory are more common in elderly group.[34]

2.6 Serotonin synthesis by gut microbiota

Approximately 90% of the serotonin in a human is synthesized in the gastrointestinal tract and serotonin acts locally to regulate gastrointestinal, cardiac, respiratory, and endocrine

functions, as well as crossing the blood-brain barrier.[37] Serotonin is derived from tryptophan by tryptophan hydroxylase followed by a decarboxylation by amino acid decarboxylase. Tryptophan is an essential amino acid. Tryptophan can be obtained from dietary or microbial sources.[38] There is both direct and indirect regulation of tryptophan and serotonin in the gut by the resident microbiota. Indirect regulation of tryptophan availability and serotonin formation by the gut microbiota is primarily via the kynurenine pathway.[39] Serum concentrations of serotonin is significantly reduced in germ-free mice raised in the absence of microbial colonization, compared to specific pathogen-free or conventionally-colonized mice.[40,41] It becomes clear that the microbial influence on tryptophan metabolism and the serotonergic system may have an important role in such regulation.[42] Germ-free mice have lower levels of tryptophan, serotonin, and indoles than conventionally raised or colonized with human microbiota animals.[43] Tryptophan is synthesized from chorismate by members of several bacterial phyla including Proteobacteria, Actinobacteria, and Firmicutes through enzymes whose genes are encoded in complex operons.[44] *Bifidobacteria* and *Escherichia coli* can synthesize chorismate, a tryptophan precursor, which acts as a branch-point for many microbial metabolic pathways.[44] The indigenous spore-forming bacteria from the mouse and human gut microbiota promoted serotonin biosynthesis in colonic enterochromaffin cells (EC) and modulated serotonin concentrations in both the colon and blood. They demonstrated that specific microbial metabolites are elevated by the spore-forming bacteria, signaling enterochromaffin cells to increase serotonin synthesis.[45]

2.7 Gut microbiota indirectly modulates serotonergic system in the brain

The indigenous microbiota has been reported to modulate the levels of serotonin in the brain, indicating a role in regulating the brain serotonergic system.[46] The brain-gut axis is a

bidirectional communication system between the central nervous system and the gastrointestinal tract. Serotonin functions as a key neurotransmitter at both terminals of this network.[38] Previous studies pointed out the critical role for the gut microbiome in regulating normal functioning of this axis. Insights about the gut-microbiome-brain axis can be well-explained by studies that used germ-free animals that are microbiota-deficient and are raised in a sterile environment. They have been used to determine that the influence of gut microbiota on normal brain development and behavior which involves tryptophan and serotonin metabolim.[42] Increased level of systemic tryptophan and decreased serotonin have been observed in germ-free animals.[46] However, significant decrease in the circulating level of tryptophan was observed when tryptophan metabolizing bacteria is introduced to their gut and this alteration has a pronounced effect on hippocampal serotonin concentrations in male germ-free animals.[46] Increased in plasma tryptophan is observed in germ-free animals and such increase is normalized upon colonization of mice right after post-weaning.[47] Male germ-free mice displayed significant changes in the central nervous system (CNS), showing increased hippocampal serotonin concentrations. These changes are irreversible even the subsequent normalization of circulating tryptophan concentrations is done with the introduction of a gut microbiota immediately post-weaning. Increased serotonin (5HT) turnover, as indicated by the 5-hydroxyindoleacetic acid (5-HIAA)/5HT ratio, is also evident in the striatum of germ-free.[48] Elevations in plasma levels of both tryptophan and serotonin in germ-free mice compared to conventional animals is also reported.[49] Plasma 5-HT levels are thought to arise mainly from the ECs of the gut.[37] Interestingly, there may be a temporal effect of the colonization process. Elevated tryptophan concentrations in germ-free mice are reduced four days following the introduction of a microbiota but not at day 30.[50] Recent study demonstrated that germ-free rats have lowered hippocampal 5HT levels but showed a similar stress-induced elevation in both

5HT and 5HIAA intermediate to their conventionally raised counterparts.[51] The modulating effects of the gut microbiota on the CNS serotonergic system is not only limited to sterile animals. Administration of the probiotic *Bifidobacterium infantis* to rats resulted in reduced 5-HIAA concentrations in the frontal cortex accompanied by a marked increase in plasma concentrations of tryptophan and kynurenic acid.[52] A balance is needed between bacterial utilization of tryptophan and the tryptophan necessary for serotonin synthesis in both enteric and central nervous systems.[42]

2.8 Probiotics and neurodegenerative disease

Aging is associated with declining cognitive performance as well as physical and social changes in human. With an increasing number of aging populations, dementia and mild cognitive impairment are recognized as major health concerns globally. Cognitive decline is also responsible for the increased risk factor for illness and death among older people. A deficit in synaptic plasticity is one of the many changes that occurs with age. The intestinal microbiota is increasingly recognized as a complex signaling network that impacts cognitive functions including learning, memory and decision-making processes aside from modulating the enteric system.[14] This has led to the idea of a gut-microbiota-brain axis, highlighting a bidirectional interaction between the central nervous system and the intestine. Recently, there is growing interest in the potential beneficial effects of probiotics on behavior, mood, and cognitive functions. Probiotics are beneficial microorganisms offering potential health benefit to the host, when provided in adequate amount and they actively interact with the endogenous microbiota.[53]

Certain Bifidobacterial strains (*B. longum* 1714*, B. breve* 1205) are able to induce some positive effects on cognition in fear-related cognitive tasks, by decreasing anxiety in mice.[13]

Administration of *Lactobacillus helveticus* NS8 in rat also improved chronic restraint stress-induced behavioral (anxiety and depression) and cognitive dysfunction by restoring hippocampal serotonin (5-HT) and norepinephrine (NE) levels, and upregulating the hippocampal BDNF mRNA expression in the brain of chronic stress rats.[14] Disturri et al.[15] demonstrated that age-related deficit in long-term potentiation (LTP) may be attenuated by changing the composition of intestinal microbiota with VSL#3, a probiotic mixture comprising 8 Gram-positive bacterial strains. VSL#3-treated aged rats showed a decrease in microglial activation markers and an increase in expression of BDNF and synapsin, demonstrating that intestinal microbiota can be manipulated to positively impact on neuronal function. Recent studies demonstrated that the promising effects of probiotic supplements on age-related disorders are due to their antioxidant and anti-inflammatory properties and neurotrophic factors modulating activity.[54] Meanwhile, Huang et al.[54] reported that *Lactobacillus paracasei* PS23 (LPPS23) delayed the progression of age-related cognitive decline in SAMP8 mice model by increasing the anti-oxidative enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) tumor necrosis factor (TNF)-α and monocyte chemotactic protein-1 (MCP1) and lowering the levels of interleukin (IL)-10 in the brain of LPPS23 fed mice. AD is a progressive and irreversible neurodegenerative disease that results in gradual cognitive deficits and eventually leads to dementia.[55] Currently, there are few studies reported linking probiotics to cognitive decline prevention. In a randomized, double-blind, and controlled clinical trial conducted by Akbari et al.[56], AD patients supplemented daily with milk containing *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium bifidum,* and *Lactobacillus fermentum* for 12 weeks showed a significant improvement in metabolic status and cognitive function as indicated by higher mini-mental state examination score (MMSE) of the treated patients compared to the control group.[56] Similarly, Kobayashi et al.[55] investigated the effects

of oral administration of *Bifidobacterium breve strain* A1 (*B. breve* A1) on behavior and physiological processes in AD model mice. They found that administration of *B. breve* A1 to AD mice prevented cognitive dysfunction as shown by Y maze and passive avoidance memory tests. They postulated that non-viable components of the bacterium or its metabolite acetate partially ameliorated the cognitive decline observed in AD mice and suppressed the hippocampal expressions of inflammation and immune-reactive genes that are induced by amyloid-β. The same group did an open-label, single-arm study to examine the effects of 24-week supplementation of *B. breve* A1 on human subjects. After 24-week of oral supplementation of *B. breve* A1, MMSE scores as well as POMS2 and GSRS rating were significantly increased in elderly patients with mild cognitive impairment. However, they further recommended a randomized, double-blind placebo-controlled studies to confirm the beneficial effects of *B. breve* A1 in managing cognitive function in mild cognitive impairment.[57] Thus, it is also anticipated that other *Lactobacillus* species probiotics may also prevent age-related cognitive decline.

2.9 Evaluation of neuroprotection and cognitive function

The pathogenesis of cognitive decline has many causes. Cholinergic dysfunction in the brain, including hydrolysis of acetylcholine from increased acetylcholinesterase (AChE) activity, is one of the indicators of neurodegenerative diseases.[58] Disturbance of the cholinergic system also disrupts hippocampal neurogenesis by modulating the mechanism involving BDNF and cyclic adenosine monophosphate response element-binding protein (CREB).[59] Oxidative stress promoted neuronal cell death and may cause cognitive impairment. Brain is highly susceptible to oxidative stress because of its high demand for oxygen. Accumulation of Aβ and phosphorylated tau protein in the brain is another possible reason of cognitive

dysfunction.[60] Increased Aβ formation and tau hyperphosphorylation are known to cause memory impairments in neurodegenerative diseases such as Alzheimer's disease.[60] These biomarkers for evaluating neuroprotection and neurodegeneration models could be used as indirect indicators of cognitive function.[61] To initiate neurodegeneration, oxidative injury leading to neuronal death is induced by neurotoxic chemicals such as glutamate, Aβ, hydrogen peroxide ($H_2O_2$) and others in cellular models. $H_2O_2$ can cause oxidative insult and apoptosis in neuronal cell types. On the other hand, human neuroblastoma SH-SY5Y cells are often used as an in vitro model in studies of neurodegenerative diseases, because the cell behaves similarly to mature human neuron when exposed to ROS.[62] In animal models, intracerebroventricular injection of Aβ and intraperitoneal injection of scopolamine and D-galactose are widely used to induce cognitive impairment. D-galactose induces aging-inducible oxidative stress in vivo, which resembles the natural aging process in mice. Hence, D-galactose treated animals have been widely studied as an ideal animal model of memory impairment for studying the molecular mechanisms involved in aging and age-associated neurodegeneration.[63] Various biomarkers are examined in these neurodegenerative models before and after treatment of neuroprotective agents. Cell viability, acetylcholine esterase level, neurotrophic factors, expression of antioxidant-related proteins such as SOD and catalase, and inflammatory cytokines are commonly measured both in cell culture and animal model to investigate neuroprotective effects.[64] Memory test has been evaluated by established behavioral tests to measure cognitive performance. Morris water maze, Barnes maze, passive avoidance Y maze test and novel object recognition tasks are among the memory and behavioral tests used to assess cognition.[65] In clinical studies, cognitive function of human subjects is usually evaluated by cognitive assessment tools. Neuroprotection in humans can be evaluated by measuring oxidative stress or other biomarkers from peripheral blood and urinary samples. In some cases,

antioxidant activities such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and $H_2O_2$ scavenging activity can be assessed to identify neuroprotective agents.[62]

## 3. Objectives

The main aim of this research was to evaluate the neuroprotective effects and cognitive decline-suppressing property of probiotics and bioactive peptides derived from fermented rice beverage and by-product. Specifically, the effect of long-term diet supplementation with *Lactobacillus* strains in preventing cognitive decline was evaluated using SAMP8 mice. The preventive effects of Amazake-derived peptides against scopolamine-induced memory impairment in mice was investigated as well as the neuroprotective effects of active peptide fractions against $H_2O_2$-induced oxidative stress using SK-N-SH neuronal cells.

## CHAPTER 2

## Long-term diet supplementation with *Lactobacillus paracasei* K71 prevents age-related cognitive decline in senescence-accelerated mouse prone 8

### 1. Abstract

This study aimed to assess the suppressive effect of long-term diet supplementation with *Lactobacillus* strains on cognitive decline in the SAMP8 model. Fourteen-week-old female SAMP8 mice were fed for 43 weeks a standard diet containing 0.1% (w/w) *Lactobacillus casei* subsp. *casei* 327 (L. 327) or *Lactobacillus paracasei* K71 (L. K71), isolated from rice grains and sake lees, respectively. SAMP8 mice that were fed a L. K71-supplemented diet had better cognitive performance compared with the control and L. 327 groups in the Barnes maze and passive avoidance tests. Long-term administration of L. K71 resulted in increased BDNF protein expression and cAMP response element binding protein phosphorylation in the hippocampus. The levels of serotonin, which stimulates BDNF expression, were also elevated in the serum and brain tissue of L. K71-fed mice. These results suggest that prolonged intake of a diet supplemented with a *Lactobacillus* strain derived from sake lees may prevent age-dependent cognitive decline by upregulating BDNF expression in hippocampus.

## 2. Introduction

Probiotics are defined as living microorganisms that confer health benefits on the host when administered in adequate amounts. The intestinal microbiota converts dietary nutrients into biologically active metabolites affecting regulatory functions in the host. Probiotics help restore gut microbial diversity and its host-beneficial functions, resulting in amelioration or prevention of gut inflammation and other intestinal or systemic disease phenotypes.[66]

Recently, interest has been growing in the potential beneficial effects of dietary probiotics on behavior, mood, and mental health. In particular, with an increasingly aging population, the risk of illness or death caused by cognitive decline among older people has been increasing. Certain *Lactobacillus* and *Bifidobacterium* families have been shown to improve gut health, as well as alleviate mood disorders and stress-induced behavioral changes.[13] Several studies have shown that ingestion of some probiotics can not only rescue stress-related disorders but also improve cognitive performance. Several *Bifidobacterial* strains (e.g., *B. longum* 1714, B. *breve* 1205) can induce positive effects on cognition in fear-related cognitive tasks by decreasing anxiety in mice.[13] Administration of *Lactobacillus helveticus* NS8 in rats also ameliorated behavioral (anxiety and depression) and cognitive dysfunction induced by chronic restraint stress.[14] Distrutti et al.[15] showed that the age-related deficit in long-term potentiation was markedly attenuated in rats that received a mixture of eight different strains of bacteria. These findings suggest that daily intake of probiotics can improve cognitive functions.

Sake lees are byproducts of Japanese rice wine production; large quantities of this residue are discarded as industrial waste. Sake lees are also known as a viable source of beneficial microorganisms, including lactic acid bacteria. Recently, *Lactobacillus paracasei* K71 (L. K71) has been isolated from rice wine lees, and Saito et al.[67] reported that L. K71 has an immunomodulatory potential. Intake of a dietary supplement containing heat-killed L. K71 has

been reported to reduce the clinical severity of atopic dermatitis in a randomized controlled trial and enhance secretory immunoglobulin A release in the saliva. *Lactobacillus casei* subsp. *casei* 327 (L. 327) is another lactic acid bacterium discovered in rice grain. Consumption of heat-killed L. 327 was effective in improving skin conditions of healthy female volunteers.[68]

The senescence-accelerated mouse is an accelerated aging model established through phenotypic selection from a common genetic pool of the AKR/J strain.[69] The unique characteristic of SAMP8 mice is a low incidence of phenotypic changes accompanying age-related cognitive impairment.[70] Therefore, SAMP8 has been widely accepted as a good animal model to investigate the effects of environmental factors, such as food intake and exercise on age-related learning and memory deficits. In the present study, we investigated whether long-term diet supplementation with Lactobacillus strains isolated from rice or rice wine lees could attenuate spatial learning deficits and memory loss in aged SAMP8 mice.

## 3 Materials and Methods

### 3.1 Animals

Fourteen-week-old female SAMP8 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were housed in groups of four per cage and the animal room was maintained at a controlled temperature (20−23°C), humidity (40−70%), and with an alternating 12 h/12 h light-dark cycle (lights on at 8:00 AM). All experiments were performed in accordance with the animal experiment protocol approved by the Institutional Animal Care and Use Committee of Shinshu University (Permit No. 270076).

### 3.2 Animal protocol

Mice were divided into 3 groups: control (n = 12), *Lactobacillus casei subsp. casei* 327-fed group (L. 327; n = 12), and *Lactobacillus paracasei* K71-fed group (L. K71; n = 12). The control group was fed an AIN-93M diet (Oriental Yeast, Tokyo, Japan) only, and the L. 327 and L. K71 groups were fed an AIN-93M diet containing 0.1% (w/w) of the respective *Lactobacillus* strain. The mice were allowed free access to food and tap water. Food intake and body weight were recorded every week. At 54- to 57-week-old, all mice were subjected to the Barnes maze, passive avoidance, and Y-maze tests to assess their cognitive performance. Mouse feces were collected and all mice were sacrificed 3-5 days after the last memory test, and the blood, hippocampus, and cerebral cortex were collected, frozen in liquid nitrogen, and kept at −80°C until analysis.

### 3.3 Barnes maze test

The Barnes maze test was performed to assess spatial learning in SAMP8 mice. The maze consisted of a gray platform (90 cm in diameter) with 20 holes (5 cm in diameter) located 3 cm from the perimeter (Muromachi Kikai Co. Ltd., Tokyo, Japan). A black escape box (EB) was

placed under one of the holes. This circular platform was mounted on top of a steel stool, 90 cm above the ground, and balanced. Visual cues were placed on the walls (triangle and square signs) of the experimental room. The maze was divided into 4 quadrants (45, 90, 135, and 180°) in clockwise and counterclockwise directions from the EB (0°) position (Figure 1A). The animals interacted with the Barnes maze in 3 phases: habituation (1 day), training (4 days), and probe (1 day).

Habituation. Mice were allowed to move freely on the platform, placed in the center of the maze, and guided to the EB, where they remained for 2 min to familiarize themselves with the maze and hidden box.

Training. All mice received 3 trials per day for 4 days. During training sessions, the mouse was placed in the middle of the maze under a box chamber for 10 s and then allowed to freely explore the platform until either it entered the EB or 5 min had elapsed. Mice were allowed to stay in the escape box for 1 min before being returned to their cages after each trial, with an inter-trial interval of 30 min. If the mouse did not enter the EB, it was returned to the maze center, gently guided to the EB, and allowed to stay in it for 1 min. Guiding mice into the EB is important to show them that it exists.

Probe test. On the probe day, the EB was removed from the maze, and mice were placed in the center of the maze under a black chamber for 10 s. Each mouse was given 2 min to explore the maze and search for the EB. The mouse was returned to its holding cage immediately after the test. During the probe test, escape latency (time to enter the EB) and stay-in-the-hole time in each quadrant were recorded.

3.4 Passive avoidance test

The passive avoidance test was conducted using a step-through test cage (Muromachi Kikai Co. Ltd.) consisting of white and black compartments separated by a sliding door. In the training phase, each mouse was placed in the light compartment and allowed to explore for 10 s. The door was opened, and the step-through latency was recorded. After the mice entered the dark compartment, the door was immediately closed and a mild foot shock of 0.2 mA was applied for 3 s. Training sessions were conducted for 2 consecutive days. The probe test was performed using the same procedure without any shock. The step-through latency to enter the dark compartment was recorded. A maximum retention latency of 300 s was allowed for mice that did not enter the dark compartment.

3.5 Y-Maze test

The Y-Maze was a 3-arm maze with equal angles between all arms (Muromachi Kikai Co. Ltd.). Mice were individually placed at the center of the maze and allowed to move freely through the maze for 5 min. Spontaneous alternations (defined as consecutive entries into all 3 arms without repetitions, in overlapping triplet sets) were recorded. The total number of arm entries was collected during the test period. The alternation percentage was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries − 2) × 100.

3.6 ELISA measurement of serotonin levels

Blood samples were centrifuged at 1,000g for 15 min at room temperature, and the serum was collected and stored at −80°C until analysis. A portion of the brain sample was weighed and homogenized in stabilization buffer (0.05 N HCl with 0.1% ascorbic acid; 1:10 brain sample: stabilization buffer). The homogenate was centrifuged at 14,000g for 20 min at 4°C, and the supernatant was passed through a 0.45 μm centrifugal filter (Ultra-free-MC-HV, Merck

Millipore, Darmstadt, Germany) and Amicon Ultra 0.5 mL centrifugal filter unit (Ultracel-10K, Merck Millipore). Serotonin levels were measured in serum and brain supernatants using a Serotonin ELISA kit (ADI-900-175, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions.

3.7 Gene expression analysis by quantitative PCR (qPCR)

Total RNA and protein were isolated using Invitrogen TRizol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The RNA samples were converted to double-stranded cDNA using ReverTra Ace (Toyobo, Osaka, Japan). Quantification was performed with a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA) and a TP850 Thermal Cycler Dice Real time system (Takara, Shiga, Japan). The forward and reverse primers for each gene of interest are summarized in Table 1. Fold changes in the relative mRNA expression level for each gene were calculated using the $2^{-\Delta\Delta Ct}$ method, and the values were normalized to that of a housekeeping gene (Actb).

**Table 1.** Sequences of the primers used in qPCR.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| *Bdnf* | TAATGCAGCATGATGGGAAA | ACACTGAGGCCACAATCATGC |
| *Tph2* | GAGCAGGGTTACTTTCGTCCATC | AAGCAGGTCGTCTTTGGGTCA |
| *Maoa* | GAGGCTCCAATTTCAATCACTCTG | ATGTAGTTTAGCAAGTCGTTCAGC |
| *Maob* | AAGCGATGTGATCGTGGTGG | CAATGAGCCAAGTGAGCGAGA |
| *Actb* | AGTGTGACGTTGACATCCGT | TGCTAGGAGCCAGAGCAGTA |

3.8 Western blotting analysis

The concentrations of proteins extracted with TRizol reagent (Thermo Fisher Scientific, Inc.) were determined using Bradford's method with bovine serum albumin as a standard. Samples containing equal protein amounts and prestained molecular weight markers were separated by Tris-SDS-PAGE and transferred onto polyvinylidene fluoride membranes (0.45 μm, Merck Millipore). The membranes were blocked with 3% BSA in Tris-buffered saline with 0.05% Tween-20 for 1 h at room temperature, and incubated overnight at 4°C with the following antibodies: rabbit polyclonal anti-brain-derived neurotrophic factor (BDNF; 1:3,000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-cAMP response element binding protein (CREB; 1:2,000; Abcam), rabbit monoclonal anti-Ser133-phosphorylated CREB (pCREB; 1:2,000; Abcam), and mouse monoclonal anti-β-actin (1:5,000; Santa Cruz Biotechnology). Subsequently, the membranes were washed and incubated for 1 h at room temperature with secondary HRP-conjugated anti-rabbit (1:5,000; Santa Cruz Biotechnology) or anti-mouse antibodies (1:10,000; Santa Cruz Biotechnology). Chemiluminescence detection was performed using the EzWestLumi plus kit (ATTO, Tokyo, Japan) and AE-9300 Ez-Capture (ATTO). Densitometric analyses were performed using the public domain NIH Image Program, ImageJ.

3.9 Immunostaining analysis

Paraffin-embedded mouse brain sections were dewaxed using xylene and hydrated in ethanol at decreasing concentration. The sections were boiled in 10mM Tris/1mM EDTA buffer (pH 9.0) for 20 min and cooled down for 30 min at room temperature for antigen retrieval. The sections were washed two times with TBS solution. After one hour blocking with 5% BSA in TBS, the sections were incubated with antibody against BDNF (1:200, Abcam) overnight at

4°C. The slides were washed two times in TBS and incubated with the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG (H&L) (1:100, Abcam). After washing two times with TBS solution, the sections were mounted with immunoselect antifading mounting medium DAPI (Dianova, Hamburg, Germany) and examined under fluorescence microscope (EVOS fl; Advanced Microscopy Group, Bothell, WA).

3.10 Statistical analysis

The GraphPad Prism 5.0 software was used to perform statistical analyses. Data are represented as the means ± SEMs. Differences between the means were evaluated using ANOVA followed by the Bonferroni post hoc test for mean comparisons.

## 4. Results

### 4.1 Effect of long-term administration of *Lactobacillus* strains on spatial learning and memory in SAMP8 mice

The cognitive performance of SAMP8 mice was assessed after 43 weeks of feeding with diets supplemented with *Lactobacillus* strains isolated from rice and rice wine lees. Spatial learning and memory were evaluated using the Barnes maze test, performance in which is dependent on hippocampal functions. In this test, mice were trained to locate an EB hidden in one of the 20 holes located around the perimeter of an open circular platform (Figure 1A). To find the EB, mice must learn, memorize, and use the relationships among the visual cues in the room. After 4 days of training sessions, L. K71 mice exhibited significantly shorter escape latency in searching the EB during the probe test compared to the control and L. 327-fed group (Figure 1B). Moreover, the L. K71-fed group spent more time in the 0° hole, where the EB was located during the training phase (Figure 1C). A test for fear-motivated passive avoidance was also employed to evaluate associative memory in aged mice. In this task, memory performance is associated with the latency to enter a dark compartment where the mouse has been exposed to an electric shock. Therefore, the greater the latency, the better the memory retention. The step-through latency of the L. K71 group was significantly higher than those of the control and L. 327 groups (Figure 2A). Further, the short-term working memory of SAMP8 mice was evaluated with the Y-maze test. However, no significant differences were found in spontaneous alternation behavior among the mouse groups (Figure 2B). These results suggest that continuous and prolonged administration of the L. K71 strain, derived from rice wine lees, can attenuate cognitive decline in SAMP8 mice.

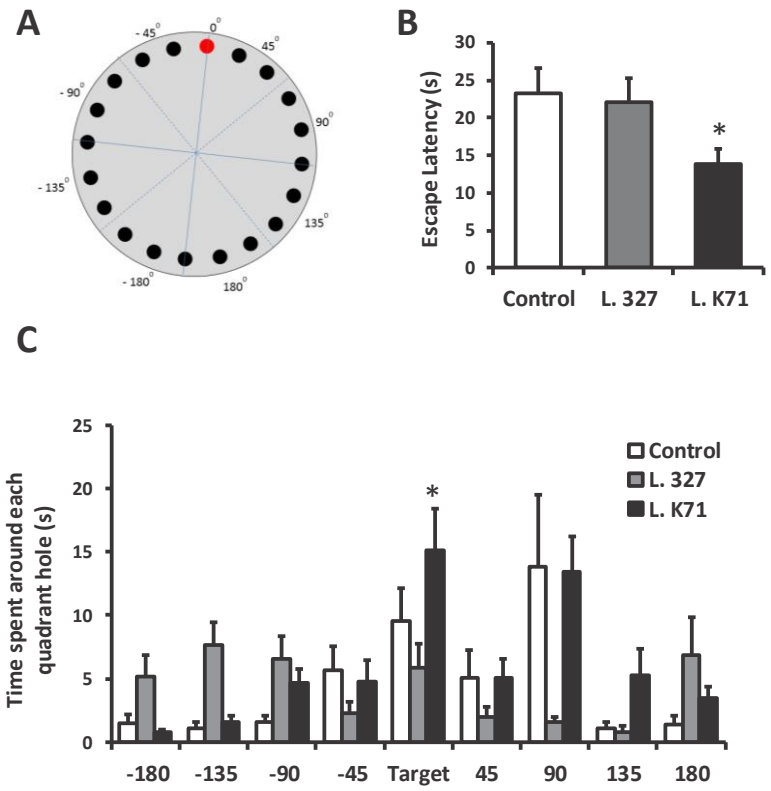


Figure 1. Effect of 43-week *Lactobacillus* strain administration on spatial learning and memory in senescence-accelerated prone 8 mice (SAMP8). (A) Barnes maze diagram used to interpret the data. (B) Escape latency during the probe test. (C) Time spent in each quadrant during the probe test. Data are presented as mean ± SEM; n = 10 mice per group; *p < 0.05 vs. the control group.

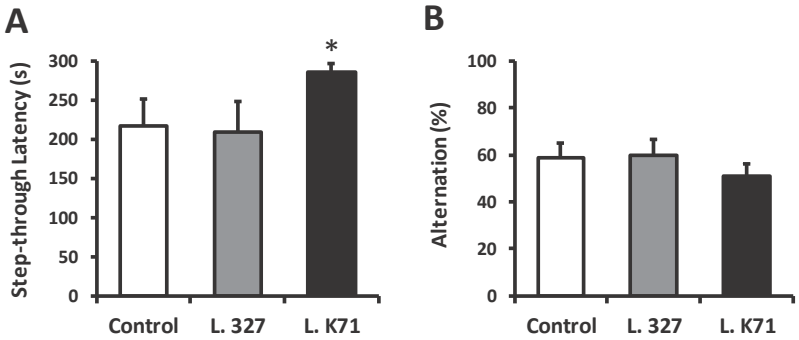


Figure 2. Effects of 43-week *Lactobacillus* strain administration on fear-motivated learning and short-term memory in SAMP8 mice. (A) Step-through latency in the passive avoidance test. (B) Spontaneous alternation behavior in the Y-maze test. Data are presented as mean ± SEM; n = 10 mice per group; *p < 0.05 vs. the control group.

4.2 Effect of *Lactobacillus* strain supplementation on serotonin levels in the blood serum and brain of SAMP8 mice

Since serotonin and its receptors play a vital role in cognitive functions including learning and memory, we then investigated the effect of long-term administration of a *Lactobacillus*-supplemented diet on the systemic and brain serotonin levels of SAMP8 mice. An ELISA analysis revealed that the serum serotonin level of L. K71-fed mice was significantly higher than that of the control and L. 327 groups (Figure 3A). A significant rise in the serotonin level was also observed in the brain extract of SAMP8 mice fed L. K71 (Figure 3B). While also increased in L. 327-fed mice, the levels of serotonin in both the blood serum and brain were not significantly different from those of the control group. We further investigated whether the observed differences in serotonin levels might be related to differences in food intake. However, the food intake and body weight dynamics did not differ among the groups throughout the feeding experiment.

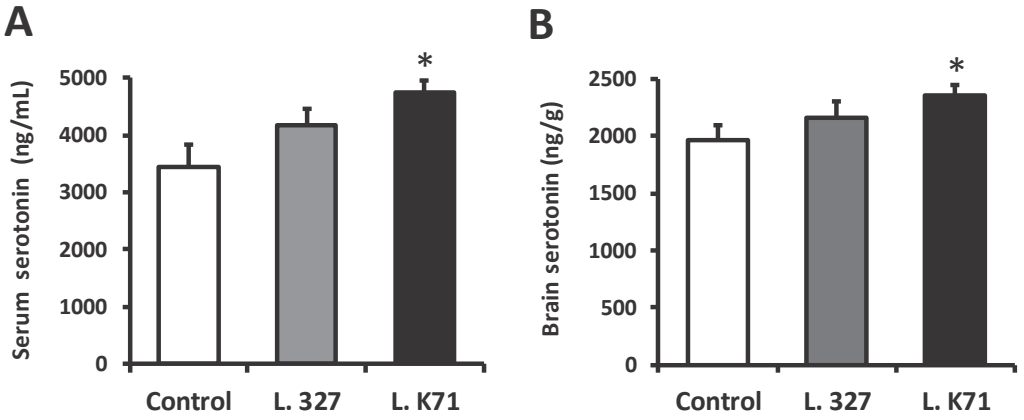


Figure 3. Effect of prolonged diet supplementation with *Lactobacillus* strains on the serotonin levels in the blood serum (A) and brain (B) of SAMP8 mice. Serotonin concentrations were measured by ELISA. Data are expressed as mean ± SEM; n = 8 mice per group; *$p < 0.05$ vs. the control group.

4.3 Effect of prolonged *Lactobacillus* supplementation on serotonin biosynthesis in the hippocampus and cortex

The effect of increased serotonin levels in the brain on serotonin synthesis and degradation enzymes was determined. The mRNA expression level of tryptophan hydroxylase 2 (TPH2), the rate limiting enzyme in serotonin biosynthesis, did not differ among the treatments. In contrast, the mRNA expression level of monoamine oxygenase A (MAO A), responsible for serotonin degradation, was significantly downregulated in the hippocampus of the L. K71 group (Figure 4A). The mRNA expression of monoamine oxygenase B isoform (MAO B) was slightly reduced in the L. K71 group, whereas no reduction in the Maoa and Maob mRNA levels was observed in the L. 327 group. In addition, a similar decrease in the Maoa mRNA level was also found in the cortex of SAMP8 mice after long-term supplementation with *Lactobacillus* strains (Figure 4B).

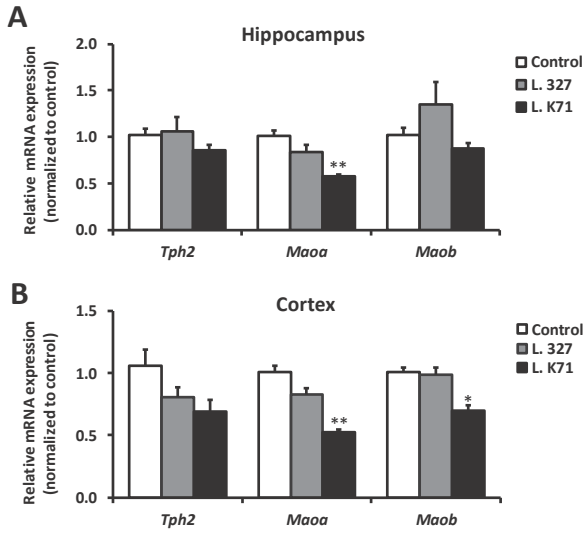


Figure 4. Expression of serotonin synthesis and degradation enzymes in the hippocampus (A) and cortex (B) of SAMP8 fed for 43 weeks with diets containing *Lactobacillus* strains. Data are expressed as mean ± SEM; n = 8 mice per group; *p < 0.05, **p < 0.01 vs. the control group. Tph2, tryptophan hydroxylase 2; Maoa, monoamine oxygenase A; Maob, monoamine oxygenase B.

4.4 Effect of *Lactobacillus* strain supplementation on BDNF expression in the hippocampus and cortex

Spatial learning and memory are dependent on the functions of the hippocampus and prefrontal cortex. Hence, we next investigated the effects of long-term administration of *Lactobacillus* strains on neuronal plasticity biomarkers, such as BDNF and CREB in the hippocampus and cortex. As shown in Figure 5A, L. K71-fed mice had significantly higher *Bdnf* mRNA levels in the hippocampus than the control group, whereas no significant increase was observed in the cortex. A similar increase was observed in BDNF protein expression (Figure 5B and 5C). Furthermore, L. K71-fed mice had higher BDNF staining within the CA3 region of the hippocampus compared to other groups (Figure 6). The expression of CREB and its activated form (pCREB) was also significantly upregulated in the hippocampus, but not in the cortex, of SAMP8 mice fed L. K71 (Figure 7A, 7B, and 7C). These results suggest that long-term supplementation of L. K71 can upregulate BDNF expression by activation of the transcription factor CREB in the hippocampus of SAMP8 mice.

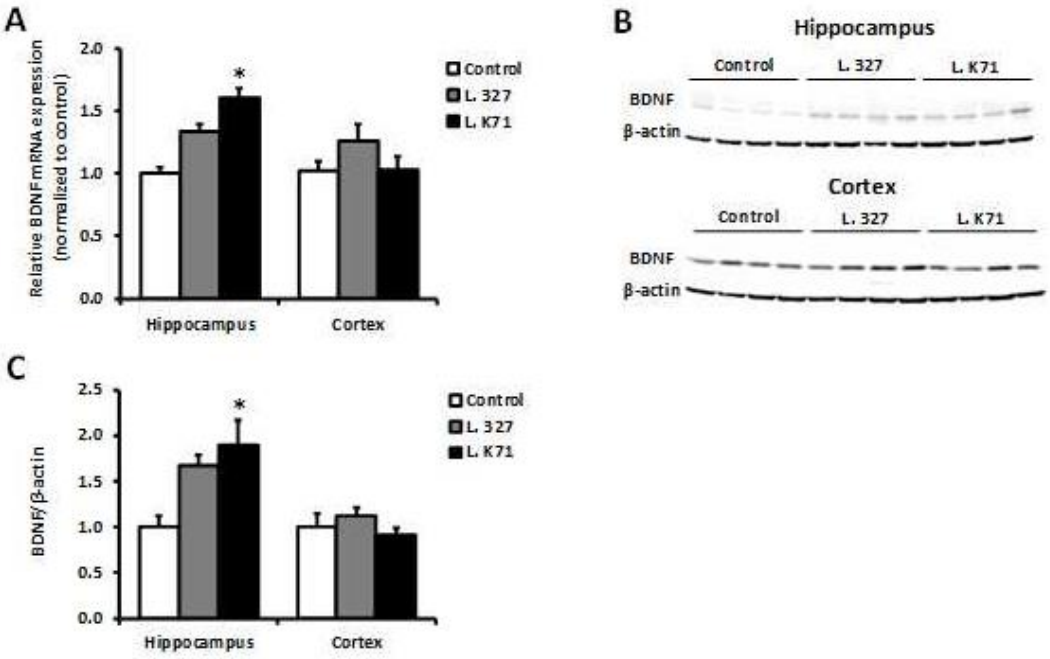


Figure 5. Effects of 43-week *Lactobacillus* strain administration on the expression levels of brain-derived neurotrophic factor (BDNF) in the hippocampus and cortex of SAMP8. (A) *Bdnf* mRNA levels measured by quantitative PCR (n = 8 mice per group). (B) Western blotting analysis (n = 4 mice per group) of the protein levels of BDNF. (C) Quantification of band intensities in (B). Data are expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$ vs. the control group.

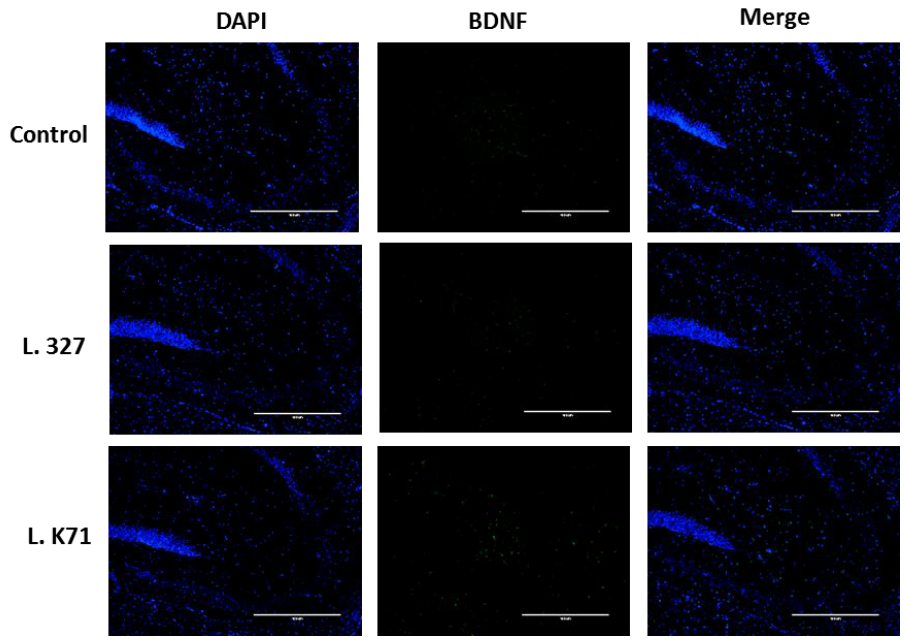


Figure 6. Immunofluorescence staining of CA3 region of hippocampus with BDNF antibody (green) and DAPI (blue). Scale bar: 400 μm.

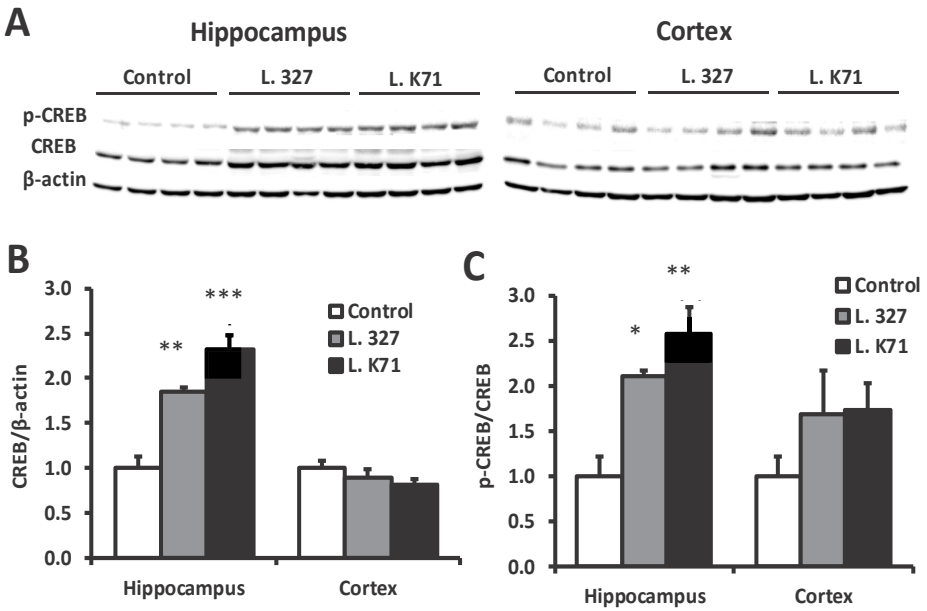


Figure 7. Effects of 43-week *Lactobacillus* strain administration on the expression levels of cAMP response element binding protein (CREB), and on CREB phosphorylation in the hippocampus and cortex of SAMP8. (A) Western blotting analysis (n = 4 mice per group) of the protein levels of CREB, and phosphorylated CREB (pCREB). (B-C) Quantification of band intensities in (A). Data are expressed as mean ± SEM, *p < 0.05, **p < 0.01,***p < 0.001 vs. the control group.

## 5. Discussion

The present study demonstrated that long-term administration of a diet supplemented with *Lactobacillus paracasei* K71, isolated from rice wine lees, could prevent age-related cognitive decline by upregulation of BDNF expression and serotonin levels in SAMP8 mice. Neurotrophic factors play key roles in neuronal development, differentiation, synaptogenesis, and survival in the brain.[71] BDNF belongs to the neurotrophin family, which has important functions in development as well as in neuronal plasticity in the adult. During development, BDNF acts as a signal for normal axonal growth[72] and is needed for the maturation and survival of different neuronal phenotypes.[73] BDNF is also involved in synaptic plasticity[74] and is crucial for cognitive processes.[75] Age-dependent cognitive decline is characterized by perturbations in neurotransmitter synthesis and dysregulation of neurotrophic factors such as serotonin and BDNF.[36] Therefore, sustained BDNF expression may be vital in preserving brain function during aging. In the present study, a significant increase in the gene and protein expression of BDNF was observed in an L. K71-fed SAMP8 group compared with animals fed a standard diet. The transcription of BDNF and other neurotrophins is tightly regulated by several intracellular signaling pathways and transcription factors, including CREB.[76,77] Taken together, these results suggest that the upregulation of BDNF expression by long-term consumption of a diet supplemented with L. K71 may be mediated by CREB and contribute to the preservation of neuronal plasticity and brain function. The results are in agreement with the findings of previous studies, such as those of long-term dietary supplementation with soy peptide[32] and green tea catechin.[78] As a brain neurotransmitter, serotonin is not only involved in mood and behavior control, and the pathophysiology of stress-related neurological disorders, but also regulates brain development and cognitive functions.[79] Indirect activation of norepinephrine and serotonin receptors by antidepressants can increase intracellular levels of cAMP and induce

CREB phosphorylation.[80] This mechanism may underlie the upregulation of BDNF expression in L. K71-fed mice: elevated brain serotonin levels may promote CREB phosphorylation, resulting in enhanced CREB-dependent transcription of target genes including *Bdnf*. Approximately 95% of serotonin in the body is produced in the gastrointestinal tract, and the remaining 5% is localized in the brain. Serotonin is synthesized from the essential amino acid tryptophan by 2 enzymes: tryptophan hydroxylase converts tryptophan into 5-hydroxytryptophan, which is decarboxylated by aromatic L-amino acid decarboxylase to produce serotonin.[81] On the other hand, monoamine oxygenases (MAOs) catalyze the degradation of monoamine neurotransmitters including serotonin, norepinephrine, dopamine, and other trace amines.[82] Both MAO A[83] and MAO B[84] isoforms increase in the brain during ageing. Moreover, the MAO B activity is also found elevated in Alzheimer's disease patient.[85] Therefore, selective inhibitors of MAOs are useful in the prevention of neurodegenerative disorders. The serotonin-degrading enzymes, particularly MAOA, was decreased in the hippocampus of SAMP8 mice fed L. K71; however, no changes were observed in the gene expression of a serotonin-synthesis-related enzyme. Tissue serotonin can be rapidly metabolized by MAO, with the A isoform having much greater affinity for the substrate compared to the B isoform.[86] Taken together, these findings show that L. K71 administration may suppress serotonin degradation without affecting serotonin synthesis in the brain.

Cognition is also affected by the activity and function of serotonin receptors. The 5-HT6 receptor is one of many serotonin receptors expressed in the hippocampus and associated with various cognitive processes. Preclinical studies of the 5-HT6 receptor revealed a role in the regulation of learning and memory.[87] However, no significant changes were found in the 5-HT6 receptor protein expression among the mouse groups in our experiments, suggesting that other serotonin receptors or combinations thereof are required for the regulation of BDNF

expression. Further study will be needed to identify the specific pathways involved in BDNF expression control following the enhancement of serotonin levels in the hippocampus.

Previously, the indigenous microbiota has been reported to modulate the hippocampal levels of serotonin, indicating a role in regulating the brain serotonergic system.[46] Previous studies have also shown the serum concentrations of serotonin to be significantly reduced in germ-free mice raised in the absence of microbial colonization, compared to specific pathogen-free or conventionally-colonized mice.[40,41] Recently, Yano et al.[88] reported that indigenous spore-forming bacteria from the mouse and human gut microbiota promoted serotonin biosynthesis in colonic enterochromaffin cells and modulated serotonin concentrations in both the colon and blood. They demonstrated that specific microbial metabolites were elevated by the spore-forming bacteria, signaling enterochromaffin cells to increase serotonin synthesis. Musumeci et al.[36] also showed that chronic administration of a high-tryptophan diet increased the brain serotonin level and prevented the reduction of BDNF protein expression in the aged rat hippocampus and frontal cortex. Thus, these findings suggest that specific microbiota can promote systemic serotonin production in the gut by enhancing the availability of tryptophan. Further studies will be necessary to reveal the underlying mechanisms of the increase in serotonin metabolism and the induction of neurotrophic factor expression in the brain.

## CHAPTER 3

**Fermented rice peptides attenuate scopolamine-induced memory impairment in mice by regulating neurotrophic signaling pathways in the hippocampus**

### 1. Abstract

This study investigated the preventive effects of fermented rice peptides (FRPs) against scopolamine-induced memory impairment in mice and their potential mechanisms. FRP pretreatment suppressed scopolamine-induced cognitive impairment in passive-avoidance test and significantly upregulated levels of BDNF and induced the phosphorylation of CREB protein and extracellular signal-regulated kinase (ERK) in the hippocampus of scopolamine-treated mice. Additionally, scopolamine-treated mice showed significantly decreased acetylcholine levels and increased acetylcholine-esterase activity in the hippocampus as compared with controls; however, these changes were suppressed by FRP pretreatment. Among the fractions separated by size-exclusion chromatography, the non-glycosylated peptide fraction of FRP suppressed $H_2O_2$-induced neuronal damage in SK-N-SH cells via upregulated BDNF levels. Results demonstrated that FRP prevented memory impairment, and that the underlying mechanism might involve regulation of the ERK/CREB/BDNF signaling pathway. These results suggest FRP as a potential agent for the prevention of age-related cognitive decline and dementia.

## 2. Introduction

As the population ages rapidly, cognitive impairment and dementia are being recognized as significant health problems worldwide based on their consideration as risk factors for illness and mortality among the elderly. Cognitive decline is related to alterations in the aging brain of levels of neurotrophic factors, such as BDNF, NGF, and NT-3, which play key roles in supporting the development, differentiation, maintenance, and plasticity of brain function.[89] Stimulation of intracellular signaling pathways is necessary for neurotrophic factors to elicit their respective effects. The CREB, a phosphorylated substrate of ERK, is critical for further stimulation of *BDNF* expression and the induction of several genes related to neuronal survival and cognition.[90,91]

Oxidative stress is another well-known causative factor involved in the pathogenesis of neurodegenerative disorders,[92,93] with excessive production of reactive oxygen species (ROS) and reactive nitrogen species resulting in damage to proteins, lipids, and nucleic acids in patients with these disorders.[94] Brain tissue is highly susceptible to oxidative stress due to its high oxygen consumption, elevated content of iron and polyunsaturated fatty acids, and low antioxidant capacity.[95] Moreover, the hippocampus and amygdala are more susceptible to oxidative injury, and extreme oxidative stress can lead to memory deficits by damaging hippocampal synaptic plasticity.[92]

Many studies report the different health-promoting effects of food-derived peptides; however, limited studies are available regarding their neuroprotective properties and capacity to suppress age-related cognitive decline. Recent studies demonstrated that peptides isolated from lantern fish (*Benthosema pterotum*),[10] chum salmon (*Oncorhynchus keta*) skin,[9] and soy peptides,[32] attenuate memory and learning deficiency in mice through activation of intracellular antioxidant-defense systems and upregulated hippocampal *BDNF* expression. Bioactive

peptides can be released by enzymatic treatment using various commercial enzymes that are usually expensive. By contrast, fermentation, regarded as an old and cost-effective method for food preservation, can naturally breakdown protein food components into smaller peptides, which in turn enhance the sensory quality and nutritional value of the product.[4,96] Moreover, fermentation represents a natural method for producing bioactive peptides from food protein with the aid of microbial proteases.[5]

Functional fermented beverages have attracted attention due to the reported health benefits of bioactive compounds present in these drinks.[4] *Amazake* is a popular Japanese fermented-rice beverage. This sweet drink is a non-alcoholic precursor to rice wine (*sake*) and undergoes a process almost identical to saccharification used for rice wine. Steamed rice mixed with rice-*koji* (*Aspergillus* spp.) and water is heated to 55°C to 60°C, and enzymes breakdown the rice into simpler compounds, such as glucose, amino acids, and peptides.[6] The health benefits of *Amazake* include suppression of liver cirrhosis and anti-obesity and anti-hypertensive effects;[7,8] however, the neuroprotective effects of fermented-rice beverages, and particularly the associated bioactive peptides, have not yet been explored.

Scopolamine is a potent muscarinic cholinergic receptor antagonist that causes damage in the cholinergic system, as well as in associated neurochemical cascades, resulting in cognitive decline.[97] Additionally, scopolamine initiates the formation of ROS and induces oxidative damage in the brain.[98] Therefore, scopolamine-induced memory impairment is a widely used model for the screening of drugs or compounds with cognitive-enhancing properties.[99] In the present study, the preventive effects of FRPs against scopolamine-induced memory impairment in mice was investigated. Moreover, the neuroprotective effects of FRP fractions against of $H_2O_2$-induced oxidative stress was evaluated using SK-N-SH neuronal cells and identified the peptide sequences by MALDI-TOF/TOF MS/MS analysis.

## 3. Materials and Methods

### 3.1 Materials

All chemicals used were of analytical grade and purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise specified. The fermented-rice beverage Amazake was kindly provided by Senjyo Brewery Co., Ltd. (Nagano, Japan).

### 3.2 Preparation of FRPs

The slurry of fermented-rice beverage was mixed with distilled water (1:1, w/w), osteorized for 30 s, and centrifuged at 7000*g* for 15 min at 4°C. The supernatant was then filtered and dialyzed using a 100- to 500-Da cellulose acetate membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) for 24 h at 4°C to remove sugars and free amino acids. The dialysate was filtered through ultrafiltration membranes with molecular-weight cut-offs of 5 kDa (Millipore Corporation, Bedford, MA, USA) to isolate FRPs and subsequently freeze-dried. For *in vitro* experiments, FRPs were further fractionated by size-exclusion chromatography using a Sephacryl S-100 column (1.2 × 90 cm; GE Healthcare Japan, Tokyo, Japan) with 50 mM ammonium bicarbonate buffer (pH 7.2). Each fraction was then freeze-dried and stored at −20°C until use.

### 3.3 Animal experiments

Male C57BL/6 mice (8-weeks old; 20–25 g) were purchased from Japan SLC, Inc. (Shizouka, Japan) and housed in a regulated environment (20–23°C, 40–70% relative humidity, 12-h light/dark cycle, light period starting at 8:00 AM) with free access to food and water. All experiments were conducted in compliance with the animal protocol approved by the Institutional Animal Care and Use Committee of Shinshu University (No. 300065).

To test the preventive effect of FRP on scopolamine-induced amnesia, male C57BL/6 mice were randomly assigned to four groups ($n$ = 5/group): control, scopolamine, and FRP treatment at 25 mg/kg and 100 mg/kg body weight. FRP samples were dissolved in PBS and administered daily by oral gavage and intraperitoneal injection for 14 and 7 days, respectively. At 24 h after administration of the final sample, mice were intraperitoneally injected with scopolamine (3 mg/kg body weight) dissolved in PBS. A passive-avoidance test was performed at 45- to 60-min post-injection, and serum and hippocampus were collected, frozen in liquid nitrogen, and kept at −80°C. The hippocampus was weighed and homogenized in 400 μL of ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA). After centrifugation at 3000 rpm for 10 min at 4°C, the supernatant was collected and further diluted with appropriate buffer solutions for the determination of acetylcholine (ACh) level, acetylcholinesterase (AChE) activity, and superoxide dismutase (SOD) activity in the hippocampus.

3.4 Passive-avoidance test

The passive-avoidance test was performed using a step-through cage (Muromachi Kikai Co. Ltd., Tokyo, Japan) consisting of white and black compartments separated by a sliding door. During the training trial, mice were placed in the white compartment for 10 s, the door was opened, and the step-through latency was recorded. When the mice entered the dark compartment, the door was closed, and an electric foot shock (0.3 mA) was delivered through stainless-steel rods for 3 s. After 24 h, a probe test was performed using the same procedure without any foot shock. The step-through latency to enter the dark was recorded. A maximum retention latency of 300 s was allowed for mice that did not enter the dark compartment.

3.5 Determination of ACh level and AChE activity

To measure the ACh concentration and AChE activity in the hippocampus, an Amplex fluorimetric acetylcholine assay kit (red fluorescence; AAT Bioquest; Sunnyvale, CA, USA) and an Amplex fluorimetric acetylcholinesterase assay kit (green fluorescence; AAT Bioquest) were used, respectively, according to manufacturer instructions.

3.6 Measurement of nitric oxide (NO) and malondialdehyde (MDA) levels and SOD activity

NO levels in serum were measured using the Griess method described by Tatsch et al. [100] MDA levels in brain tissues homogenized in 0.1 M potassium phosphate buffer (pH 7.4) were determined according to the protocol described by Prabhakar et al.[101] SOD activity in the hippocampus was determined using an assay kit (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer instructions.

3.7 Measurement of cell viability during $H_2O_2$-induced oxidative stress in SK-N-SH cells

Human neuroblastoma SK-N-SH cells were grown in modified Eagle medium-α supplemented with 10% (v/v) fetal bovine serum and 50 U/mL to 100 U/mL penicillin/streptomycin in plastic 75-$cm^2$ flasks at 37°C and an atmosphere of 5% $CO_2$. The cells were allowed to grow for 5 to 6 days until sub-confluent, with medium replaced every 2 or 3 days. To determine the protective properties of FRPs against $H_2O_2$-induced oxidative stress, 200 µL of cell suspension at a density of $1 \times 10^5$ cells/mL was transferred to standard 96-well plates and incubated at 37°C in an atmosphere of 5% $CO_2$ until sub-confluence. The cells were preincubated with various concentrations of FRP subfractions for 24 h and exposed to 500 µM $H_2O_2$ (200 µL) for another 4 h at 37°C and an atmosphere of 5% $CO_2$. After washing with PBS, 50 µL of MTT (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) dissolved in culture medium was added to the cells and incubated for 4 h at 37°C. The formazan products formed following

MTT administration were dissolved in 250 μL of DMSO, and the absorbance was measured at 570 nm using a Bio Rad Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The results were expressed as the percentage of viable cells relative to control cells.

3.8 RNA extraction and real-time quantitative PCR (qPCR)

Total RNA in the hippocampus and FRP-pretreated cells was isolated using Invitrogen Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. RNA samples were converted to double-stranded cDNA using ReverTra Ace (Toyobo, Osaka, Japan). Quantification was performed with a Kapa SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA) and a TP850 Thermal Cycler Dice real-time system (Takara, Shiga, Japan). Primer sequences for each gene of interest are summarized in Table 2. Fold changes in the relative mRNA-expression level of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method, with the values normalized to that of a housekeeping gene (*β-actin*).

Table 2. Primer sequences used for qPCR.

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| *ChAT (m)* | CTTGGATGGTCCAGGCAC | GTCATACCAACGATTCGCTCC |
| *mChAR1 (m)* | AGTGGCATTCATCGGGATCA | CTTGAGCTCTGTGTTGACCTTGA |
| *mChAR2 (m)* | CATGCCTGGTGGTGATGGTG | GGCCCAGGGAAGTGGAAAC |
| *mChAR3 (m)* | TTATGAACCGCTGGGCTCTG | AATCATCACACCGGCTCGTT |
| *mChAR4 (m)* | GCTAGTTCCGCCGTCTGTCC | CAGGTGGTTGTGGGCTGTTG |
| *mChAR5 (m)* | GCATGGCTGGTCTCCTTCATC | CCCGGTAGATCCGGCAGTAG |
| *Actb (m)* | AGTGTGACGTTGACATCCGT | TGCTAGGAGCCAGAGCAGTA |
| *BCL2 (h)* | ACGACTTCTCCCGCCGCTAC | CTGAAGAGCTCCTCCACCAC |
| *BDNF (h)* | TAACGGCGGAGACAAAAAGA | GAAGTATTGCTTCAGTTGGCCT |

Table 2 (continued)

| Gene | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| *NGF (h)* | CCATCCCATCTTCCACAG | CTCTCCCAACACCATCAC |
| *GST (h)* | ACTAAAGCCAGCCTGACCTTCCTT | AATGCTGCTCCTTCATGCAACACG |
| *HO1 (h)* | ATGGCCTCCCTGTACCACATC | TGTTGCGCTCAATCTCCTCCT |
| *SOD2 (h)* | ACAGGCCTTATTCCACTGCT | CAGCATAACGATCGTGGTTT |
| *ACTB (h)* | AGTGTGACGTGGACATCCGCA | GCCAGGGCAGTGATCTCCTTCT |

3.9 Western blot analysis

Hippocampal tissues and SK-N-SH cells were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology) in an ice bath, and the homogenates were centrifuged at 12,000*g* for 15 min at 4°C. The total protein concentration of the extract was measured using the BCA assay. Proteins (10 μg) were separated by electrophoresis on 10% or 12.5% polyacrylamide gels and transferred electrophoretically onto a polyvinylidene difluoride immobilon-P membrane (0.45-μm pore size; Millipore) for 1 h. After three washes in PBS containing 0.1% Tween-20 (PBST) for 5 min each, membranes were blocked with 3% BSA in PBST for 60 min and incubated with primary antibodies against BDNF (1:2500; Abcam, Cambridge, UK), NGF (1:3000; Abcam), postsynaptic density protein 95 (PSD95; 1:5000; Abcam), CREB (1:2000; Abcam), phosphorylated (p)CREB (1:2000; Abcam), ERK (1:3000; Abcam), pERK (1:2000; Abcam), SOD2 (1:3000; GeneTex, Irvine, CA, USA), glutathione S-transferase (GST; 1:3000; Nacalai Tesque, Kyoto, Japan), β-tubulin (1:5000; Abcam), and β-actin (1:5000; Santa Cruz Biotechnology) overnight at 4°C. Membranes were washed with PBST and incubated with secondary anti-rabbit horseradish peroxide (HRP)-conjugated (1:5000; Santa Cruz Biotechnology) or anti-mouse HRP-conjugated antibodies (1:10,000, Santa Cruz Biotechnology) for 60 min at room temperature. Detection was performed using EzWest Lumi

plus reagent (ATTO, Osaka, Japan) and AE-9300 Ez-Capture reagent (ATTO). Protein bands were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized against β-actin or tubulin.

3.10 Immunostaining analysis

The collected brain samples were fixed in 10% paraformaldehyde, embedded in paraffin, and cut into 4-μm longitudinal sections, which were heated to 100°C in Tris-EDTA buffer [10 mM Tris and 1 mM EDTA (pH 9.0)] for 20 min to aid in antigen presentation before cooling at room temperature for 30 min. Sections were blocked with TBST containing 10% normal goat serum and 1% BSA for 2 h and were then incubated with a rabbit anti-BDNF (1:200; Abcam) antibody overnight at 4°C. After washing with TBST, sections were then incubated with a secondary antibody conjugated to Alexa Fluor 488 (goat anti-rabbit IgG; 1:200; Abcam) for 2 h. The slides were washed with PBS and mounted in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (ImmunoSelect Antifading Mounting Medium; Dianova, Hamburg, Germany). Images were captured at 10× magnification using an EVOS fl fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA).

3.11 Purification and identification of FRP sequences

The FRP subfraction was dissolved in distilled water and further separated using HPLC (JASCO, Tokyo, Japan) on an ODS C18 column (5 μm, 250 × 4.6 mm; Inertsil GL Science, Tokyo, Japan) using a linear gradient of acetonitrile (0–50% for 30 min) containing 0.1% TFA at a flow rate of 1.0 mL/min for 60 min at 40°C. Peaks were detected at 235 nm, and the fraction associated with the major peak was collected and freeze-dried. Mass spectra were acquired using a MALDI-TOF/TOF MS/MS system (AB SCIEX TOF/TOF 5800; SCIEX, Redwood City, CA, USA) equipped with a 355-nm pulsed or ultraviolet nitrogen laser and a

dual microchannel detector. The fraction associated with the major peak was dissolved in matrix solution (5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA), and 1 μL of the mixture was spotted and dried onto the target plate at room temperature. A TOF/TOF mass standard kit (SCIEX) containing components with known masses was used to calibrate the instrument. Spectra were obtained using at an accelerating voltage of 20 kV in the range of 400 Da to 3000 Da. *De novo* peptide sequencing of the peptides associated with the major peaks was performed using DeNovo Explorer (SCIEX).

3.12 Statistical analysis

Statistical analyses were performed using SAS software (v.9.1.3; SAS Institute, Cary, NC, USA) or GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA), and data are represented as the mean ± SD or SEM. Differences between the means were evaluated by analysis of variance, followed by Tukey's honestly significant difference or Fisher's least significant difference post hoc test for mean comparison.

## 4. Results

### 4.1 Modulating effects of FRP on upregulated BDNF levels in the mouse hippocampus

FRP was administered to normal mice orally for 14 days, respectively, resulting in slight increases in hippocampal levels of BDNF, although this was not statistically significant relative to levels in control mice (Figure 8A). By contrast, BDNF levels increased significantly following intraperitoneal injection of FRP at 100 mg/kg body weight for 7 days as compared with levels in control mice (Figure 8B). Additionally, immunostaining of brain sections revealed elevated BDNF levels in the dentate gyrus (DG), cornu ammonis (CA)1, and CA3 (Figure 8C), with the number of BDNF-positive cells significantly higher in the CA3 region of the hippocampus of mice treated with FRP at 100 mg/kg body weight (Figure 8D).

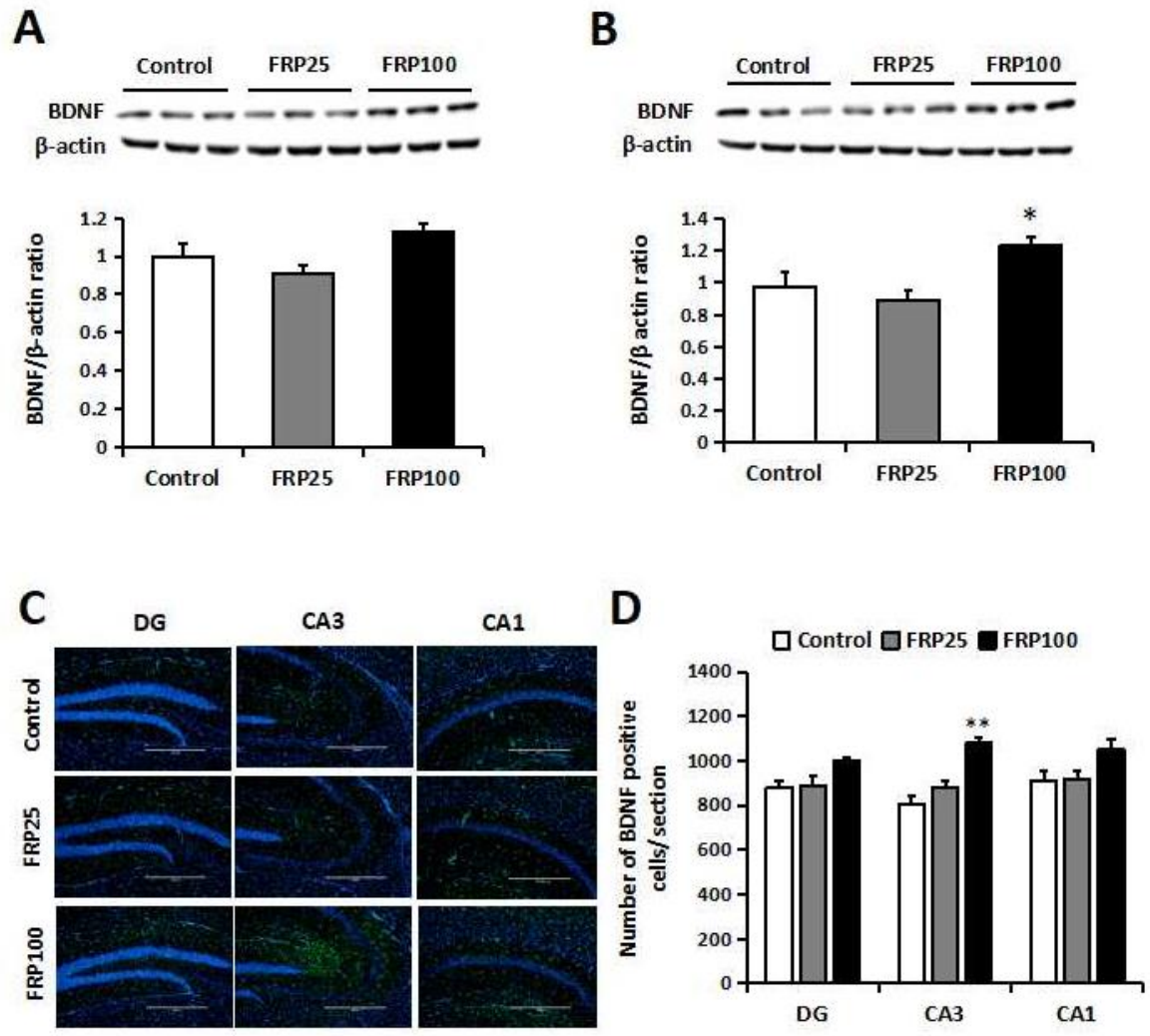


Figure 8. Effect of oral and intraperitoneal FRP administration on BDNF levels in the hippocampus. Western blot analysis of BDNF levels in the hippocampus of mice treated with FRP (25 and 100 mg/kg body weight) daily via (A) oral gavage or (B) intraperitoneal injection for 14 and 7 days, respectively. (C) Representative images of hippocampal DG, CA3, and CA1 regions stained with DAPI (blue) and BDNF (green), Scale bar: 400 μm. (D) Quantification of BDNF-positive cells in different regions of the hippocampus of mice intraperitoneally injected with FRP for 7 days. Values are expressed as the mean ± SEM (n = 6). *P < 0.05; **P < 0.01 compared with control mice.

4.2 Suppressive effects of FRP against scopolamine-induced memory impairment

In the training trial of the passive-avoidance test, there was no significant difference in step-through latency among mouse groups, whereas in the test trial, step-through latency was significantly decreased following scopolamine treatment (Figure 9A). Oral administration of FRP for 14 days resulted in slight increases in step-through latency in a dose-dependent manner, although we did not observe a statistically significant difference. On the other hand, intraperitoneal administration of FRP at 100 mg/kg significantly increased the step-through latency relative to that observed in the scopolamine-treated group (Figure 9B).

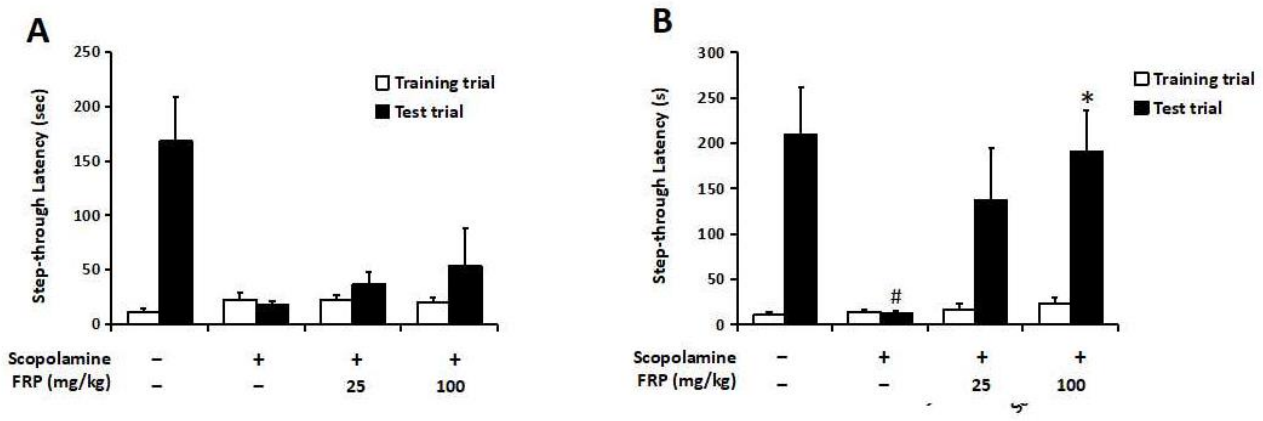


Figure 9. Effect of FRP on the step-through latency time of scopolamine-treated mice during the passive-avoidance test. Following FRP pretreatment via (A) oral gavage for 14 days or (B) intraperitoneal injection for 7 days, mice were intraperitoneally injected with scopolamine. Data represent the mean ± SEM (n = 5). #P < 0.05 compared with control mice; *P < 0.05 compared with scopolamine-treated mice.

4.3 Effects of FRP on cholinergic activity in scopolamine-treated mice

Scopolamine treatment significantly increased AChE activity and decreased ACh concentration in the hippocampus relative to controls; however, these changes were attenuated by FRP pretreatment (Figure 10A and B). Additionally, FRP administration prevented reductions in Ach-synthesizing enzyme (*ChAT*), muscarinic ACh receptor1 (*mAChR1*), and muscarinic ACh receptor3 (*mAChR3*) in the hippocampus (Fig. 10C).

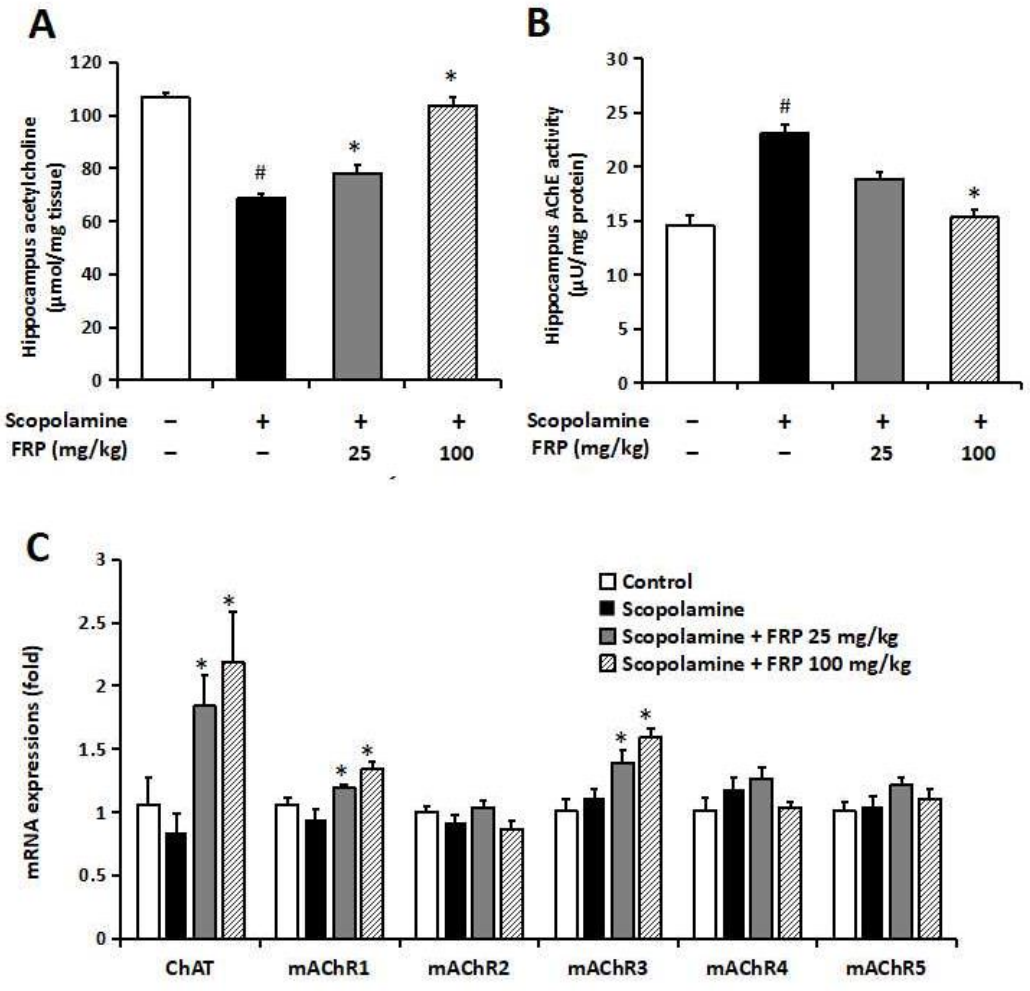


Figure 10. Effects of FRP on cholinergic activity in the hippocampus of scopolamine-treated mice. (A) ACh levels, (B) AChE activity, and (C) mRNA levels of ChAT and mChARs. Data represent the mean ± SEM (n = 5). #P < 0.05 compared with control mice; *P < 0.05 compared with scopolamine-treated mice.

4.4 Effects of FRP on oxidative stress in scopolamine-treated mice

Levels of MDA, a metabolite of lipid oxidation, were significantly increased in the brains of scopolamine-treated mice as compared with levels in control mice (Figure 11A); however, FRP pretreatment decreased MDA levels relative to those in the scopolamine-treated group. Additionally, SOD activity in the hippocampus and serum was attenuated following scopolamine treatment; however, FRP pretreatment increased SOD activity relative to that observed in scopolamine-treated mice (Figure 11B and C). Moreover, serum NO levels, which are associated with oxidative stress, were markedly increased following scopolamine treatment, whereas FRP pretreatment reduced serum NO levels relative to those observed in scopolamine-treated mice (Figure 11D).

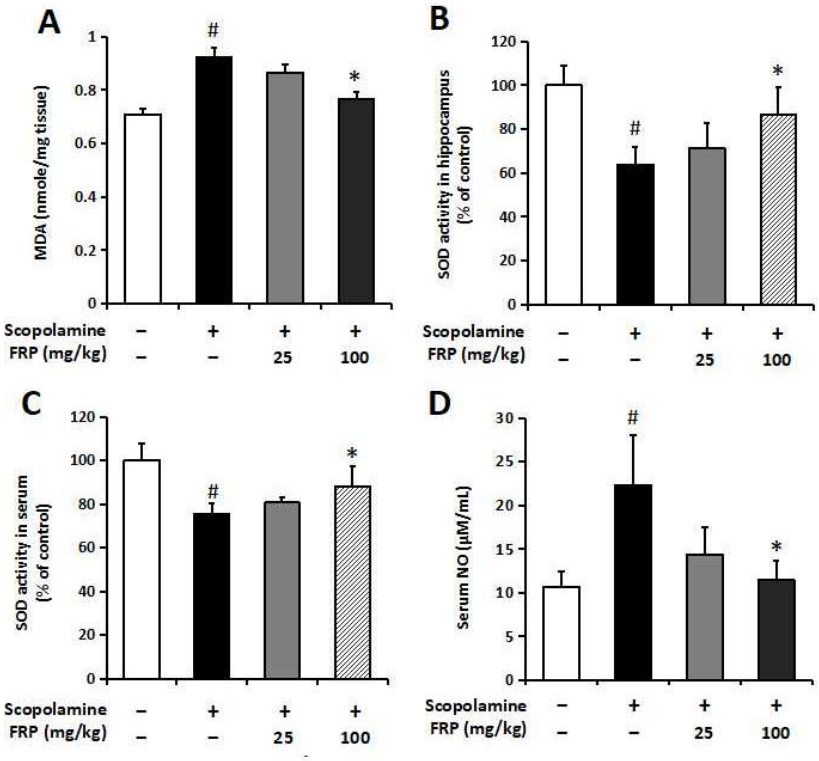


Figure 11. Effects of FRP on oxidative stress in scopolamine-treated mice. (A) MDA levels in the brain, (B) SOD activity in the hippocampus, (C) serum SOD activity, (D) and serum NO levels. Data represent the mean ± SEM (n = 5). #P < 0.05 compared with control mice; *P < 0.05 compared with scopolamine-treated mice.

4.5 Effects of FRP on levels of neuroplasticity proteins in scopolamine-treated mice

Scopolamine treatment markedly reduced levels of pCREB and pERK, as well as those of BDNF and PSD95 in the hippocampus (Figures 12A–D). By contrast, FRP pretreatment effectively prevented these scopolamine-induced reductions in levels of neuroprotective and synaptic plasticity biomarkers.

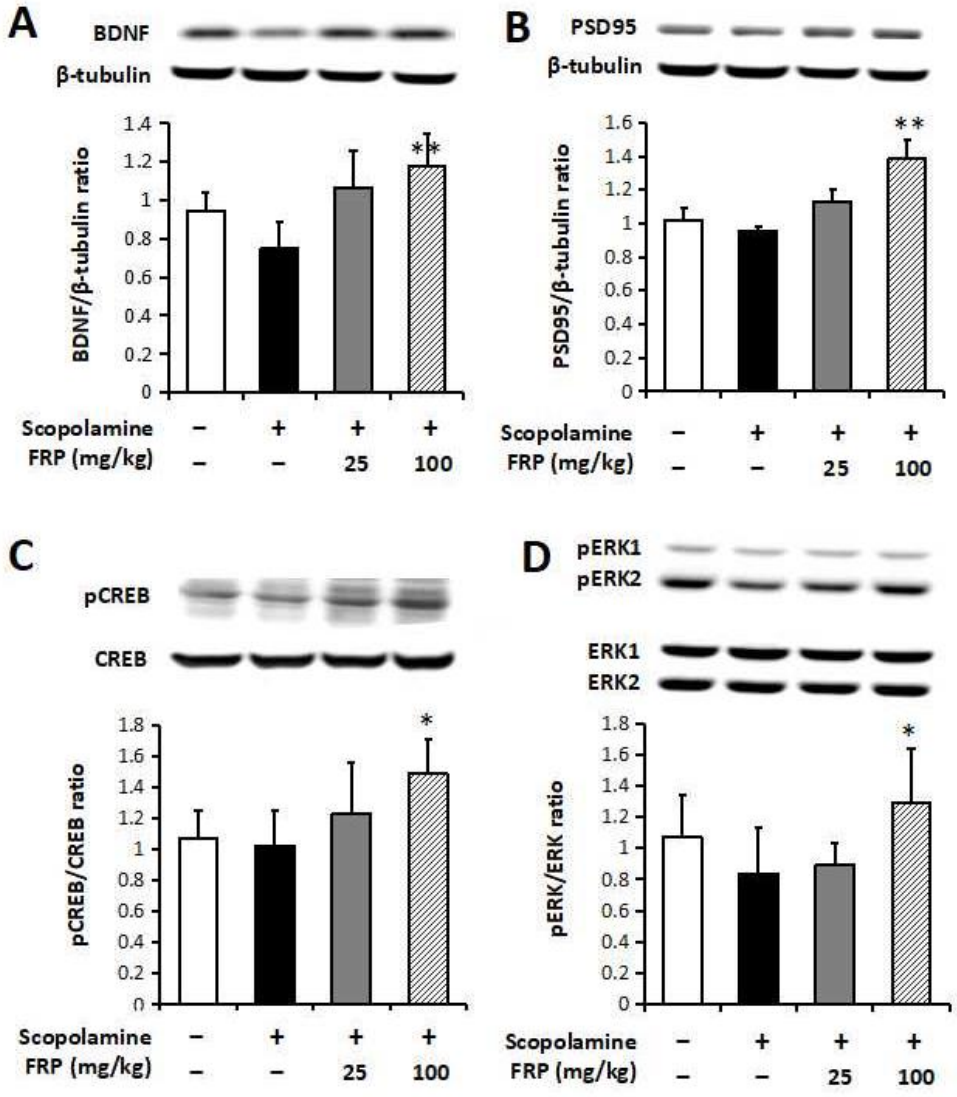


Figure 12. Effect of FRP on levels of neurotrophic factors and synaptic plasticity protein biomarkers in the hippocampus of scopolamine-treated mice. Western blot analysis of protein levels of (A) BDNF, (B) PSD95, (C) pCREB, and (D) pERK. Data represent the mean ± SD (n = 3). *P < 0.05; **P < 0.01 compared with scopolamine-treated mice.

4.6 The protective effects of FRP subfractions on $H_2O_2$ -induced oxidative stress in SK-N-SH cells

FRP was separated by size-exclusion chromatography, ultimately yielding two major fractions designated as F1 and F2, respectively (Figure 13A). Result of analysis suggested that the F1 fraction might represent a melanoidin compound due to its high total sugar content and strong absorbance at 325 nm and 405 nm (Figure 13B). These findings indicated that the F1 and F2 fractions represented glycosylated and non-glycosylated subfractions, respectively.

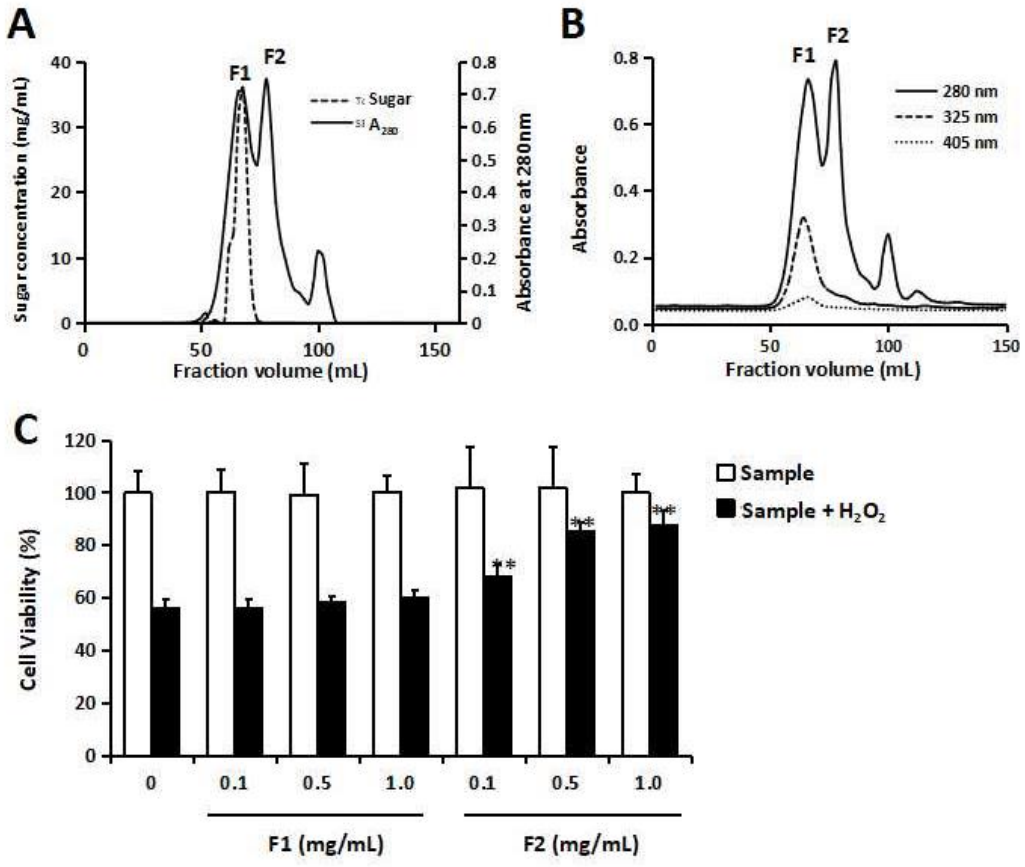


Figure 13. Size-exclusion chromatogram of FRP and the effect of FRP subfractions on SK-N-SH cell viability in the presence of H2O2. (A) Total sugar concentration and absorbance at 280 nm. (B) Absorbance at 280 nm, 325 nm, and 405 nm. (C) SK-N-SH cells were pre-incubated with FRP subfractions for 24 h, followed by exposure to 500 μM H2O2 for 4 h and evaluation of cell viability by MTT assay. Data represent the mean ± SD (n = 6). **P < 0.01 compared with H2O2-treated cells.

Pre-incubation with the F2 subfraction significantly attenuated cell death caused by $H_2O_2$-induced oxidative stress (Figure 13C), and the viability of cells pretreated with the F2 subfraction for 24 h was significantly higher than that observed in cells pre-incubated with the F1 fraction or without FRP pretreatment. Moreover, mRNA levels of B-cell lymphoma 2 (*BCL2*), *BDNF*, *NGF*, *GST*, heme oxygenase-1 (*HO-1*), and *SOD2* were significantly upregulated by treatment with the F2 subfraction (Figure 14A), which agreed with significant increases in protein levels of BDNF, NGF, and GST following treatment with 1.0 mg/mL of the F2 subfraction, whereas no significant change was observed in SOD2 level (Figures 14B-E).

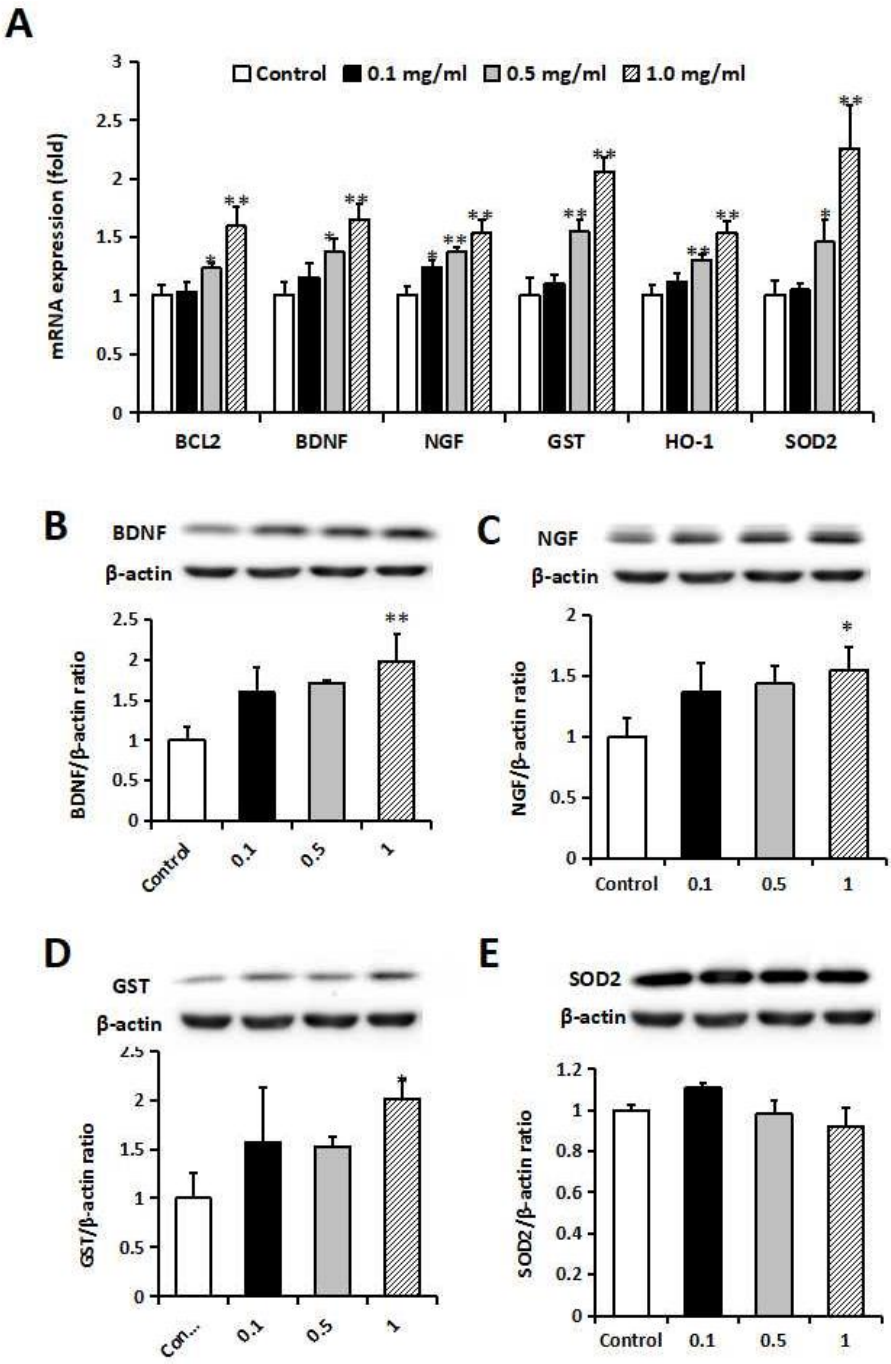


Figure 14. Effects of the F2 subfraction on mRNA and protein levels of neuroprotective factors in SK-N-SH cells. (A) mRNA levels of BCL2, BDNF, NGF, GST, HO-1, and SOD2. Protein levels of (B) BDNF, (C) NFG, (D) GST, and (E) SOD2. Data represent the mean ± SD (n = 3). *P < 0.05; **P < 0.01 compared with H2O2-treated cells. Identification of the peptide sequence of the F2 subfraction.

The amino acid sequence of the major peptide in the F2 subfraction was identified as His-Ser-Met-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Thr-Val-His-Thr, with a molecular weight of 1848.87 Da (Figures 15A–C).

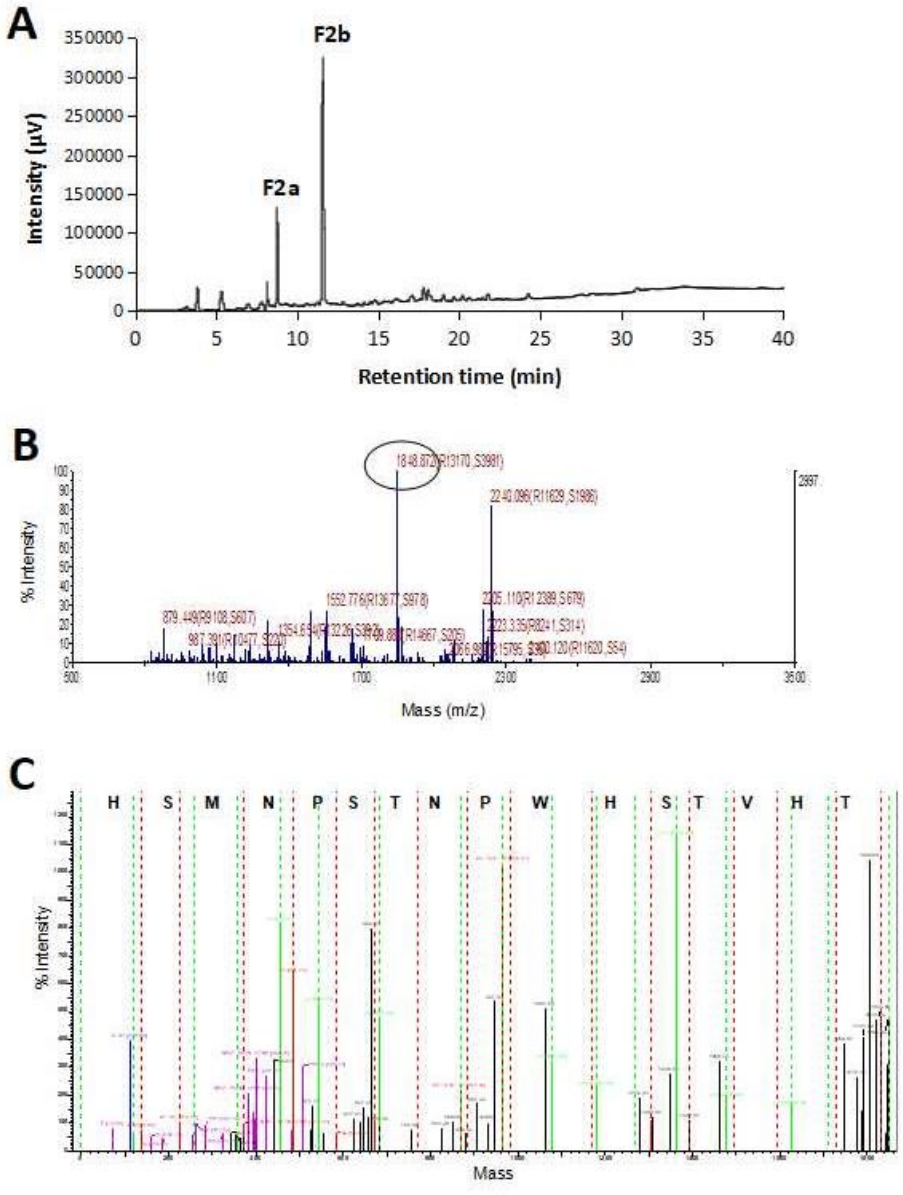


Figure 15. Identification of the F2 peptide sequence. (A) RP-HPLC chromatogram of two FRP subfractions. (B) MALDI-TOF/TOF MS/MS spectra of the F2 subfraction (F2b). The molecular mass associated with the highest active peak was determined as 1848.87 Da. (B) MALDI-TOF/TOF MS/MS spectra showing fragmentation of the peptide associated with the F2b subfraction. (C) Schematic representation of the peptide sequence.

## 5. Discussion

In this study, we demonstrated that intraperitoneal administration of FRP upregulated BDNF levels in the hippocampus of mice and prevented scopolamine-induced cognitive deficits according to passive-avoidance tasks, which are dependent upon hippocampal function [102]. Because BDNF is involved in the survival, differentiation, and function of neuronal cells and crucial for learning and memory, BDNF upregulation following FRP pretreatment might contribute to the attenuation of scopolamine-induced damage.

Scopolamine-induced amnesia model rodents have been widely used to screen therapeutic agents against cognitive impairment.[99] Scopolamine induces cognitive impairment in animal models by disturbing cholinergic neurotransmission [97] and promoting oxidative stress and damage in the brain.[98] Neurodegenerative diseases, such as Alzheimer's disease, are characterized by the loss of learning and memory abilities, which correlate with the loss of cholinergic neurons.[103] Therefore, the cholinergic system is implicated in the maintenance of synaptic plasticity and memory.[104] In cholinergic neurons, the ACh neurotransmitter is synthesized from choline and acetyl-CoA by the ChAT enzyme, followed by ACh transport into vesicles and release into the synaptic cleft, where it can bind to mAChRs and/or nicotinic ACh receptors. Within the synapse, ACh is hydrolyzed into choline and acetic acid by AChE.[105] Results revealed that scopolamine treatment increased AChE activity and prevented the upregulation of ChAT levels in the hippocampus as compared with controls, and that FRP administration suppressed decreases in ACh levels in the hippocampus. The actions of ACh involved in learning and memory are mainly mediated by mAChRs.[106] Result showed that FRP pretreatment might be mediated by mAChR1 and mAChR3. Moreover, activation of mAChRs by ACh facilitates long-term potentiation (LTP) in brain regions, such as the hippocampus,[107] and previous studies demonstrated mAChR1 and mAChR3 involvement in ACh-based

cognition effects.[108] Results demonstrated that FRP-mediated inhibition of AChE activity, and that activation of ChAT activity maintained ACh levels, which might have activated mAChRs to enhance LTP in the hippocampus. Overall, these findings suggested that FRP administration might play a protective role against ACh degradation and enhance cholinergic neurotransmission.

In addition to its effects on cholinergic neurotransmission, scopolamine induces oxidative stress in the mouse brain, resulting in neuronal injury or neuronal cell death. Therefore, we examined the antioxidative effects of FRP in the hippocampus, given that oxidative stress contributes to the pathogenesis of neurodegenerative disorders and associated histological changes.[109] As expected, scopolamine injection induced oxidative stress, as indicated by reduced SOD activity, elevated MDA level in the brain, and increased NO level in serum. These scopolamine-induced alterations in antioxidant activity were significantly attenuated by FRP pretreatment, demonstrating that FRP might ameliorate the cognitive deficits caused by scopolamine through inhibition of oxidative stress in the mouse hippocampus.

The anti-amnesic effects of FRP administration was also determined by analyzing several proteins related to brain plasticity and cognitive functions, including BDNF, PSD95, CREB, pCREB, ERK, and pERK, in the hippocampus. Memory improvement is regulated at the molecular level in neurons and by major signaling pathways, with the BDNF/ERK/CREB signaling pathway involved in many neuronal aspects, including neuronal survival and synaptic plasticity.[110] This pathway activates CREB binding of the promoter regions of various genes related to memory and synaptic plasticity. Additionally, CREB levels can also be upregulated ERK activation.[111] A previous study showed that CREB activity is specifically associated with memory formation and regulates levels of BDNF, which is responsible for neuron growth and cellular differentiation and proliferation in the central nervous system.[112] BDNF is implicated

in synaptic plasticity and memory formation and plays a crucial role in hippocampus-dependent long-term memory.[113] Moreover, elevated levels of BDNF in the brain can activate synaptogenesis and improve cognitive function.[114,115] FRP administration also inhibited scopolamine-induced reductions in BDNF, PSD95, pERK, and pCREB levels in the mouse hippocampus. A previous study used an *in vivo* experiment to show that (N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxyphenyl)-acrylamide), a novel cyclic derivative of squamosamide from *Annona glabra*, exerted neuroprotective effects by enhancing the BDNF/tropomyosin receptor kinase B/CREB signaling pathway and inhibiting neuronal apoptosis by increasing the BCL2/BCL2-associated X protein ratio in the hippocampus of amyloid precursor protein/presenilin-1 double transgenic mice.[116] These results implied that treatment with FRP might ameliorate scopolamine-induced learning and memory impairment by activating BDNF/ERK/CREB signaling in the mouse hippocampus in order to promote neuron survival and synaptic plasticity.

The neuroprotective effects of FRP was verified in an $H_2O_2$-induced injury model using SK-N-SH cells in vitro. $H_2O_2$ is an ROS generated during cellular metabolism and that activates several downstream signaling pathways involved in cell survival or apoptosis in various cell types during oxidative insult.[117] Results showed that exposure of SK-N-SH cells to $H_2O_2$ significantly decreased cell viability; however, pre-incubation of cells with the F2 subfraction of FRP reduced $H_2O_2$-induced cell death. This was attributed to upregulation of not only neuroprotective agents, such BDNF and NGF, but also the antioxidant enzyme GST, which is necessary for maintaining the balance of cellular redox. Li et al.[118] reported that rice protein significantly suppresses ROS production *in vivo* through its ROS-scavenging capacity in Wistar rats fed a cholesterol-free or high-cholesterol diet and by inducing the expression of genes encoding antioxidant/detoxification proteins, such as NAD(P)H quinone dehydrogenase-

1, HO-1, glutamate cysteine ligase (GCL) catalytic subunit, GCL modulatory subunit, and GST, by activating the Nrf2 transcription factor. These findings suggest that food-derived peptides could represent potent modulators of antioxidant/detoxification enzymes.

Subsequently, the peptide sequence of the F2 subfraction was identified using MALDI-TOF/TOF MS/MS via *de novo* sequencing. The sequence showed a high degree of homology with glutelin protein and was capable of either directly or indirectly upregulating levels of BDNF in the mouse hippocampus and SK-N-SH neuronal cells. However, the mechanisms associated with this activity warrant further investigation.

# CHAPTER 4

## SUMMARY AND CONCLUSION

This study was to conducted to assess the neuroprotective and cognitive decline-suppressing activities of biomaterials derived from fermented beverages. In the first study, the suppressive effects of long-term diet supplementation with *Lactobacillus* strains derived from rice wine lees on cognitive decline was evaluated using SAMP8 mice. Results of Barnes maze and passive avoidance test showed that long-term administration of *Lactobacillus paracasei* K71 (L. K71) prevented cognitive impairment in aged SAMP8 mice. RT qPCR and western blot analysis revealed that prolong diet supplementation with L. K71 resulted in increased BDNF protein expression in the hippocampus of aged SAMP8 mice. Since age-related cognitive decline is associated with the reduction of neurotrophic factors, a sustained BDNF expression would be important in maintaining brain function during aging. The expression of CREB and its activated form (pCREB) was also significantly upregulated in the hippocampus of SAMP8 mice fed L. K71. The transcription of BDNF is tightly controlled by several intracellular signaling pathways and transcription factors, including CREB. Thus, upregulation of BDNF expression by long-term consumption of a diet supplemented with L. K71 may be mediated by CREB transcription factor and contribute to the sustained neuronal plasticity and brain cognitive functions. Neurotransmitter serotonin not only regulates mood and behavior but also plays key role in brain development and cognitive functions. An increased of serotonin level was observed in the serum and brain extract of L. K71-fed mice. Intracellular levels of cAMP and CREB phosphorylation could be increased and induced through indirect stimulation of norepinephrine and serotonin receptors by antidepressants via cAMP-Protein kinase A (PKA) pathway. This mechanism may underlie the upregulation of

BDNF expression in L. K71-fed mice. Elevated brain serotonin levels may promote CREB phosphorylation, resulting in enhanced CREB-dependent transcription of target genes including *Bdnf*. The expression level of serotonin-degrading enzymes, particularly MAOA, was decreased in the hippocampus of SAMP8 mice fed L. K71 but no changes were observed in the gene expression of a serotonin-synthesis-related enzyme. MAOs are usually increased during aging. Serotonin in the tissues can be rapidly metabolized by MAO, with the A isoform having much greater affinity for the substrate compared to the B isoform. This indicated that L. K71 administration may suppress serotonin degradation without affecting serotonin synthesis in the brain.

For the second study, the preventive effects of fermented rice peptides, FRPs against scopolamine-induced memory impairment in mice was investigated. Results showed that intraperitoneal administration of FRP upregulated BDNF levels in the hippocampus of mice and prevented scopolamine-induced cognitive deficits according to passive-avoidance tasks, which are dependent upon hippocampal function. Because BDNF is involved in the survival, differentiation, and function of neuronal cells and crucial for learning and memory, BDNF upregulation following FRP pretreatment might contribute to the attenuation of scopolamine-induced damage. Scopolamine induces cognitive impairment in animal models by disturbing cholinergic neurotransmission and promoting oxidative stress and damage in the brain. We found that scopolamine treatment significantly increased AChE activity and decreased ACh concentration in the hippocampus relative to controls; however, these changes were attenuated by FRP pretreatment Moreover, FRP administration prevented reductions in Ach-synthesizing enzyme *ChAT*, muscarinic ACh receptor1 (*mAChR1*), and muscarinic ACh receptor3 (*mAChR3*) in the hippocampus. Since the activation of mAChRs by ACh facilitates long-term potentiation (LTP) in the hippocampus, our findings suggested that FRP-mediated inhibition

of AChE activity, and that activation of ChAT activity maintained ACh levels, which might have activated mAChRs in the postsynaptic site to enhance LTP in the hippocampus. Therefore, FRP administration might play a protective role against ACh degradation and enhance cholinergic neurotransmission. Scopolamine injection induced oxidative stress, as indicated by reduced SOD activity, elevated MDA level in the brain, and increased NO level in serum but these scopolamine-induced alterations in antioxidant activity were significantly attenuated by FRP pretreatment, demonstrating that FRP might ameliorate the cognitive deficits caused by scopolamine through inhibition of oxidative stress in the mouse hippocampus. The anti-amnesic effects of FRP administration was assessed by analyzing several proteins related to brain plasticity and cognitive functions and results showed that FRP administration inhibited scopolamine-induced reductions in BDNF, PSD95, pERK, and pCREB levels in the mouse hippocampus, suggesting that FRP might ameliorate scopolamine-induced learning and memory impairment by activating ERK/CREB/BDNF signaling in the mouse hippocampus in order to promote neuron survival and synaptic plasticity. The neuroprotective effects of FRP were further verified in an $H_2O_2$-induced injury model using SK-N-SH cells in vitro. Results revealed that pre-incubation of cells with the F2 subfraction of FRP reduced $H_2O_2$-induced cell death and upregulated not only neuroprotective agents, such BDNF and NGF, but also the antioxidant enzyme GST, which is necessary for maintaining the balance of cellular redox. These findings suggest that FRP could represent potent modulators of antioxidant/detoxification enzymes. Finally, the amino acid sequence of the F2 subfraction was identified using MALDI TOF/TOF MS/MS via de novo sequencing. The amino acid sequence had a high degree of homology with glutelin protein and was capable of either directly or indirectly upregulating levels of BDNF in the mouse hippocampus and SK-N-SH neuronal

cells. However, further studies are needed to identify the minimum active sequence of this peptide and the more detailed mechanisms associated with this activity.

In conclusion, long-term administration of a diet supplemented with *Lactobacillus paracasei* K71, isolated from rice wine lees, prevents age-related cognitive decline in the SAMP8 mouse model by enhancing serotonin levels and inducing BDNF expression in the hippocampus which contributed to sustained neuronal plasticity. Moreover, FRP upregulated BDNF levels in the hippocampus via intraperitoneal administration and prevented scopolamine-induced amnesia in mice through activation of the cholinergic system, antioxidant activity, and ERK/CREB/BDNF signaling in the mouse hippocampus. Regular intake of *Lactobacillus paracasei* K71 and fermented rice peptides might promote beneficial health-related effects particularly associated with the prevention of cognitive impairment and dementia in the elderly.

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