Collagen peptides enhance hippocampal neurogenesis and reduce anxiety-related behavior in mice

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ABSTRACT

The present study examined the effects of enzymatically hydrolyzed collagen peptides on the level of hippocampal neurogenesis and emotional behavior in adult mice. For this purpose, two kinds of enzymatically hydrolyzed collagen peptides, the lower or higher molecular weight peptides (LP: below 2,000, HP: about 30,000) were administered orally to C57BL/6 mice for 4 weeks. As a result, the density of proliferating cells in subgranular zone of hippocampus showed a 1.2-fold increase in LP mice as compared with HP mice. Additionally, LP mice spent less time in closed arms than HP mice in elevated plus maze test to examine anxiety-related behavior. These results suggest that oral administration of the lower molecular weight peptides derived from collagen enhanced the hippocampal neurogenesis and exerted emotional behavior in adult mice.

A variety of health issues such as neurodegenerative diseases and mood disorders face modern society (16, 22, 26, 27). These neurological disorders can be treated by an enhancement of adult neurogenesis, which helps to improve spatial memory and synaptic plasticity and moderate depression (3, 5). Interestingly, the level of adult neurogenesis depends on environmental conditions such as exercise, learning, enriched environment and chronic stress (6, 12, 13, 17, 20). Therefore, manipulating environmental factors could regulate the level of neurogenesis for maintenance or improvement of human health.

In this study, we set out to examine whether ingestion of nutrients, considered as one of the major environmental variables, might affect the level of adult neurogenesis and its associated behavior. It has been reported that the dietary restriction enhances hippocampal neurogenesis in adult mice (14), and we attempted to investigate the effects of food ingestion with no influence of dietary restriction. Several studies have shown that some nutrients including arachidonic acid and docosahexaenoic acid affect brain functions, such as neurogenesis in the hippocampus or neuronal differentiation of neural stem cells (10, 11, 15). Moreover, it has been reported that protein-derived peptides (e.g. soybean peptide) have the beneficial effects on brain functions including not only an increase in the number of neural stem cells but also an improvement of memory and emotional state (4, 7, 8).

Among different nutrients available in nature, we focused on peptides from collagen. As well as being the most abundant protein in animal tissues, collagen is an extracellular matrix protein and often served as a protein source of human diet. Low molecular weight collagen peptides degraded by enzymes are more efficiently absorbed through intestinal epithelium than collagen which is a very stable and high molecular weight protein. Although the primary structure of collagen is rather simple and generally expressed as tripeptide repeats of Gly-X-Y (where X and Y are any amino acid residues), some collagen-derived peptides are known to exert physiolog-
nal effects such as neural protective effects by Gly-Pro-Glu and anti-inflammatory effects by Pro-Gly-Pro (1). However, the effects of collagen peptides on adult neurogenesis and behavior have been unknown.

In the present study, we examine the effect of the peptide on the hippocampal neurogenesis facilitation, antianxiety action and antidepressant activity.

MATERIALS AND METHODS

Peptide. We have prepared two kinds of peptide samples having different molecular weights by the proteolytic digestion of pig skin gelatin with a mixture of collagenase I (Washington Biochemical Corporation, NJ, USA) and protease N (Amano Enzyme Inc, Aichi, Japan). The mean molecular weights as determined by gel filtration chromatography using AKTA purifier and Superdex Peptide 10/300 GL (Amersham Biosciences, Buckinghamshire, U.K.) were 367.5 (ranging from 300 to 2,000, mostly consisted of tripeptides) for the lower molecular weight peptides (LP) and about 30,000 for the higher molecular weight peptides (HP). HP was a less absorbable peptide sample and used as a control. LP and HP were the same in amino acid composition. We have also analyzed some of the major components of LP by amino acid sequencing, and showed that the highest constituent is Gly-Pro-Hyp which accounted for 8 percent in mass.

Mice and feeding procedure. Five-week-old male C57BL/6J mice were divided into two groups, and each received LP or HP-containing water ad libitum for 4 weeks. After performing behavioral studies in 8-week-old mice, we evaluated the level of adult neurogenesis in hippocampus at 9-week old. Mice were housed at 23°C under 12-h light/12-h dark cycles, and handled in accordance with the institutional animal care guidelines.

Immunocytochemistry and measurement of BrdU-positive cells in dentate gyrus. To evaluate the level of adult neurogenesis in hippocampus, the proliferating cells were labeled by the intraperitoneal injection of 5-bromo-2-deoxyuridine (BrdU, 100 mg/kg) for 2 h, and BrdU-positive cells were detected by the immunofluorescence staining, as described previously (25). Briefly, coronal brain sections of 40 μm in thickness were collected every 200 μm in the region of hippocampus, and free-floating sections were reacted with primary antibodies for BrdU (1/400, rat monoclonal IgG; Abcam, Tokyo, Japan) and doublecortin (1/400, rabbit polyclonal IgG; Abcam) and then with Cy3- or Cy5-conjugated secondary antibodies against rat or rabbit IgG (1/200, donkey polyclonal IgG; Jackson ImmunoResearch, Tokyo, Japan). Cell nuclei were stained with Syto13 green (1/2,000; Molecular Probes, CA, USA). Doublecortin was used to stain premature neurons. Images were acquired by a LSM510 microscope (Carl Zeiss Microscope, Tokyo, Japan) with ×10 or ×20 objective lens at 40-μm optical thickness under the same settings. The average linear density of BrdU-positive cells (the number of cells per mm of subgranular zone, averaging of 8 independent slices) was used as an indicator for hippocampal cell gene-

Behavioral analysis. We then performed behavioral studies on 8-week-old male mice after intake of LP or HP for 4 weeks (12 mice per group). All the behavioral apparatus and data acquisition/analysis software were designed and manufactured by O’Hara & Co., Tokyo, Japan. The software used for the behavioral analysis (Image TS, EP and OF) was based on Image J.

Tail suspension test. The level of depression-like behavior was examined by tail suspension test (23). Mice were suspended 30 cm above the floor with an adhesive tape placed ~1 cm from the tip of the tail, and their behavior was recorded for 5 min. The periods of immobile status was automatically analyzed by Image TS software.

Elevated plus maze test. In clinical cases of mood disorders, depression often accompanies anxiety or vice versa (19). Anxiety-related behavior was examined by the elevated plus maze test. When mice are placed onto a cross-shaped elevated platform (55 cm above the floor level) under ambient illumination at 300 lx to induce natural aversion to height and open space, mice tend to travel within the safer closed arms (a pair of 5 × 45 cm white plexiglass surrounded by clear 15-cm high side walls) rather than the riskier open arms (a pair of 5 × 45 cm white plexiglass, no side wall). Each mouse was placed on the
central platform facing one of open arms and the activities were recorded for 10 min. The number of entries into open and closed arms, the stay time on the open and closed arms (s), and total distance traveled (cm) were automatically analyzed using Image EP software.

Open field test. Anxiety-related behavior was also analyzed by the open field test. Mice were placed in the defined corner of the open-field apparatus (white plexiglass: 50 × 50 × 30 cm) under illumination at 500 lx, and the activities were recorded with a video camera for 10 min. Total distance (cm) and time spent in the central part of the open field (s) and move episode in the open field (number of times) were automatically analyzed using Image OF software.

Statistical analysis. All the presented data are expressed as mean ± standard deviation. Statistical analyses were performed with unpaired Student’s t-test unless otherwise specified, where a value of $P < 0.05$ was considered to be significant.

RESULTS

The consumption of peptides (amount of drinking), food intake and the body weight of animals were similar between LP and HP ingested groups during 3 weeks of ingestion before performing behavioral studies (amount of drinking per day: $6.7 \pm 0.7$ g in LP mice and $6.7 \pm 0.7$ g in HP mice, $P = 0.99$; food intake per day: $3.4 \pm 1.3$ g in LP mice and $3.6 \pm 1.4$ g in HP mice, $P = 0.65$; the body weight of animals: $23.2 \pm 0.9$ g in LP mice and $23.9 \pm 1.3$ g in HP mice, $P = 0.09$).

In contrast, the brain weight of LP mice was increased by 3% compared with that of HP mice when the brain was removed to evaluate the level of adult neurogenesis in hippocampus (LP mice: $454.9 \pm 11.6$ mg, HP mice: $440.1 \pm 3.9$ mg, $P = 0.01$).

Measurement of BrdU-positive cells in dentate gyrus

We observed most of BrdU-positive cells in subgranular zone (SGZ) of dentate gyrus where neural progenitor cells (NPCs) are enriched. We counted the number of BrdU-positive cells along SGZ, and found that the density of proliferating cells in LP mice was 1.2-fold higher than that in HP mice (LP mice: $8.7 \pm 1.1$ cells/mm SGZ, HP mice: $7.4 \pm 0.7$ cells/mm SGZ, $P = 0.04$) (Fig. 1, 2C and 2D). In addition, doublecortin-staining for premature neurons in LP mice were consistently stronger than HP mice (Fig. 2A and 2B). These results suggest that ingestion of the lower molecular weight collagen peptides for 4 weeks efficiently enhances hippocampal neurogenesis in adult mice.

Behavioral analysis

Tail suspension test. The total moved distance had no significant difference between the two groups (LP mice: $799.4 \pm 212.9$ mm, HP mice: $805.6 \pm 188.0$ mm, $P = 0.94$) (Fig. 3). Although both group moved at a similar extent during the initial 2 min, we found LP mice continue to move more frequently than HP mice ($F(4,84) = 3.119$, $P = 0.02$) (Fig. 4).

The total freeze time (percent) had no significant difference between the two groups (LP mice: $34.2 \pm 22.0$ %, HP mice: $31.8 \pm 18.7$ %, $P = 0.80$).

Elevated plus maze test. LP mice spent less time in closed arms than HP mice (LP mice: $264.3 \pm 37.8$ s, HP mice: $312.6 \pm 55.3$ s, $P = 0.03$) (Fig. 5), whereas the total moved distance during the 10-min test period was similar (LP mice: $2127.4 \pm 325.2$ cm, HP mice: $2127.4 \pm 325.2$ cm, $P = 0.36$) (Fig. 6). Thus, this result suggests that LP mice reduced anxiety-like behavior than HP mice.

Open field test. When mice were placed in the apparatus, LP mice more frequently traveled between the center and peripheral area than HP mice (LP: $197.3 \pm 11.2$ times, HP: $185.4 \pm 12.6$ times, $P = 0.03$) while the time spent in the center area was similar (LP mice: $111.5 \pm 18.3$ s, HP mice: $104.7 \pm 13.9$ s).
adult mice. Since the level of adult neurogenesis, especially in hippocampus, is susceptible to various environmental stimuli (17), we have expected that intake of different nutrients under no calorie restriction might also affect the degree of hippocampal neurogenesis.

We used collagen as an sample which was the most abundant protein in animal tissues and served as protein source for human diet. A mixture of collagen peptide we tested has a mean molecular weight of 367.5, mainly consisted of tri-peptides (Gly-Pro-Hyp is the major component accounted for 8% in mass). Part of the proline in collagen is hydroxylated by the enzyme reaction in vivo. Proteins

DISCUSSION
In the present study, we found that oral administration of the lower molecular weight peptides derived from collagen enhanced hippocampal neurogenesis and exerted anxiolytic and antidepressant effects in adult mice. Since the level of adult neurogenesis, especially in hippocampus, is susceptible to various environmental stimuli (17), we have expected that intake of different nutrients under no calorie restriction might also affect the degree of hippocampal neurogenesis.

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$P = 0.84$). The total distance traveled during the 10-min test period was not significantly different between groups (LP mice: 4829.8 ± 488.1 cm, HP mice: 4596.2 ± 673.2 cm, $P = 0.36$). These results suggest that although the differences turned out to be small in this behavioral paradigm, LP-ingested mice showed a tendency of reduced anxiety-like behavior.

Fig. 2  A, B Fluorescent images of nuclear-staining (green), doublecortin-staining (blue) and BrdU-positive cells-staining (red) in dentate gyrus of hippocampus (left: LP mice, right: HP mice). Especially within the circles of figures, doublecortin-staining for premature neurons in LP mice (A) are stronger than HP mice (B). C, D Distribution of BrdU-positive cells (white) in the same area. Most of BrdU-positive cells were found in subgranular zone (SGZ). The linear density of BrdU-positive cells along SGZ (cells/mm SGZ) is higher in LP mice (C) as compared with HP mice (D). In the presented images, there are twenty BrdU-positive cells per 1858.2 μm subgranular zone in LP, and seven BrdU-positive cells per 1748.8 μm subgranular zone in HP. Scale bar: 200 μm.
are absorbed in the intestinal tract after they are degraded to peptides or amino acids. It has been demonstrated in the animal experiment that only lower molecular weight collagen peptides (less than 15,000) can be absorbed (18). Therefore, we considered LP used in this study (mean molecular weights of 367.5) served as efficient substrates compared with LP (mean molecular weights of about 30,000). In general, small molecules less than 500 tend to cross blood-brain barrier (BBB) (24). For example, peripheral administration of Gly-Pro-Glu is known to cross BBB in adult rats (2). Thus, it suggests that these peptides may be able to permeate into the brain tissue to take effect.

However, we still do not know whether these peptides affect the neural progenitor cells (NPCs) in a direct or indirect manner. For example, some growth factors like brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) directly act on NPCs to promote its proliferation for adult neurogenesis (21, 25). Therefore, in...
vitro analysis using NPC cell culture would be necessary to address this issue.

Moreover, it remains to be clarified which specific peptides are required for adult neurogenesis. Our preliminary study in cell culture suggests that the application of synthetic peptides of GPVGAR in the presence of 50 ng/mL NGF enhances the differentiation of PC12 cells into neurite-bearing cells. Therefore, these peptides might enhance hippocampal neurogenesis and produce antidepressant- and anxiolytic-like effects that we have observed with lower molecular weight collagen peptides. Further investigation is required to address this issue.

This study also provided novel evidence that ingestion of the lower molecular weight peptides derived from collagen influenced mood-related behavior. The correlation of hippocampal neurogenesis and clinical depression has been suggested, in which a reduced level of neurogenesis may provide a ground for the progress of this mental illness. As we expected, antidepressant trend was observed from the result of the tail suspension test. Interestingly, we also found that anxiety-related behavior was improved in the elevated plus maze test and the open field test, consistent with the fact that clinical depression often accompanies an increased level of anxiety (9). However, it still remains unclear whether there is a common neural pathway for depression and anxiety, and how hippocampal neurogenesis influences these emotional behavior.

In conclusion, we demonstrated that intake of the lower molecular weight peptides derived from collagen was able to influence the level of hippocampal neurogenesis and emotional behavior in a correlative manner. Our data indicate that food intake without dietary restriction also influences the level of adult neurogenesis and emotional behavior in a correlated manner. There may be a variety of natural substances yet to be discovered that contributes to the improvement of human health and prevention of neurological disorders.

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