Prolyl-hydroxyproline, a collagen-derived dipeptide, enhances hippocampal cell proliferation, which leads to antidepressant-like effects in mice

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Abstract
Depression has been a mental health issue worldwide. We previously reported that ginger-degraded collagen hydrolysate (GDCH) suppressed depression-like behavior in mice. Furthermore, prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG) were detected in the circulating blood after the oral administration of GDCH. In the present study, PO, but not OG, was detected in the cerebrospinal fluid of rats after the oral administration of GDCH, suggesting that PO is transported from blood to the brain. We then investigated the effects of PO and OG on the depression-like behavior of mice. The oral administration of PO significantly decreased depression-like behavior in the forced swim test. OG had no antidepressant-like effect. In addition, proline and hydroxyproline, components of PO, also had no antidepressant-like effect after their oral administration. PO significantly increased the gene expression of brain-derived neurotrophic factor and nerve growth factor in the hippocampus, and promoted the proliferation of neural progenitor cells in vivo and in vitro. PO also increased the dopamine concentration in the prefrontal cortex. Thus, PO-dependent regulation of neurotrophic function and neurotransmitter may be the mechanism for antidepressant-like behavior. Together, these results demonstrate that PO is an antidepressant bioactive peptide accompanying the proliferation of hippocampal neural progenitor cells.

KEYWORDS
corticosterone, depression, mental stress, neural stem cell, peptide

ABBREVIATIONS: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle medium; EGF, epidermal growth factor; GDCH, ginger-degraded collagen hydrolysate; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; NGF, nerve growth factor; NT-3, neurotrophin-3; OG, hydroxyprolyl-glycine; PBS, phosphate-buffered salts; PO, prolyl-hydroxyproline.

Dai Nogimura and Takafumi Mizushige contributed equally to this work.

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1 | INTRODUCTION

Collagen, a major component of the extracellular matrix, is one of the most abundant proteins in vertebrates, accounting for one-third of all proteins.1 The amino acid sequence of Gly-X-Y (X, Y: arbitrary amino acid) is repeated approximately 330 times. In many cases, the X is proline (Pro) and the Y is hydroxyproline (Hyp). Previous studies reported that Pro-Hyp (PO) and then Hyp-Gly (OG) were detected in the circulating blood after the oral ingestion of collagen hydrolysate.2-7

Depression is a prevalent health issue worldwide.8,9 The efficacy of antidepressant drugs is limited by a number of factors including the necessity for long-term administration, tolerance, and side effects. We previously reported that ginger-degraded collagen hydrolysate (GDCH) suppressed depression-like behavior in mice.10 Ginger protease exhibits potent activity and cleaves peptide bonds with Pro and Hyp in the second position (P2). Therefore, GDCH includes abundant amounts of X-Hyp-Gly- and Gly-Pro-Y-type tripeptides.3,11 The oral administration of GDCH resulted in higher circulating PO and OG concentrations than that of gastrointestinal enzyme-degraded collagen hydrolysate.3

A recent study demonstrated using in situ perfusion experiments that dipeptides, such as glycyl-sarcosine (Gly-Sar), Gly-Pro, and Tyr-Pro, penetrated the cerebrospinal fluid barrier in mice.12 The dipeptide form was transferred to the brain. This finding suggests that dietary dipeptides migrate to the brain and affect the brain function. In the present study, we investigated whether PO and OG were transferred to the cerebrospinal fluid after the oral administration of GDCH. PO, but not OG, was detected in the cerebrospinal fluid, suggesting that PO is transported from blood to the brain. In the present study, we investigated the effects of PO and OG on depression-like behavior using the forced swim test, a paradigm used in the development of antidepressants.

Neurogenesis is a physiological phenomenon in which neural stem cells proliferate and differentiate into neurons, and plays an important role in the brain formation and development. Neurogenesis in the hippocampus was previously shown to be essential in the formation of memory and learning.13,14 Recently, it has been reported that depressive behavior is involved in hippocampal neurogenesis. Depression with chronic stress decreased hippocampal neurogenesis, while the administration of antidepressants exerted the opposite effects.9,15,16 We previously reported that GDCH promoted neural stem cell proliferation in the hippocampus.10 Neurotrophic and nerve growth factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3), regulate the proliferation and differentiation of neural stem cells.17,18 In the present study, we investigated the effects of PO on the proliferation of hippocampal neural progenitor cells in vivo and in vitro as well as the gene expression of neurotrophic and growth factors in the hippocampus.

We previously demonstrated that the antidepressant-like activity of GDCH was abolished after blocking dopamine D1 receptors, but not serotonin 5-HT1A receptors,10 suggesting that dopamine signaling is important for the antidepressant-like activity of collagen hydrolysate. Dopamine concentration in the prefrontal cortex and hippocampus was found to be lower in the depression model mice and an antidepressant drug treatment recovered this decrease,19,20 indicating that dopamine release in the prefrontal cortex and hippocampus is related to antidepressant behavior. We determined the concentration of dopamine in the hippocampus and prefrontal cortex after the oral administration of PO.

2 | MATERIALS AND METHODS

2.1 | Animals

Male ddY mice (SLC, Shizuoka, Japan) at 5 weeks of age were raised in plastic cages in an environment-controlled room with a 12-hour light-dark cycle and at a constant temperature (23 ± 1°C) and humidity (50 ± 10%). Animals were group-housed for 5 days to acclimate them to the environment and were provided regular tap water and commercial solid chow (MF; Oriental Yeast, Osaka, Japan) ad libitum. The present study was conducted in accordance with the ethical guidelines of the University of Tsukuba Animal Experimentation Committee (Approval No. A17-0007 and A18-0003) and was in complete compliance with the National Institutes of Health: Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to limit experimentation to what was necessary to produce reliable scientific information.

Regarding the LC-MS analysis, the animal study was approved by the Experimental Ethical Committee of the Institute for Animal Reproduction and Nippon Research Institute of Biomatrix (Approval No. 16004). Five-week-old male Wistar-Imamichi rats (Institute for Animal Reproduction) were fed a collagen-free diet, AIN-93M (Oriental Yeast, Tokyo, Japan) ad libitum. The present study was conducted in accordance with the ethical guidelines of the University of Tsukuba Animal Experimentation Committee (Approval No. A17-0007 and A18-0003) and was in complete compliance with the National Institutes of Health: Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to limit experimentation to what was necessary to produce reliable scientific information.

Plasma and cerebrospinal fluid samples from rats after the oral administration of GDCH were prepared by the Institute for Animal Reproduction (Ibaraki, Japan). Rats were divided into six groups with six rats in each group. GDCH dissolved
in distilled water was orally administered at a dose of 600 mg/kg for five groups, and plasma and cerebrospinal fluid were collected 0.5, 1, 2, 4, or 6 hours later. In the remaining group, plasma and cerebrospinal fluid were collected without the administration of GDCH as a control (0 hour). Several cerebrospinal fluids and corresponding plasma samples were excluded because the mixing of blood in cerebrospinal fluid was visually observed. A previously developed internal standard mixture of collagen-derived amino acids and oligopeptides was mixed into plasma and cerebrospinal fluid samples, and samples were then deproteinized by the addition of three volumes of ethanol. The ethanol-soluble fraction was dried using the centrifugal evaporator CVE-3100 (EYELA, Tokyo, Japan) and reconstituted with 0.1% formic acid. PO and OG in samples were analyzed by LC-MS in the multiple reaction monitoring mode using a 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Foster City, CA, USA) coupled with an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA), as described previously.

2.3 | Forced swim test

The depression-like behavior of mice was assessed according to the method of Porsolt et al. Briefly, mice were individually forced to swim in an open cylindrical container (diameter, 10 cm; height, 20 cm) containing 10 cm of water at 25 ± 1°C. Water was changed after each trial. The forced swim test was performed during the light phase of the light/dark cycle. The total immobility time (s) was measured during a single 6-min test session. Mice were considered to be immobile when they made no attempts to escape as reflected by their movements to keep their heads above the water. A decrease in the immobility time was considered to be an indicator of an antidepressant-like effect. After 5 days of acclimatization, mice were divided into the required number of groups. Prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG) were purchased from Bachem AG (Bubendorf, Switzerland). Pro and Hyp were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Each reagent dissolved in saline was administered orally once a day (10:00-11:00) to each group for three consecutive days. On Day 3, the forced swim test was started 3 hours after oral administration.

2.4 | Immunohistochemistry for the neural cell proliferation assay in vivo

Mice were orally administered PO once a day for three consecutive days. On Day 3, mice were transcardially perfused with PBS followed by 4% paraformaldehyde under isoflurane anesthesia 3 hours after the administration of PO. After perfusion, each brain was collected and postfixed in 4% paraformaldehyde at room temperature for 1 hours, and then replaced in 30% sucrose at 4°C for 3 days. Brain sections were embedded in OCT compound and then cut to a thickness of 40 μm on a freezing microtome. After frozen sections had been washed with PBS, they were blocked in 3% normal donkey serum (Thermo Fisher Scientific Inc, Waltham, MA, USA) for 1 hours. Sections were incubated overnight with anti-Ki67 (1:100; rabbit monoclonal; GeneTex, Irvine, CA, USA) at 4°C. Sections were washed with PBS and incubated with anti-rabbit Alexa 488 (1:500; donkey polyclonal; Thermo Fisher Scientific Inc, Waltham, MA, USA) and DAPI (1:1000, Thermo Fisher Scientific Inc) at room temperature for 2 hours. Ki67-labeled cells at the hippocampal dentate gyrus were counted under the microscope (Leica microsystems GmbH, Wetzlar, Germany).

2.5 | Cell culture and neurosphere assay in vitro

Primary neural stem cells were isolated from the hippocampus of ddY mice at P1. The hippocampus was enzymatically treated with 0.25% trypsin/HBSS at 37°C for 5 min. Cells were then washed with 0.1% aprotinin and replaced with culture medium. The culture medium was composed of Dulbecco’s modified Eagle medium (DMEM)/Ham’s F12 (1:1; Thermo Fisher Scientific Inc), 2% B27 supplement (Thermo Fisher Scientific Inc), 0.5 mM L-glutamine (Wako, Tokyo, Japan), 1% penicillin-streptomycin (Thermo Fisher Scientific Inc), 10 ng/mL of basic fibroblast growth factor (bFGF) (PeproTech Inc., Rocky Hill, NJ, USA), and 20 ng/mL of epidermal growth factor (EGF) (PeproTech Inc). Cells were then suspended and passed through a 25-μm mesh, plated at the required amount (5000 cells/well), and cultured (37°C, 5% CO2). PO at a concentration of 1 mM was added on Day 4. Corticosterone (Sigma-Aldrich., St. Louis, MO, USA) at a concentration of 0.5 μM was continuously added from Days 4 to 8. PO was added 30 minutes before the addition of corticosterone. The area of the neurosphere was measured with ImageJ and calculated the fold change from PBS control.

2.6 | Real-time reverse transcription-polymerase chain reaction for the measurement of gene expression

Mice were divided into the required number of groups. The reagent dissolved in saline was administered orally once a day (10:00-11:00) to each group for three consecutive
On Day 3, mice were euthanized by decapitation and the hippocampus was collected. Total RNA was extracted from each hippocampus using the QIAzol Lysis Reagent (QIAGEN Sciences Inc) and purified using the RNeasy Mini Kit (QIAGEN Sciences Inc, Chatsworth, CA, USA). Total RNA was reversely transcribed to cDNA with random 6-mers using the ReverTra Ace qPCR RT Master Mix with the gDNA Remover (Toyobo Co., Osaka, Japan). In quantitative PCR, we amplified cDNA using the LightCycler 96 System (Roche Diagnostics Co., Mannheim, Germany) with the THUNDERBIRD qPCR Mix (Toyobo Co) and primers specific for mouse BDNF, NGF, NT-3, and β-actin according to the manufacturer’s instructions. These primer sequences are shown in Table 1. Reactions were cycled 45 times with denaturation at 95°C for 10 seconds, followed by annealing and elongation at 65°C for 60 seconds. The relative expression level of each mRNA was normalized using the mRNA level of β-actin.

### 2.7 Measurement of dopamine and metabolites

Dopamine in the hippocampus and prefrontal cortex was assessed using the HPLC electrochemical detector (ECD) system HTEC-500 (Eicom Co., Ltd., Kyoto, Japan). Brain samples were homogenized in 2.5 volumes of 0.2 M perchloric acid containing 0.1 mM EDTA 2Na (Dojindo Lab., Kumamoto, Japan) and 5 ng isoproterenol (Sigma-Aldrich). The homogenate was centrifuged at 20 000g for 15 minutes. Thirty microliters of the supernatant was added to 1.5 μL of 1 M sodium acetate and filtered through a centrifuge filter (0.22 μm; Merck Millipore, Billerica, MA, USA). The HTEC-500 system consisted of a reversed-phase column (Eicompack SC-50DS, 3 mm id × 150 mm; Eicom Co., Ltd.) and a graphite carbon working electrode (EICOM WE-3G; 12 mm id) with an Ag/AgCl reference electrode. The ECD potential was set at +750 mV for the working electrode. The acetate citrate buffer (pH 3.5) used for separation (flow rate, 0.5 mL/min at 4°C) contained 0.053 M citric acid, 0.047 M sodium acetate, 5 mg/L of EDTA, 220 mg/L of sodium octyl sulfonate (Nacalai Tesque, Inc, Kyoto, Japan), and 17% methanol (v/v).

### 2.8 Statistical analysis

Data are expressed as means ± standard errors (SE). Statistical comparisons between groups were performed by a one-way analysis of variance (ANOVA) followed by Tukey-Kramer’s test or the unpaired Student’s t test. The significance of differences was defined as $P < .05$. Statistical calculations were performed using SPSS version 24 software.

### 3 RESULTS

We measured PO and OG concentrations in plasma and cerebrospinal fluid after the oral administration of GDCH at a dose of 600 mg/kg to rats. PO was detected in plasma and cerebrospinal fluid 30 and 60 minutes after the administration of GDCH over the endogenous basal level (Figure 1A,B). PO concentrations in plasma and cerebrospinal fluid peaked 30 minutes after the administration (Figure 1A,B). Moreover, the concentration of OG in plasma was approximately one-twelfth that of PO (Figure 1A). OG was not detected in cerebrospinal fluid. PO might be efficiently generated from GDCH and penetrated the brain following the oral administration of GDCH.

We then investigated the effects of PO and OG on antidepressant-like activity in mice in the forced swim test. The oral administration of 10 mg/kg of PO resulted in a significantly shorter immobility time than that of control (Figure 2A). The shorter immobility time of PO seems to be
in a dose-dependent manner. In contrast, OG did not significantly affect immobility times (Figure 2B). Pro, Hyp, and the mixture of Pro and Hyp at a dose of 5 mg/kg also had no significant effect on immobility times (Figure 2C).

We evaluated neuronal proliferation in the hippocampus by counting ki67-positive cells in the dentate gyrus. The number of ki67-positive cells in the dentate gyrus was significantly higher following the oral administration of 10 mg/kg of PO than in the control (Figure 3A-C). We examined the effects of PO on neuronal proliferation in vitro. The area of the neurosphere in cultured neural stem cells was significantly wider following the administration of PO at a concentration of 1 mM than with the vehicle (Figure 4A-C). The area of neurosphere in cultured neural stem cells was significantly lower following the administration of corticosterone at a concentration of 0.5 μM than with the vehicle (Figure 4D). PO fully restored proliferation in corticosterone-treated cells (Figure 4D).

We also investigated the effects of PO on the mRNA expression of neurotrophic and growth factors in the hippocampus. Hippocampal BDNF and NGF mRNA expression were significantly stronger in the PO group than in the control group (Figure 5A,B). Hippocampal NT-3 mRNA expression was slightly stronger in the PO group than in the control group (Figure 5C).

We measured the concentration of dopamine and the metabolite in the hippocampus and prefrontal cortex when PO was orally administered to mice. Dopamine...
concentrations in the prefrontal cortex but not in the hippocampus were higher in PO-treated mice than in control mice (Figure 6A,B). No significant differences were observed in the concentration of dopamine metabolites in the prefrontal cortex and hippocampus (Table 2).

4 | DISCUSSION

In the present study, PO was detected in cerebrospinal fluid when GDCH was orally administered to rats. Previous studies reported that PO and OG were detected in circulating blood after the oral administration of collagen hydrolysate.\(^2\)\(^-\)\(^7\) PO and OG were previously shown to be more efficiently transported into the blood after the oral administration of GDCH than control collagen hydrolysate.\(^3\) In the present study, PO concentrations in plasma were
approximately 12-fold higher than those of OG 30 minutes after the oral administration of GDCH. OG was below the detection limit in cerebrospinal fluid. Dipeptides, such as Gly-Sar, Gly-Pro, and Tyr-Pro, have shown to penetrate the cerebrospinal fluid barrier of mice in in situ perfusion experiments.\textsuperscript{12} We revealed that a dipeptide generated from dietary protein hydrolysate was transferred to cerebrospinal fluid. PO may be transferred into the brain parenchyma in its dipeptide form. Further studies are needed to elucidate the mechanisms by which PO is transferred into cerebrospinal fluid and brain parenchyma.

Endogenous collagen may affect the brain function. Previous studies reported that the knockout of collagen type 6 in mice increased cell death and neurotoxicity with amyloid beta in the hippocampus and resulted in cognitive decline\textsuperscript{23,24} Collagen type 6 also improved the ultraviolet-induced inhibition of axon extension.\textsuperscript{25} Thus, central collagen type 6 may play an important role in maintaining the survival of neurons. Furthermore, collagen hydrolysate has been shown to inhibit the expression of matrix metalloproteinases (MMP), which are extracellular matrix enzymes involved in collagen degradation.\textsuperscript{26,27} In the present study, functional collagen may have been increased by inhibiting the expression of MMP and suppressing the degradation of central collagen by PO. Moreover, PO in the CSF may have been derived from the administered hydrolysate. Further studies are needed to clarify whether the administration of PO changes the amount of collagen and if fluorescent-labeled PO may be detected in the brain after its administration.

In the present study, antidepressant-like activity was observed following the oral administration of PO. Oral administration of OG, Pro, and Hyp had no effect, suggesting that the peptide form of PO is important for antidepressant-like activity. Dipeptides such as pyroglutamyl-leucine and leucyl-isoleucine exhibit antidepressant-like activity.\textsuperscript{28,29} Emotional behavior may be regulated by dipeptides derived from food proteins.

Depressive behavior is closely associated with hippocampal neurogenesis. The administration of antidepressants, such as imipramine and fluoxetine, was shown to promote the proliferation of hippocampal neural progenitor cells.\textsuperscript{9,15,16,30} Tunc-Ozcan et al and Hill et al previously reported that the inhibition of newly generated hippocampal neurons increased depression-like behavior in mice.\textsuperscript{31,32} Moreover, stressed conditions such as corticosterone administration and chronic unpredictable mild stress suppressed the proliferation of hippocampal neural progenitor cells.\textsuperscript{33,34} The proliferation of cultured neural progenitor cells was shown to be inhibited by the addition of corticosterone.\textsuperscript{35} Corticosterone regulated the proliferation of neural progenitor cells via the signal pathway with glucocorticoid receptor, GR.\textsuperscript{36,37} In the present study, PO also directly promoted neural progenitor cell proliferation and improved the suppression of proliferation caused by the addition of corticosterone. Further studies are needed to identify the target molecule and signal pathways in neural progenitor cells and brain tissue.

Neurotrophic and nerve growth factors, such as BDNF and NGF, regulate the proliferation and differentiation of neural progenitor cells.\textsuperscript{17,18} Previous studies reported that the gene expression of BDNF in the hippocampus decreased with chronic stress.\textsuperscript{38-40} Furukawa-Hibi et al and Deltheil et al demonstrated that the antidepressant-like activity of antidepressants disappeared in BDNF knockout mice.\textsuperscript{28,41} Deltheil et al also reported that the intraventricular administration of BDNF exhibited antidepressant activity.\textsuperscript{41} Thus, BDNF regulates neural progenitor cell proliferation and differentiation and exerts neuroprotective effects. Moreover, the gene expression of NGF decreased in the hippocampus of chronic stress model mice.\textsuperscript{42,43} NGF regulates neuronal growth and maturation. Based on these findings, BDNF and NGF are regarded as major factors that control depressive behavior through the proliferation of neural progenitor cells. In the present study, BDNF and NGF mRNA expression levels in the hippocampus were higher in mice after the oral administration of PO than in controls. These results suggest that PO promotes the expression of BDNF and NGF in the hippocampus and subsequently induced the proliferation of hippocampal neural progenitor cells. Hippocampal neurotrophic factor and neurogenesis regulate short-term memory and space perception. Further studies are needed to examine the cognitive function as a novel function and the related neurotransmitter such as acetylcholine after the administration of collagen peptides.

In the present study, we found that orally administered PO increased the concentration of dopamine in the prefrontal cortex. Antidepressants have been shown to increase the dopamine concentration in the hippocampus and prefrontal cortex.\textsuperscript{44,45} Dopamine concentration was decreased in the prefrontal cortex in the chronic stress model mice and recovered by the administration of antidepressants.\textsuperscript{46,47} In addition, depression-like behavior was induced by the knockdown of dopamine receptors in the prefrontal cortex.\textsuperscript{47} An increase in the dopamine concentration in the prefrontal cortex may be important for the control of depression-like behavior. We previously reported that the antidepressant-like activity of GDCH disappeared following the inhibition of dopamine D\textsubscript{1} receptors, but not serotonin 5-HT\textsubscript{1A} receptors.\textsuperscript{10} Thus, the antidepressant-like activity of collagen hydrolysate and PO may be dependent on neural proliferation in the hippocampus and the facilitation of dopamine release in the prefrontal cortex.

Collectively, the present results demonstrated for the first time that collagen-derived PO was transported from blood to cerebrospinal fluid in the form of a peptide. Additionally, the oral administration of PO exhibited antidepressant-like
activity. PO may be the active substance for the antidepressant-like activity of GDCH. This activity may be dependent on neural progenitor cell proliferation in the hippocampus and dopamine activation in the prefrontal cortex.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
T. Mizushige supervised and designed the experiments. D. Nogimura, Y. Taga, A. Nagai, S. Shoji, and N. Azuma, with help from T. Mizushige, M. Kusubata, S. Adachi, F. Yoshizawa, and Y. Kabuyama, performed the experiments and analyzed the data. D. Nogimura and T. Mizushige wrote the paper. All authors discussed the results and manuscript.

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