Ginger-Degraded Collagen Hydrolysate Exhibits Antidepressant Activity in Mice

Takafumi Mizushima1, Dai Nogimura1, Akitoshi Nagai1, Haruka Mitsuhashi1, Yuki Taga2, Masashi Kusubata2, Shunji Hattori2 and Yukihito Kabuyama1

1 Department of Applied Biological Chemistry, Faculty of Agriculture, Utsunomiya University, 350 Minemachi, Utsunomiya, Tochigi 321–8505, Japan
2 Nippi Research Institute of Biomatrix, 520–11 Kuwabara, Toride, Ibaraki 302–0017, Japan
(Received October 8, 2018)

Summary Collagen is the most abundant protein in animals. Collagen hydrolysate has been found to have multiple functions in the skin, bones, joints, muscles, and blood vessels. Recently, it has been reported that the low molecular weight fraction of collagen hydrolysate exhibited anxiolytic activity, suggesting that collagen peptides affect brain functions. In the present study, we found that oral administration of ginger-degraded collagen hydrolysate (GDCH) significantly decreased depression-like behavior in a forced swim test, suggesting that GDCH exhibited antidepressant activity in mice. The antidepressant activity of GDCH was abolished by pre-treatment with an antagonist of the dopamine receptor, but not treatment with a serotonin receptor antagonist. GDCH significantly increased gene expression of glial cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) in the hippocampus, molecules that affect the differentiation and survival of neurons, relative to that in the control condition. Meanwhile, there were no changes in the gene expression of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3, major factors related to depression-like behavior. We also found that GDCH exhibited antidepressant activity in corticosterone-administered mice in a model of stress. In addition, GDCH increased GDNF and CNTF expression in the stressed condition, suggesting that mechanisms of the antidepressant activity of GDCH were the same in unstressed and stressed conditions. These results imply that GDCH exhibits antidepressant activity in unstressed and stressed conditions in mice. The upregulation of neurotrophic genes in the hippocampus may contribute to the reduction of depression-like behavior via a dopamine signal pathway modulated by GDCH.

Key Words food-derived peptide, depression, mental stress, hippocampus, monoamine neurotransmitter, neurotrophic factor, neural stem cell, corticosterone

Collagen is the most abundant animal protein and is the main constituent of skin, bone, cartilage, and the extracellular matrix. One-third of collagen’s constituent amino acids are glycine in a repeating Gly-X-Y sequence, where X and Y are other amino acids. However, there is no tryptophan or cysteine in collagen. Hence, collagen protein has low nutritional value. However, it was reported that collagen hydrolysate with collagenase exhibited various efficacious functions in the skin, bones, joints, muscles, and blood vessels. Recently, Kakoi et al. reported that the low molecular weight fraction of collagen peptides exhibited anxiolytic activity (1), suggesting that collagen peptides affect brain function. It has been reported that ginger enzyme has potent protease activity and cleaves peptide bonds with proline at the P2 position, that is, hydrolyzes collagen protein, which consists of approximately 20% proline. Moreover, oral administration of collagen hydrolysate prepared with ginger enzyme is efficiently absorbed in the form of short-chain peptides like di- and tripeptides (2, 3). We expected that this ginger-degraded collagen hydrolysate (GDCH) might have potent activity in the brain.

Major depressive disorder, one of the most common psychiatric diseases, is characterized by dysregulation of emotion and mood with abnormalities of cognitive function, sleep, appetite, and metabolism, among others (4, 5). Depression is an epidemic health problem all over the world. It is estimated that there are around 322 million depressed patients in the world according to the World Health Organization. In the current study, we investigated whether collagen hydrolysate exhibits a fast-acting antidepressant-like effect by examining its effects in the forced swim test, a paradigm used in the development of antidepressants. It is known that chronic stress decreases hippocampal neurogenesis and this neurogenesis can be increased by the administration of antidepressants (5–7). Neurotrophic and nerve growth factors such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and nerve growth factor (NGF) regulate proliferation and differentiation of neural stem cells. We thus examined whether collagen hydrolysates exhibited antidepressant activity and
increased neurogenesis in vivo, and whether the expressions of genes for neurotrophic and growth factors were changed in the hippocampus.

The hypothalamic-pituitary-adrenal (HPA) axis activates in response to stress. In general, corticosterone secreted from the adrenal glands has a negative feedback effect, downregulating the HPA axis. However, chronic high concentrations of corticosterone induce attenuation of neurogenesis, abnormal function and morphology of neurons, and behavioral abnormalities (8, 9). It has been reported that corticosterone administration increases the expression of depression-like behaviors (10–13). Thus, we investigated whether collagen hydrolysate is effective in a stress-induced mouse model of depression where mice were administered corticosterone for 21 d.

MATERIALS AND METHODS

Animals. Male ddY mice (Japan SLC, Inc., Shizuoka, Japan) at 5 wk of age were raised in plastic cages in an environment-controlled room with a 12-h light-dark cycle (dark phase: 17:00–05:00), maintained at a constant temperature (23 ± 1˚C). The animals were group-housed for 5 d to acclimate them to the environment. Animals were provided regular tap water and a commercial solid chow (MF; Oriental Yeast Co., Ltd., Osaka, Japan) ad libitum. This study was conducted in accordance with the ethical guidelines of the Utsunomiya University Animal Experimentation Committee (Approval No. A17-0007) 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.

Reagents. WAY100135 dihydrochloride, a serotonin 5-HT1A receptor antagonist was purchased from Tocris Bioscience (Bristol, UK). R(+)-SCH-23390 hydrochloride, a dopamine D1 receptor antagonist was purchased from Sigma Aldrich (St. Louis, MO, USA).

Preparation of collagen hydrolysate. Collagen hydrolysate was prepared from bovine bone gelatin using ginger rhizome according to a previously described method (2), with a slight modification for industrial production. The average molecular weight of the collagen hydrolysate (GDCH) was 816 Da (14).

Forced swim test. The depression-like behavior of mice was assessed according to the method of Porsolt et al. (15). 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. 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 immobile when they made no attempts to escape reflected in their movements to keep their heads above the water. A decrease in the immobility time was considered an indicator of an antidepressant-like effect. After 5 d of acclimatization, GDCH dissolved in saline were administered orally once a day (10:00–11:00) at a dose of 0, 30, or 100 mg/kg body weight in each group for 3 consecutive days. On Day 3, the forced swim test was started 3 h after oral administration. WAY100135 dihydrochloride (10 mg/kg) or R(+) -SCH-23390 hydrochloride (30 μg/kg) was administered intraperitoneally once 30 min before the administration of the hydrolysate or saline.

Y-maze test. GDCH dissolved in saline were administered orally once a day (10:00–11:00) at a dose of 0 or 100 mg/kg body weight in each group for 3 consecutive days. On Day 3, the forced swim test was started 3 h after oral administration. WAY100135 dihydrochloride (10 mg/kg) or R(+)-SCH-23390 hydrochloride (30 μg/kg) was administered intraperitoneally once 30 min before the administration of the hydrolysate or saline.

### Table 1. Primer sequences used in the present study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences</th>
<th>Product size (bp)</th>
<th>NCBI ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>sense TCAGTTGGCCTTTTGATAACC</td>
<td>85</td>
<td>NM_007540</td>
</tr>
<tr>
<td></td>
<td>antisense GGGCAGATAAAAAAGACTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>sense CAGGCAGAACCTACACAGA</td>
<td>91</td>
<td>NM_013609</td>
</tr>
<tr>
<td></td>
<td>antisense CTGTGTCAAGGAAATGCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT-3</td>
<td>sense TGGCCGAAAGCTCTCCTCAAAT</td>
<td>87</td>
<td>NM_001164034</td>
</tr>
<tr>
<td></td>
<td>antisense CATCCACCATCTGTGTTGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDNF</td>
<td>sense GGCTGACTTCATCAGGCAAT</td>
<td>99</td>
<td>NM_010275</td>
</tr>
<tr>
<td></td>
<td>antisense TTCCAGAGGCCCATTAACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFα</td>
<td>sense GAGAGAGGGCGAAGTCCCTTTT</td>
<td>96</td>
<td>NM_001287056</td>
</tr>
<tr>
<td></td>
<td>antisense GGAATTGGTTTTGCTGTGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td>sense GGCTGCTGGTCTAAGTGT</td>
<td>87</td>
<td>NM_008006</td>
</tr>
<tr>
<td></td>
<td>antisense TCCGTCACCCTAAGTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>sense GTGCCACTTGTCCATGTCATC</td>
<td>91</td>
<td>NM_010514</td>
</tr>
<tr>
<td></td>
<td>antisense AACCTTTGAGATTTTGGCCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>sense TGCTTCTAGGGCGAAGTCACTG</td>
<td>68</td>
<td>NM_007393</td>
</tr>
<tr>
<td></td>
<td>antisense CTGCACAGTATGTTTGTGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
arm. Mice were allowed to move freely for 10 min. An arm entry was counted when the hind paws of mouse were entering into the arm completely. Total arm entering was recorded as index of the spontaneous behavior.

Real-time reverse transcription polymerase chain reaction (RT-PCR). GDCH dissolved in saline were administered orally once a day (10:00–11:00) at the dose of 0 or 100 mg/kg body weight in each group for 3 consecutive days. On Day 3, the mice were euthanized by decapitation, and the hippocampus was removed. Total RNA was extracted from the hippocampus using the QIAzol Lysis Reagent (QIAGEN Sciences Inc., Germantown, MA) and RNeasy Mini Kit (QIAGEN Sciences Inc.). The total RNA was transcribed to cDNA with random 6-mers using PrimeScript™RT Master Mix (Takara Bio Inc., Shiga, Japan). For quantitative PCR, we amplified the cDNA using the LightCycler® 96 System (Roche Diagnostics Co., Mannheim, Germany) with THUNDERBIRD® qPCR Mix (Toyobo Co., Osaka, Japan) and primers specific for mouse brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), vascular endothelial growth factor α (VEGFα), fibroblast growth factor 2 (FGF2), insulin like growth factor 2 (IGF2), and β-actin according to the manufacturer’s instructions. These primer sequences are shown in Table 1. The reactions were cycled 45 times with denaturation at 95˚C for 10 s, followed by annealing and elongation at 65˚C for 60 s. The relative expression level of each mRNA was normalized using the mRNA level of β-actin.

Immunohistochemistry for neural cell proliferation assay. Mice were divided into two groups. GDCH dissolved in saline was orally administered once a day (10:00–11:00) at a dose of 0 or 100 mg/kg body weight in each group for 3 consecutive days. On Day 3, the mice were euthanized by decapitation, and the brain was removed. Each brain was fixed with 4% PFA 3 h after the administration and then immunostained with anti-ki67 (1 : 100; rabbit monoclonal; GeneTex, Inc., Irvine, CA, USA), anti-rabbit, alexa 488 (1 : 500; donkey polyclonal; Thermo Fisher Scientific Inc., Waltham, MA, USA) antibodies, and DAPI (Thermo Fisher Scientific, Rockford, IL, USA). Proliferating cells in the hippocampus were counted under the microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Corticosterone treatment. Corticosterone dissolved in saline including 0.1% Tween 80 and 0.1% dimethyl sulfoxide (DMSO) was subcutaneously administered once a day. 3 h after the oral administration of the hydrolysate or vehicle at a dose of 20 mg/kg body weight in the corticosterone-treated group for 21 consecutive days.

Statistical analysis. Data are expressed as mean± standard errors. Statistical significance was defined as p<0.05. In Fig. 1, statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett’s test. In Figs. 2 and 3, statistical comparisons between groups were performed using unpaired t-test. In Fig. 4A, statistical comparisons between groups were performed using two-way ANOVA. In Fig. 4B, statistical comparisons between groups were performed using two-way ANOVA followed by Tukey’s test. In Figs. 5 and 6, statistical comparisons between groups were performed using one-way ANOVA followed by Tukey’s test. Statistical calculations were performed using SPSS version 24 software.

RESULTS

We examined the effect of collagen hydrolysate on...
depression-like behavior as reflected in a forced swim test in mice. Immobility time in the GDCH group (30 and 100 mg/kg body weight) was significantly lower than in the control group (Fig. 1). There was no effect on spontaneous behavior in the Y-maze task (Data not shown). These results show that GDCH exhibited antidepressant-like activity.

Next, we examined the effect of collagen hydrolysates on the mRNA expression of neurotrophic and growth factors in the mice hippocampus. GDNF and CNTF mRNA expressions in the GDCH group (100 mg/kg body weight) were significantly higher than in the control group (Fig. 2A and 2B). These changes were approximately 1.5-fold larger than in the control group. There were no significant differences between the control and GDCH groups regarding the mRNA expressions of BDNF, NGF, NT-3, VEGFα, FGF2, or IGF2 in the hippocampus (Table 2). These results suggest that GDCH up-regulated GDNF and CNTF expression in the hippocampus and might have induced a neurotrophic effect on neural stem cells, astrocytes, and neurons.

To examine the proliferative activity in hippocampal neural stem cells, we counted ki67-positive cells in the dentate gyrus of the hippocampus. The number of hippocampal ki67-positive cells was significantly higher than in the control group (Fig. 3). This change was approximately 1.4-fold larger than in the control group. This result suggests that GDCH promoted the proliferation of hippocampal neural stem cells.

Serotonin and dopamine are major neurotransmitters related to depressive behavior. To clarify whether

<table>
<thead>
<tr>
<th>Genes</th>
<th>Control</th>
<th>GDCH</th>
<th>target gene mRNA/β-actin mRNA (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>1.00±0.03</td>
<td>1.08±0.05</td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>1.00±0.03</td>
<td>1.31±0.17</td>
<td></td>
</tr>
<tr>
<td>NT-3</td>
<td>1.00±0.04</td>
<td>1.12±0.07</td>
<td></td>
</tr>
<tr>
<td>VEGFα</td>
<td>1.00±0.05</td>
<td>1.09±0.09</td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td>1.00±0.06</td>
<td>1.38±0.23</td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>1.00±0.07</td>
<td>1.10±0.07</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean±SE, n=7–8.

---

Fig. 3. Effects of collagen hydrolysate on the proliferations of neural stem cell in the hippocampal dentate gyrus. Mice were administered saline or collagen hydrolysate for 3 d. The hippocampi were removed on Day 3 and were immunostained for ki67 protein. The hippocampal puncta were counted to evaluate the proliferation of neural stem cell. Each value represents the mean±SE (n=6–7). Asterisks (*) indicate a significant difference in comparison with the control mice (p<0.05).

Fig. 4. Dopamine receptor antagonist administration attenuated the antidepressant activity of collagen hydrolysate (A) but not serotonin receptor antagonist administration (B). Each value represents the mean±SE (A: n=5–6, B: n=8–9). Different characters represent significant differences (p<0.05) among each group.
the serotonin and dopamine pathways were involved in the antidepressant activity of GDCH, we investigated the antidepressant activity of GDCH after administration of receptor antagonists. The administration of SCH23390, an antagonist of the D1 dopamine receptor, reversed the antidepressant activity of GDCH (Fig 4A, two-way ANOVA, GDCH: not significant, SCH23390: not significant, GDCH*SCH23390: p<0.05). On the other hand, the administration of WAY100135, an antagonist of the 5-HT1A serotonin receptor, did not affect the antidepressant activity of GDCH (Fig 4B, two-way ANOVA, GDCH: p<0.05, WAY100135: not significant, GDCH*WAY100135: not significant).

Next, we investigated the antidepressant activity of GDCH in corticosterone-administered mice as model of stress conditions. Corticosterone administration for 21 d significantly increased immobility time in the forced swim test (Fig. 5). In the corticosterone-administered mice, immobility time in the GDCH group was significantly lower than in the control group (Fig. 5). These results suggest that GDCH suppressed the depression-like behavior induced by corticosterone administration.

In addition, in the corticosterone-administered mice, GDNF and CNTF mRNA expressions in the GDCH group were significantly higher than in the control group (Fig. 6). However, there were no significant differences in GDNF and CNTF mRNA expression between the vehicle-control and corticosterone-control groups (Fig. 6), suggesting that GDNF and CNTF might not be involved in the depressant effect induced by corticosterone administration. Taken together, GDNF and CNTF expressions were up-regulated by GDCH in both normal and stress conditions.

**DISCUSSION**

In the present study, we investigated the effect of GDCH on depression-like behavior in mice. We found that GDCH decreased immobility time in the forced swim test and had no effect on spontaneous behavior in the Y-maze task, showing that GDCH exhibited antidepressant activity in mice.

Kakoi et al. reported that collagen hydrolysate, which has a mean molecular weight of 367.5 Da, enhanced neurogenesis in the hippocampus and exhibited anxiolytic activity in mice (1). Dipeptides or tripeptides produced from collagen might have important actions on the brain. However, it has been unclear whether these peptides penetrate into the brain. Ginger has a cysteine-type protease that cleaves peptide bonds with Pro at the P2 position. Taga et al. reported that the GDCH used in the present study included the Gly-Pro-Y-type dipeptides and Gly-Pro-X-hydroxyproline (Hyp)-Gly-type tripeptides and is effectively absorbed in the circulatory system (2). We have reported that several kinds of di- and tri-peptides are absorbed into the circulatory system from gastrointestinal tract when collagen hydrolysate is administered orally (16). It is possible that low-molecular-weight peptides such as di- and tri-peptides affect the antidepressant activity of GDCH. Further investigation will elucidate the structure and active site of the peptide exhibiting antidepressant activity after intestinal absorption and hepatic metabolism.

It has been reported that hydrolysates of food protein and low molecular weight of peptides derived from food proteins exhibit antidepressant activity. Yamamoto et al. reported that pyroglutamyl leucine and pyroglutamyl glutaminyl leucine derived from wheat glutenin and gliadin exhibit antidepressant activity and enhance hippocampal neurogenesis after intraperitoneal and intracerebroventricular administration in mice (17). Mori et al. reported that soybean β-conglycinin hydrolysate with thermolysin had antidepressant activity via the vagus nerve in mice (18). Several groups reported that egg protein hydrolysate promoted mood and emotional function in humans (19–21). These results suggest that regulation of incentive may be impacted by food-derived peptides.

It has been reported that neurotrophic and nerve growth factors were associated with regulation of the depression-like behavior. It is reported that a release of...
the glial cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor superfamily promoted after treatment with antidepressants such as amitriptyline, clomipramine, mianserin, fluoxetine, and paroxetine in cultured glial cells (22). In addition, Liu et al. and Wan et al. reported that antidepressants such as clomipramine and schisandrin increased GDNF expression in the hippocampus of model animals where depression was induced by chronic unpredictable mild stress (23, 24). Meanwhile, ciliary neurotrophic factor (CNTF), which regulates neuronal development and neuroprotection, is also related to depression-behavior. Peruga et al. reported that CNTF-knockout mice increased more depression-like behaviors than normal mice (25). In the present study, we found that GDNF and CTNF mRNA expression in the hippocampus of mice that were orally administered GDCH was significantly higher than in the control animals. These results suggest that GDCH promotes the gene expression of GDNF and CNTF in the hippocampus, which are both related to depression-behavior in mice.

We found that the antidepressant effect of GDCH was abolished after blocking dopamine receptors, but not serotonin receptors. It has been reported that the dopamine level in the hippocampus was lower in depressive model mice, and that antidepressant drug treatment recovered the decrease in the hippocampal dopamine level (26–28), suggesting that hippocampal dopamine was related to antidepressant behavior. Intriguingly, GDNF treatment of cultured brain slices promoted differentiation and survival of dopaminergic neurons (29). Moreover, intracerebroventricular administration of GDNF increased the release of dopamine in the nucleus accumbens (30). Further investigation will elucidate the relationships between dopamine signaling, GDNF expression, and depression-like behavior. Previous studies have highlighted brain-derived neurotrophic factor (BDNF) as a major factor in relation to depression (31–35). Unexpectedly, there was no change in the expression of BDNF in GDCH-treated mice.

A treatment with corticosterone for 3 wk was used to induce a model of depression in mice, which highlights the potential for depression to be activated by the stress response (10–13). Consistent with previous reports (10–13), our results show that corticosterone treatment increases the expression of depression-like behaviors. Corticosterone administration did not affect GDNF or CNTF expression, suggesting that corticosterone-induced depression-like behavior is independent of GDNF and CNTF. GDCH treatment recovered the increase in depression-like behavior caused by corticosterone treatment in the stress-induced depressive model mice. In addition, GDCH administration of corticosterone-treated mice significantly increased the gene expression of GDNF and CNTF, similar to that seen in the unstressed condition. These results suggest that GDCH has an effective antidepressant action in stressed condition that has the same mechanism as in normal conditions.

In summary, the present study suggests that GDCH exhibits antidepressant activity in normal and corticosterone-administered mice. In addition, GDCH increases the mRNA expression of GDNF and CNTF, which promote the dopaminergic differentiation of neural stem cells. The antidepressant activity of GDCH was inhibited by the administration of a dopamine receptor antagonist. These results imply that GDCH exhibits its antidepressant activity via the dopaminergic signaling pathway.

Acknowledgments
This work was partially supported by JSPS KAKENHI Grant Number 16K07731 and the Nutrition and Food Science Fund of Japan Society of Nutrition and Food Science. Experiments were partially performed at the Center for Bioscience Research and Education, Utsunomiya University.

REFERENCES
13) Ali SH, Madhana RM, K V A, Kasala ER, Bodduluru LN,


27) Sehar N, Agarwal NB, Vohora D, Raisuddin S. 2015. Atorvastatin prevents development of kindling by modulating hippocampal levels of dopamine, glutamate, and GABA in mice. Epilepsy Behav 42: 48–53.


34) Jha S, Dong B, Sakata K. 2011. Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV. Transl Psychiatry 1: e40.