Effects of a Tea Cultivar "MK5601" on Behaviors and Hippocampal Neurotrophin-3 Levels in Middle-Aged Mice

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Summary Dietary factors are thought to play an important role in the prevention of cognition diseases and depression in late life. In the present study, we compared the effects between the theogallin-rich tea cultivar, "MK5601" and a common Japanese tea cultivar, "Yabukita" on behaviors and hippocampal neurotrophin levels in experimental animals. Middle-aged mice (aged 8 mo) were given either of the tea infusions or water ad libitum for 4 mo. In the novel object location test, the middle-aged mice drinking water or "Yabukita" performed worse than young mice (aged 2–3 mo) although the middle-aged mice drinking "MK5601" retained spatial memory at the same level as the young mice. We also found that the middle-aged mice drinking "MK5601" showed high levels of neurotrophin-3 in the hippocampus. In conclusion, the "MK5601" tea infusion appears to be effective in preventing age-related changes in cognitive function, as compared with a common Japanese tea cultivar.

Key Words "MK5601" tea cultivar, theogallin, cognitive function, neurotrophin-3, middle-aged mice

Cognition diseases as well as depression in late life are recognized as serious public health problems. Interventions against cognitive impairment and mood disturbance should be effective in reducing the social and economic burden. Dietary factors are thought to play an important role in the prevention of these diseases. The tea plant, *Camellia sinensis* L., has been cultivated for thousands of years, and its leaves were used for medicinal purposes. Recently, a considerable number of epidemiological studies have shown an inverse association between tea consumption and cognitive impairment (1, 2). Another systematic review of epidemiological studies that focused on green tea supported the hypothesis that green tea consumption might decrease the incidence of cognitive impairment (3). Moreover, several epidemiological studies have found that tea consumption may also improve mental performance and reduce the risk of depression (4). One pilot intervention study (5) with normal green tea powder suggested the potential effects of typical green tea consumption on cognitive dysfunction. Since the design of the pilot study was a before-after trial, an additional study was conducted as a randomized, placebo-controlled trial (6). However, the results showed that green tea consumption did not significantly affect cognitive function in the elderly. Thus, the effects of continuous consumption of typical Japanese green tea on cognitive function are inconclusive, and some fortifications of green tea should be needed to obtain clear evidence.

Green tea has demonstrated its multiple bioactivities (7) as well as the preventive effects for cognitive impairment. We previously reported anti-atherogenic (8) and anti-diabetic (9) effects of flavonol-rich tea cultivars. The results from both studies suggest higher efficacy of the flavonol-rich tea cultivar, as compared with a common Japanese cultivar, "Yabukita." Therefore, selecting distinct tea cultivars depending on its ingredient composition is expected to enhance cognitive effects of green tea. One of the Japanese cultivars, Cha Chuukanbohon Nou 6, was found to be high in polyphenolic compounds, such as theogallin, and 1,2-di-O-galloyl-4,6-O-(S)-hexahydroxy-diphenoyl-β-d-glucopyranose (G-strictinin) (10). Another tea cultivar, "MK5601," which is bred from Cha Chuukanbohon Nou 6, also keeps these levels as high compared with common Japanese varieties. In the present study, therefore, we compared the cognitive effects between the theogallin-rich "MK5601" and "Yabukita" cultivar in experimental animals.

Although severe cognitive impairment notably occurs in dementia and neurodegenerative diseases, mild cognitive deficits are also prevalent in healthy older subjects in humans, even in middle-aged subjects (11), as well as in animal models. Batteries of behavioral tests with C57BL/6 wild-type mice (12, 13) demonstrated the age-related changes during the early stages of life, and from 8 to 12-mo-old group exhibited pronounced changes in most behavior, including deficits in spatial memory, as compared with 2–3-mo-old young group. Habitual green tea consumption is considered more effective to prevent mild cognitive deficits during the early stages of life, rather than to suppress the patho-

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logical changes in brain of dementia. In the present study, therefore, we evaluate the effects of tea infusions on behaviors in middle-aged mice. The mice were given the tea infusion of “MK5601” from 8 to 12 mo of age when the early changes in behaviors due to aging were observed. We also assessed its effects on hippocampal levels of neurotrophins and growth factors that are associated with the mechanisms underlying the behavioral changes in experimental animals.

MATERIALS AND METHODS

Green tea samples. “MK5601” and control “Yabukita” green tea leaves were obtained from the NARO plantations in Makurazaki (Kagoshima, Japan) and Kanaya (Shizuoka, Japan). The dried green tea leaves of each cultivar were crushed into fine powders with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and separately steeped in cold water for 1 h. To simulate leaf tea infusions, the tea suspensions were centrifuged (10 min, 4°C, 1,200 × g) and the resultant supernatants were used in animal experiments. Aliquored stock solutions were stored at −20°C and diluted with water before being administered to the mice. Dilution factors were determined based on the results from our pilot studies in which we estimated the maximum dose of tea ingredients that would not induce reduction of voluntary daily intake. Fresh tea infusion was provided to all mice every 2 d. High performance liquid chromatography (HPLC) was used to measure the levels of caffeine, L-theanine and catechins in the stock solutions, as described previously (14). Epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epicatechin (EC) were detected at 272 nm, and epigallocatechin (EGC) and gallatechin (GC) were detected at 242 nm. Theogallin, strictinin and G-strictinin were detected at 272 nm. The average preservation rates of the ingredients during frozen storage of stock solutions were as follows, caffeine: 99.4%, L-theanine: 97.0%, theogallin: 98.3%, strictinin: 98.7%, G-strictinin: 99.4%, EGCG: 99.5%, ECG: 99.6%, EGC: 99.4%, GC: 98.5%, EC: 99.4%.

Animals. C57BL/6J mice (male), aged 1 mo for young mice and 6 mo for middle-aged mice, were procured from Japan SLC, Inc. (Shizuoka, Japan). The mice were given a standard laboratory diet (CRF-1, Charles River Laboratories Inc., Wilmington, MA, USA). The related compounds were not detected in the standard diet, and the detection limits for the compounds were as follows, caffeine: 13.9 μg/g, L-theanine: 85.6 μg/g, theogallin: 15.9 μg/g, strictinin: 20.0 μg/g, G-strictinin: 21.0 μg/g, EGCG: 27.9 μg/g, ECG: 23.4 μg/g, EGC: 30.2 μg/g, GC: 27.8 μg/g, EC: 62.5 μg/g. The mice were maintained in a humidity- and temperature-controlled animal facility on a 12-h light/dark cycle (lights on at 7:00 AM, lights off at 7:00 PM) with ad libitum access to food and water. This study was approved by the Ethical Committee on Animal Experiments in the NARO Institute of Fruit Tree and Tea Science (No. 2016-02, No. 2017-02 and No. 2018-5), and all animal experiments were conducted according to Law No. 105 and Notification No. 6 of the Government of Japan.

To investigate whether either tea infusion could affect brain functions, “MK5601” (aged-MK601 group) and “Yabukita” (aged-Yabukita group) were administered to middle-aged mice (from 8 mo of age) as the only drinking fluid for 4 mo. Similar middle-aged mice were given water instead of tea infusions as controls (aged-water group). At the end of the experiment (aged 12 mo), the mice in each group were subjected to behavioral tests. After the behavioral test, the mice were applied for various growth factor quantifications in hippocampi. Young mice (aged 2–3 mo) were included as young controls (young group). The body weight and food consumption were measured weekly. The sample size for each analysis is described in the “Results” section and the figure legends.

Behavioral testing. Animals were allowed to acclimate to the behavior laboratory for at least 30 min before testing. All tests were conducted between 7:00 PM and 0:00 AM (during the dark period). Each test occurred at the same time of the day. The illumination in the center of the test field was approximately 500-lux. Behavioral testing was completed in the following order: Y-maze test, elevated plus-maze (EPM) test, open field test and novel object location test (NOLT). The experimental apparatus was thoroughly cleaned after each trial to minimize the effects of odor. Animals were subjected to one test per day, with at least a day between tests. Sessions were recorded and analyzed with Limelight-4, a video-tracking system (Actimetrics Inc., Wilmette, IL, USA).

The Y-maze test was used to assess the effects of tea ingestion on working memory. The Y-maze apparatus consists of three identical arms (40×5×12 cm) joined in the middle. Each mouse was placed at the start arm of the maze. After that, the mouse explored the apparatus for 8 min. The total number of arm entries and the sequence of arm entries were measured. Percent alternations were calculated as [number of consecutive entries into three different compartments/total alternations (number of arm entries − 2)]×100 (%). The EPM test relies on the animal’s preference for dark and enclosed spaces over bright and exposed spaces and involves a conflict between the desire to explore and the anxiety related to exposure and height (16). The EPM consisted of two open arms (30×5 cm, with 3-mm high ledges) and two closed arms (30×5 cm, with 12-cm high walls) of the same size. The arms and central square (5×5 cm) were elevated to a height of 50 cm above the floor. The arms of the same type were arranged on sides opposite to each other. Each mouse was placed in the central square and was observed for a 5-min period. The number of entries into the open arms and the percentage of time spent in the open arms were calculated.

Locomotor activity of the mice was assessed by the open field test (16). A rectangular, plastic box (45×30×22.5 cm) was divided into 24 zones in a 6×4 grid formation. Each zone was 7.5×7.5 cm. Mice were placed in the corner closest to the operator facing
toward the wall and allowed to explore the field for 3 min. The numbers of zone entries (horizontal exploration) and rears on hind-limbs (vertical exploration) were recorded.

NOLT is based on the rodents’ natural behavior (novelty preference), an innate instinct that drives animals to learn about their environment (17). It has been reported that the performance of animals in this task depends on their hippocampal function (18). The experimental apparatus used in the test was the open-field box (45 × 30 × 22.5 cm). The objects employed were two woody blocks with the same texture, size, shape, and color. The NOLT consisted of a period of habituation, a familiarization phase, and a test phase. During habituation, each mouse was allowed to freely explore the apparatus without objects for 5 min per day for 3 consecutive days just before the familiarization trial. In the familiarization phase, the objects were placed equidistant from the two corners. Each mouse was allowed to explore the objects for 5 min, and its behavior was recorded. The mouse was then removed from the apparatus and returned to its home cage. The test phase was conducted an hour after the familiarization trial. In the test phase, one of the objects was moved to a different location, and the other object was retained in the same position as in the familiarization phase. The location of the displaced object was counterbalanced for each animal. The mouse was reintroduced into the experimental apparatus for 3 min, and its behavior was recorded. The amount of object exploration (defined as pointing the nose toward the object at a distance <2 cm and/or touching it with the nose) was scored. The results of the test phase were reported as novel object location indices (100% × displaced object exploration/total object exploration). The location index for the familiarization phase was also calculated. Mice were excluded from the analysis if they showed an imbalanced preference in the familiarization phase (less than 25% or more than 75%) or if they did not touch the objects at all in either phase. Based on this criterion, 11 mice were excluded (young group: 2/20, aged-water group: 1/18, aged-Yabukita group: 4/18, aged-MK5601 group: 4/18).

Two-bottle preference test. To confirm the possible effects of flavor of the tea infusions on brain functions, the preferences were tested in separated mice (male C57BL/6j aged 12 mo, n = 11) in the two-bottle choice test, as described previously (19). The mice (3 or 4 mice per cage, 3 cages) that were never given any fluid except for water were first simultaneously administered with 5% sucrose and water for 10 min to train the mice for selective intake of the preferred fluid of the two presented in the two-bottle preference test. After the mice had learned to select a significant amount of the sucrose solution for 3 consecutive days, they were tested for their preference for each tea infusion vs. water. In all experiments, the mice were deprived of food and water for 1 h before the tests.

Preparation of hippocampus samples and enzyme-linked immunosorbent assay (ELISA). A couple of days after the final behavioral test, the brains were quickly removed from each animal under isoflurane anesthesia. The hippocampi were dissected from the brains on ice and snap-frozen in liquid nitrogen and stored at −80°C. Tissue samples were harvested from the mice of each group in random order, between 9:00 AM and 0:00 PM.

The hippocampi were thawed under ice-cooling, and the following items were added per 10 mg of their wet weight: 100 μL of ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, pH 7.5) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Tissue homogenates were prepared by sonication on ice with a probe sonicator in short bursts (5–7 s), followed by incubation on ice for 30 min. Then, the sonication and incubation processes were repeated. After centrifugation (30 min at 15,000 × g, 4°C), the protein concentration of the resultant supernatant was measured using a bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Quantification of nerve growth factor (NGF), precursor brain-derived neurotrophic factor (proBDNF), and mature BDNF in the supernatants were performed using the NGF, proBDNF, and mature BDNF Rapid ELISA Kit (Biosensis Pty Ltd., Thebarton, SA, Australia), respectively, following the manufacturer’s protocol. Neurotrophin-3 (NT-3) levels were quantified using the ELISA Kit for NT-3 (Cloud-Clone Corp., Katy, TX, USA), and the levels of other growth factors, insulin-like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF), were quantified using Quantikine ELISA kits (R&D Systems Inc., Minneapolis, MN, USA), following the manufacturer’s protocol. The results were standardized to the protein concentration of each sample.

Statistical analysis. All values are expressed as mean ± standard error of mean. Differences in the daily intake of the tea ingredients between “Yabukita” and “MK5601” were compared using Student’s unpaired t-test. Differences in the fluid intake of two-bottle preference test were compared using Student’s paired t-test. When the data were not normally distributed, Welch’s t-test was applied. Tea infusion-induced changes in middle-aged mice were compared using one-way factorial analysis of variance (ANOVA), followed by Dunnett’s post hoc test comparing the aged-Yabukita or the aged-MK5601 group to the aged-water group. When the data were not normally distributed, the Kruskal-Wallis test and Steel’s multiple comparison test were applied. For behavioral studies with NOLT, changes within a group between trials were analyzed using the Wilcoxon signed-rank test. One-sample t-tests were used to determine whether the location index was different from chance performance (50%). Differences with p-values less than 0.05 were considered significant.
RESULTS

Body weight and food and fluid intakes during the experimental period

The body weight in the young group (24.6 ± 0.6 g, aged 2–3 mo, n = 20) was lesser than that in the aged groups (the aged-water group: 34.9 ± 0.9 g, n = 18, the aged-Yabukita group: 35.0 ± 0.4 g, n = 18, the aged-MK5601 group: 34.7 ± 0.6 g, n = 18, aged 12 mo). There were no significant differences in body weight among the aged groups during the 4-mo experimental period. Daily food and fluid intakes in the aged-Yabukita group were similar to those in the aged-MK5601 group (food and fluid intakes; the aged-Yabukita group: 3.57 ± 0.08 g/d/mouse and 5.40 ± 0.27 g/d/mouse, the aged-MK5601 group: 3.34 ± 0.04 g/d/mouse and 4.67 ± 0.38 g/d/mouse, respectively). Daily intakes of the tea ingredients were calculated based on the fluid intakes (Table 1). The aged-Yabukita and the aged-MK5601 groups had similar caffeine intakes. The intake of theogallin in the aged-MK5601 group was eight times higher than that in the aged-Yabukita group, whereas l-theanine intake in the aged-MK5601 group was lower than that in the aged-Yabukita group. The intake of strictinin in the aged-MK5601 group was five times higher than that in the aged-Yabukita group, and G-strictinin was detected only in the MK5601 tea infusion (the detection limit for G-strictinin: 0.021 μM).

The intake of gallate-type catechin (EGCG and ECG) in the aged-MK5601 group was higher than that in the aged-Yabukita group, whereas non-gallate-type EGC intake in the aged-MK5601 group was lower than that in the aged-Yabukita group. There was no significant difference in the total intake of catechins between the aged-MK5601 and the aged-Yabukita groups.

Behavioral testing

To assess the effects of tea ingestion on working memory in middle-aged mice, spontaneous behaviors in the Y-maze were measured (young: n = 20; aged-water: n = 18; aged-Yabukita: n = 18; aged-MK5601: n = 18). The aged-water, the aged-Yabukita, and the aged-MK5601 groups showed lower total number of arm entries than the young group (Fig. 1A). In contrast, the percent alternation in the aged groups were the same levels as that in the young group (Fig. 1B), suggesting that working memory was not impaired in the middle-aged mice. Neither the total number of arm entries nor the percent alternation was affected by the tea infusions (Fig. 1A and Fig. 1B).

The number of entries into open arms and the percentage of time spent in the open arms in the EPM test were measured to assess the effects of tea ingestion on anxiety-like behavior in middle-aged mice (young: n =

Table 1. The daily intakes of the ingredients from each tea infusion during the experimental period.

|            | Yabukita     | MK5601      |
|------------|--------------|-------------|
| Caffeine   | 4.80 ± 0.29  | 4.32 ± 0.32 |
| l-Theanine | 2.88 ± 0.16  | 1.88 ± 0.13*** |
| Theogallin | 0.55 ± 0.03  | 4.50 ± 0.32*** |
| Strictinin | 0.059 ± 0.004| 0.31 ± 0.02***** |
| G-strictinin| ND           | 0.46 ± 0.03  |
| Total catechins | 11.2 ± 0.6 | 12.6 ± 0.9  |
| EGCG       | 2.43 ± 0.15  | 3.91 ± 0.27** |
| ECG        | 0.40 ± 0.02  | 1.19 ± 0.08**** |
| EGC        | 6.19 ± 0.37  | 4.82 ± 0.34* |
| GC         | 0.28 ± 0.02  | 0.35 ± 0.03  |
| EC         | 1.87 ± 0.09  | 2.25 ± 0.19  |

(μmol/mouse/d)

Data are represented as mean ± standard error of mean (n = 4).

Statistical analysis of the Yabukita group vs. the MK5601 group was conducted using Student’s unpaired t-test (*p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001). ND: not detected (the detection limit for G-strictinin: 0.021 μM).

EGCG: epigallocatechin gallate. ECG: epicatechin gallate. EGC: epigallocatechin, GC: gallocatechin, EC: epicatechin.
The middle-aged MK5601 group showed lower numbers of entries into the open arms compared with the young group (Fig. 2A). Among the three middle-aged groups, the number of entries tended to be higher in the aged-MK5601 group, as compared with that in the aged-water group ($p = 0.091$, the Kruskal-Wallis test and Steel’s multiple comparison test). The aged groups also showed remarkably lower percentages of time spent in the open arms compared with the young group (Fig. 2B). There were no significant differences in the percentages among the aged groups.

To assess the effects of tea ingestion on locomotor activities in middle-aged mice, spontaneous behaviors in the open field were measured (young: $n=10$; aged-water: $n=9$; aged-Yabukita: $n=9$; aged-MK5601: $n=9$). The aged-water, the aged-Yabukita, and the aged-MK5601 groups showed lower numbers of zone entries (horizontal exploration) than the young group (Fig. 3A). The aged groups also showed much lower numbers of rear (vertical exploration) than the young group (Fig. 3B). Neither the number of zone entries nor rears on hind-limbs was affected by the tea infusions (Fig. 3A and Fig. 3B).

The effects of tea ingestion on spatial memory in the middle-aged mice were assessed using the NOLT (young: $n=18$; aged-water: $n=17$; aged-Yabukita: $n=14$; aged-MK5601: $n=14$). The young group showed a significantly higher location index in the test phase than that in the familiarization phase (Fig. 4A, $p<0.005$). The aged-water group performed worse than the young group and showed a similar location index in the test phase as that in the familiarization phase. Although there was no significant difference between the location indices of the familiarization and test phases in the aged-Yabukita group, the aged-MK5601 group showed a significantly higher location index in the test phase ($p=0.041$) and retained spatial memory at the same level as the young group. The aged-water and the
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Fig. 4. Effects of the tea infusions on spatial memory in the novel object placement test. (A) Location indices in the familiarization and test phases. The aged-water and the aged-Yabukita groups showed impaired spatial memory reflected by the same location indices in the test phase as those in the familiarization phase. The aged-MK5601 group retained spatial memory similar to the young group, reflected by significantly high location indices in the test phase (Wilcoxon signed-rank test). *p<0.05, ***p<0.005 vs. familiarization phase. The location indices of the young and the aged-MK5601 groups were significantly higher than the chance level (50%) in the test phase (one-sample t-test). #p<0.05 vs. the chance level in the test phase. Dotted line indicates the chance level. (B, C) Time spent exploring both objects during the familiarization (B) and test phases (C). There were no significant differences among the groups. Data are represented as mean±standard error of mean (young: n=18; aged-water: n=17; aged-Yabukita: n=14; aged-MK5601: n=14).

Fig. 5. Preference for “Yabukita” and “MK5601” infusions in the two-bottle test. (A) “Yabukita” infusion vs. water. (B) “MK5601” infusion vs. water. No significant preference for these test fluids was observed in mice (n=3).

-aged-Yabukita groups presented location indices that did not differ from the chance level (Fig. 4A), while the aged-MK5601 group showed a location index significantly higher than the chance level (p=0.023), similar to the young group (p=0.010). There were no statistically significant differences in total exploration time among the three middle-aged groups in both phases (Fig. 4B and Fig. 4C).

Two-bottle preference test

Intact mice consumed equivalent amount of each test fluid in the two-bottle preference test (n=3). Comparison between “Yabukita” infusion and water (Fig. 5A) or “MK5601” infusion and water (Fig. 5B) indicated no significant preference for these test fluids in the mice.

Neurotrophin and growth factor levels in the hippocampus

Several neurotrophins and growth factors were quantified in the hippocampus samples using commercial
Table 2. Effects of the tea infusions on hippocampal neurotrophin and growth factor levels in middle-aged mice.

|               | Young Water | Young Yabukita | Young MK5601 | Aged Water | Aged Yabukita | Aged MK5601 |
|---------------|-------------|---------------|--------------|------------|---------------|-------------|
| NGF           | 167.8±4.8   | 168.7±4.5     | 166.1±4.7    | 174.8±7.3  |
| ProBDNF       | 594.6±32.2  | 576.4±21.2    | 670.0±20.6*  | 652.6±31.7*|
| Mature BDNF   | 2.446±76    | 2.465±69      | 2.558±99     | 2.651±156  |
| NT-3          | 435.2±21.7  | 483.3±20.0    | 477.5±12.2   | 558.8±23.1*|
| FGF-2         | 375.4±10.5  | 360.3±4.8     | 375.2±8.6    | 387.6±14.5 |
| IGF-1         | 33.10±1.12  | 35.82±1.16    | 35.57±0.80   | 37.45±1.44 |
| VEGF          | 18.78±0.31  | 20.22±0.89    | 19.80±0.74   | 19.32±0.58 |

Growth factor levels were quantified by enzyme-linked immunosorbent assay and standardized to the protein concentration of the extracts (pg/mg protein). The differences in NT-3 level reached statistical significance (p<0.01, one-way factorial analysis of variance [ANOVA] followed by Dunnett’s post hoc test). *p<0.05 vs. middle-aged water group. The levels of proBDNF were significantly higher in the aged-Yabukita group and tended to be higher in the aged-MK5601 group (p=0.05, one-way ANOVA followed by Dunnett’s post hoc test)*. \(p<0.05, \text{vs. aged-water group. Data are represented as mean±standard error of mean (young group: n=10, aged-water group: n=9, aged-Yabukita group: n=9, aged-MK5601 group: n=9).}\)

NGF: nerve growth factor, BDNF: brain-derived neurotrophic factor, NT-3: neurotrophin-3, FGF-2: fibroblast growth factor-2, IGF-1: insulin-like growth factor-1, VEGF: vascular endothelial growth factor.

In the present study, we evaluated the effects of “MK5601” tea cultivar, on brain functions in middle-aged mice. In the Y-maze, the percent alternation between the aged groups (12 mo) and the young group (2–3 mo) were equivalent (Fig. 1), suggesting that working memory was not impaired in the middle-aged mice. Since the percent alternation was not affected by both the tea infusions (Fig. 1A and Fig. 1B), neither could improve memory further when the brain function is not impaired. Decreased total arm entries in the Y-maze indicated decreased spontaneous locomotor activity with aging. Similarly, the middle-aged mice showed a smaller number of zone entries and rears on hind-limbs than the young mice in the open test, indicating that spontaneous locomotor activity decreased. These results agree with previous studies with C57BL/6 mice (12, 20–22) that reported the age-related decrease in spontaneous locomotor activities. In the present study, although the middle-aged mice showed a much smaller number of entries and shorter time spent in the open arms than the young mice in the EPM (Fig. 2), the scores were considered to be affected by an age-related decrease in locomotor activities in addition to an increase in anxiety-like behaviors.

ELISA kits (Table 2, young: n=10; aged-water: n=9; aged-Yabukita: n=9; aged-MK5601: n=9). One-way ANOVA followed by Dunnett’s test comparing the three middle-aged groups revealed that the level of proBDNF in the hippocampus was significantly higher in the middle-aged groups than in the young group (p=0.026). The level of proBDNF tended to be higher in the aged-MK5601 group, as compared with that in the aged-water group (p=0.075). The aged-MK5601 group also showed a higher level of NT-3, as compared with that in the aged-water group (p=0.018).

**DISCUSSION**

In the present study, we evaluated the effects of “MK5601” tea cultivar, on brain functions in middle-aged mice. In the Y-maze, the percent alternation between the aged groups (12 mo) and the young group (2–3 mo) were equivalent (Fig. 1), suggesting that working memory was not impaired in the middle-aged mice. Since the percent alternation was not affected by both the tea infusions (Fig. 1A and Fig. 1B), neither could improve memory further when the brain function is not impaired. Decreased total arm entries in the Y-maze indicated decreased spontaneous locomotor activity with aging. Similarly, the middle-aged mice showed a smaller number of zone entries and rears on hind-limbs than the young mice in the open test, indicating that spontaneous locomotor activity decreased. These results agree with previous studies with C57BL/6 mice (12, 20–22) that reported the age-related decrease in spontaneous locomotor activities. In the present study, although the middle-aged mice showed a much smaller number of entries and shorter time spent in the open arms than the young mice in the EPM (Fig. 2), the scores were considered to be affected by an age-related decrease in locomotor activities in addition to an increase in anxiety-like behaviors. The previous studies with C57BL/6J mice (12, 20, 21) reported inconsistent results on age-related changes in anxiety-like behaviors observed in the EPM. The percentages of time on the open arms were reported to be low (21) or high (12) in older mice, while there were no significant changes between young and aged mice (20). These reports suggest that the behavioral indices in the EPM reflect different sides of anxiety-like behavior under each condition. The number of entries into open arms tended to be higher in the aged-MK5601 group without the changes in locomotor activities evaluated by the open field and Y-maze tests under the present study’s conditions. Thus, there is the possibility that “MK5601” would be more effective to anxiety although other types of behavioral studies are needed to confirm the effects on mood changes in the middle-aged mice.

The previous data of the Barnes maze test (12, 20) indicate that aged mice showed impaired spatial memory. Another study with adult (5 mo) and aged (15 mo) mice (22) reported the age-related deficit assessed by the NOLT, in parallel with the Barnes maze test. In the present study, the aged-water group performed worse than the young group and showed a similar location index in the test phase as that in the familiarization phase in the NOLT (Fig. 3). However, the aged-MK5601 group showed a significantly higher location index in the test phase and retained spatial memory at the same level as the young group. These results indicate that “MK5601” prevents age-related deficits in spatial memory. Among these previous studies (12, 20, 22) that assessed the cognitive decline in aged C57BL/6J mice by the Barnes maze test, behavioral tests were performed during the dark phase of the light/dark cycle under illu-
mination (900-lux) in one study (22), while during the light phase in the other two studies (12, 20). Although all studies concluded that aged mice displayed deficits of spatial memory, there were some differences in the result from the Barnes maze test, e.g., in latency to target and number of errors, on the acquisition (training session) and retention (probe test). Thus, illumination conditions might have influences on the results of behavioral tests during the dark phase, as well as other testing conditions.

A previous study (13) reported that long-term treatment with an antidepressant, vortioxetine, improved spatial memory assessed by the NOLT; however, hippocampal growth factor levels, including NT-3, were inconsistent with behavioral outcomes in middle-aged mice (12 mo). In the present study, we found that the aged-MK5601 group showed a high level of NT-3 in the hippocampus concurrently with the preservation of spatial memory in the aged-MK5601 group. Although the relationship between the NT-3 level in the hippocampus and spatial memory remains undetermined, there are some pieces of evidence that one would change in parallel with the other. Environmental enrichment, which is the experience of animals in a complex environment, was demonstrated to reduce age-related impairments in spatial memory (23) and upregulate NT-3 mRNA expression in the brains of middle-aged mice (24). Moreover, intracerebroventricular infusion of NT-3 reversed spatial memory impairments in aged rats (25), and transgenic mice showing high NT-3 expression in the hippocampus exhibited an improvement in spatial learning, as compared with age-matched non-transgenic mice (26). Taken together, the high NT-3 protein level in the hippocampus may be associated with the prevention against age-related deficits in spatial memory in the aged-MK5601 group. Since a conditional mutant line in which the NT-3 gene is deleted in the brain, exhibited deficits in spatial memory tasks at 2–4 mo of age (27), NT-3 is required to maintain normal cognitive function also in young adult mice. However, a study on transgenic mice (26) showed that overexpression of NT-3 did not affect spatial memory in young adult mice. The results from the Y-maze test in the present study suggest that tea infusions had no effects to improve memory further. Therefore, it’s unlikely that the consumption of “MK5601” tea infusion or enhanced NT-3 expression in the hippocampus has a positive impact on spatial memory in young healthy subjects. In addition, postmortem brain analysis indicated that the expressions of certain neurotrophic factors are stimulated under conditions of progressive neurodegeneration in humans, for example in Alzheimer’s disease patients (28). Animal studies with dementia models are needed to clarify whether “MK5601” tea infusion is effective to prevent histological changes, such as brain atrophy or neural degeneration.

The cold brew infusion of “MK5601” is characterized by the high theogallin content (Table 1). Theogallin was originally identified as an umami-enhancing compound in green tea infusion (29). While biological effects related to theogallin have rarely been reported, an in vitro investigation using hippocampal slice preparation provided evidence that theogallin increases long-term potentiation, commonly taken as representative for enhancement of spatial and time dependent memory (30). The stimulation inducing long-term potentiation in hippocampal slices is known to evoke significant increases in both BDNF and NT-3 gene expressions (31). An in vivo electropharmacogram study (32) also indicated that after oral administration of L-theanine- and theogallin-enriched decaffeinated green tea extract or theogallin alone, electrical brain activities changed in freely moving rats within a few hours. Comparison with known drug effects on electrical brain activities revealed that the changes by theogallin were similar to those by antidepressive and stimulating drugs. These results agree with an electroencephalogram study with healthy volunteers (33). It is hypothesized that the effect of continuous ingestion of “MK5601” tea infusion on cognitive function and hippocampal NT-3 levels were related to the repeated induction of changes in electrical brain activities by theogallin, although there is no clear evidence for the access of orally administered theogallin to the brain via the blood-brain barrier. There is another possibility that the characteristic flavors of tea infusions could affect cognitive function because an exposure of mice to novel pleasant odors for a few weeks was demonstrated to increase the neuronal cell numbers in the hippocampus (34). In the present study, however, the mice showed no preference for the tea infusions, as compared to water, suggesting that the flavors would not make enough strong impact to lead the alterations in the hippocampus. To clarify the mechanisms under the effects of “MK5601” on cognitive function, the long-term effect of isolated theogallin should be investigated. The effects of continuous theogallin consumption and the relationship with its bioavailability have never been reported perhaps because the material was too limited to allow these experiments due to its low content in typical green tea (29). The theogallin-rich tea cultivar “MK5601” is necessary for the experiments as the material.

Green tea contains several chemical compounds, other than theogallin, that may affect brain function. Among them, greater efforts have been put into the investigations of caffeine, L-theanine, and catechins (35). L-Theanine administration (0.4% in drinking water) was demonstrated to ameliorate the impairment of contextual memory in transgenic mice with Alzheimer’s disease (AD) (36). The content of L-theanine in drinking water was extremely high compared with those in “Yabukita” and “MK5601” infusions (more than 40 times), indicating that L-theanine is thought to hardly contribute to the improvement of cognitive function in the present study. Caffeine also counteracted the age-related decline in recognition memory when rodents received caffeine (1 mg/mL in drinking water) during aging (37, 38). It is unlikely that caffeine in “Yabukita” and “MK5601” infusions played a signifi-
cant role in preventing the age-related changes because the content of caffeine in the tea infusions was low compared with that in the caffeine-containing water (more than five times). Concerning catechins, the intake of EGCG in the aged-MK5601 group was significantly higher than that in the aged-Yabukita group, while there was no significant difference in the total intake of catechins between them (Table 1). In a previous report (39), supplementation with 0.05% or 0.1% green tea catechins (GTC, w/v) in drinking water was demonstrated to prevent age-related spatial memory decline in female C57BL/6 mice. Since both “Yabukita” and “MK5601” infusions in the present study contained EGCG within the range of 0.05% to 0.1% GTC solution in the previous report, both infusions have the potential to affect age-related cognitive changes. A shorter period for administering green tea, conditions of behavioral tests, and the animal’s age and sex could cause the differences between the studies. Thus, there is the possibility that slightly high EGCG content in “MK5601” infusion involves in its effects on cognitive function.

The better performance of GTC-treated mice in the behavioral test was associated with increased protein levels of BDNF in the hippocampus in the previous report (39). In the present study, there were no significant differences in the hippocampal levels of mature BDNF among the middle-aged mice; however, the level of its precursor, proBDNF, was significantly higher in the aged-Yabukita group and tended to be higher in the aged-MK5601 group than in the aged-water group. Therefore, it is presumed that the administration of these tea infusions may affect BDNF processing in the hippocampus. Simultaneously with the neurotrophins, hippocampal levels of their receptors were measured in some of the previous reports with aged rodents (13, 37, 38). The studies revealed that middle-aged mice (13) and rats (38) had lower levels of TrkB (Ntrk2, the receptor for BDNF) in the hippocampus, compared with young adults. Since the balance in the expression levels of neurotrophins and their receptors is critical for cognitive output, there is also the possibility that “MK5601” consumption could induce changes in the receptor expressions in middle-aged mice. More detailed studies are needed for understanding these mechanisms about neurotrophins.

In conclusion, a unique “MK5601” cultivar appears to be more effective for preventing cognitive impairment in middle-aged mice, as compared to a common tea cultivar. Further studies are needed for future application to humans.

Authorship
Research conception and design: SN, MM and AN; experiments and data collection: SN, MM, KE, KY, SY and AO; statistical analysis of the data: SN and KE; writing of the manuscript: SN and MM.

Disclosure of state of COI
No conflicts of interest to be declared.

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