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Original Article

Active Component in Green Tea Catechins and Effective Intake Period for Prevention of Age-related Brain Dysfunction

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Abstract

Objective: We previously found that green tea catechins (GT-catechin) decrease oxidative damage to DNA and suppress brain dysfunction in aged senescence-accelerated mice (SAMP10). To clarify the effect of GT-catechin on suppression of brain dysfunction, we compare the effect on learning ability among several catechins and examined the essential intake period for prevention of brain dysfunction.
Methods: Male SAMP10 mice were allowed free access to water containing epigallocatechin gallate (EGCG, 0.06 mg/ml), epigallocatechin (EGC, 0.03 mg/ml), GT-catechin (0.2 mg/ml), or green tea extract (0.66 mg/ml). Learning ability of mice was measured using a step-through passive avoidance task.
Results: SAMP10 mice exhibit brain dysfunction with aging. However, learning ability was significantly higher in mice that drank GT-catechin and EGCG than same-aged control mice that drank water. EGCG was an important component, but EGC had no effect on learning ability. The learning ability was significantly improved in mice that ingested EGCG for >5 months, and tended to improve in mice that ingested EGCG for 2 or 3 months. Next, the level of synaptophysin, a marker of presynapse, tended to be higher in mice that ingested EGCG but not in mice that ingested EGC. The levels of synaptophysin were significantly higher in mice ingested GT-catechin and green tea extract than control mice.
Conclusion: The intake of EGCG, the major catechin in green tea, but not EGC, suppressed age-related brain dysfunction. The effective intake period of EGCG was > 5 months for suppression of brain dysfunction.

KEY WORDS: green tea catechin, brain dysfunction, epigallocatechin gallate, intake period, synaptic plasticity

Introduction

Aging is the outcome of a balance between damage and repair, and is probably related to a multi-factorial process [1,2]. Although a modest level of reactive oxygen species (ROS) generated under physiological conditions participate in cell signal transduction cascades to regulate cell growth and differentiation [3], in contrast, severe ROS cause oxidative damage of cellular DNA, protein and lipids, resulting in the initiation or development of various diseases such as neurodegenerative diseases, cancer and type 2 diabetes mellitus [4,5]. Endogenous antioxidants and antioxidative enzymes are engaged in the detoxification of ROS, and additionally, numerous dietary antioxidants are thought to be involved in the antioxidative defense system [6-8].

We have found that the production of superoxide anion increased with aging in the brain of mice, rats and birds [9]. The level was higher in the brain of senescence-accelerated mice (SAMP10) than the same-aged control mice (SAMR1) of normal longevity. Although the activity of superoxide dismutase (SOD) did not change, the activity of glutathione peroxidase was lower in the brain of SAMP10 at 12 months [10]. These results suggested that oxidative damage increased with aging. Actually, DNA oxidative damage in aged SAMP10 was higher than same-aged control mice [11,12]. However, we have previously found that green tea catechins (GT-catechin), potent antioxidants, decrease oxidative damage to DNA and suppress brain dysfunction in aged SAMP10 when ingested from the age of 1 month to the age of 12 months [11,12].

GT-catechin is composed of many kinds of catechins such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) (***Fig. 1***). To clarify the mechanism of GT-catechin in brain, we examined whether the suppressive effect of GT-catechins on lowered learning ability was caused by an active component in GT-catechin or a summed ability of several catechins. Next, we investigated the consumption period of catechin for suppression of brain regression. The starting age and continuing periods of catechin intake were variously changed and the effects were compared.

The loss of synapse has been observed in aged SAMP10 as a reason for cognitive dysfunction [13]. A correlation between loss of synapse and cognitive impairment has been observed in human patients with Alzheimer's disease and mild cognitive impairment [14], and aged rhesus monkeys [15]. To investigate the reason for the preventing effect of catechin on age-related brain dysfunction, the level of synaptophysin, a marker of presynapse, was measured. We found that the level of synaptophysin was higher in age-matched mice that drank GT-catechin for a longer period [16]. In this study, the level of synaptophysin in the cerebral cortex of mice that ingested EGCG, EGC or green tea extract was compared.

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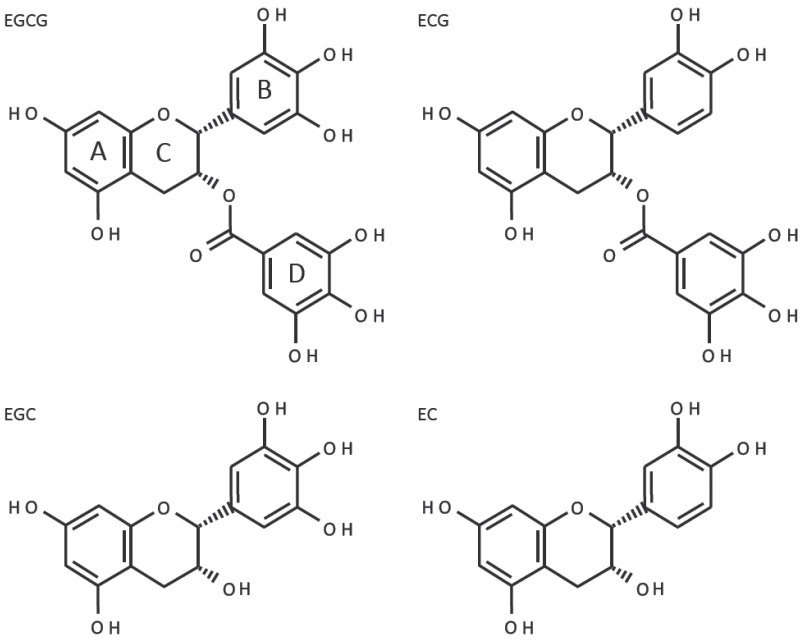

Fig. 1. **Structures of green tea catechins.**

Materials and methods

Animals

All experimental protocols were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka. Male SAMP10/TaSlc (SAMP10) mice, which are senescence-prone, were purchased from Japan SLC Co., Ltd. (Hamamatsu-city, Shizuoka, Japan) and bred under conventional conditions in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Experimental mice had free access to a normal diet (CE-2; Clea Co Ltd., Meguro-ku, Tokyo, Japan) and tap water containing green tea catechins. Catechin water was freshly prepared twice a week. Control mice were given a normal diet and tap water.

Experimental design

GT-catechin (Polyphenon 70S, Mitsui Norin Co., Ltd, Minato-ku, Tokyo, Japan) is composed of several kinds of green tea catechins such as EGCG, EGC, ECG, EC and other catechins (***Fig. 1***). Although EGCG is the most abundant catechin in green tea, accounting for 32% of GT-catechin, EGC is also abundant and ranked second, accounting for 16% of GT-catechin. Since the concentration of EGCG and EGC in GT-catechin solution (0.2 mg/ml) was 0.06 and 0.03 mg/ml, respectively, both the effects of EGCG and EGC were compared with that of GT-catechin at these concentrations.

Ninety mice were prepared and divided into five groups containing a control. Six mice were housed per cage. The first four groups of 18 mice consumed (all dissolved in water) 0.20 mg/ml GT-catechin (Polyphenon 70S), 0.06 mg/ml EGCG (Sunphenon EGCg, Taiyo Kagaku Co. Ltd., Yokkaichi-city, Mie, Japan), 0.03 mg/ml EGC (Sunphenon EGC, Taiyo Kagaku Co. Ltd.) or 0.66 mg/ml green tea extract (Camellia extract 30S, Taiyo Kagaku Co. Ltd.), respectively from 6 to 12 months. The fifth group of 18 mice drank tap water as a control.

Another 60 mice were prepared and divided into 8 groups containing a control (control; n=18, other 7 groups; n=6). The mice consumed EGCG in water at a concentration of 0.06 mg/ml. To investigate the effect of the starting age of ingestion and intake period of EGCG, the starting and continuing periods were variously changed. The learning abilities of mice were measured when mice were 11 months of age.

Green tea catechins

Polyphenon 70S contains about 70% GT-catechin and no caffeine. GT-catechin consists of 31.7% EGCG, 15.7% EGC, 10.0% ECG and 8.5% EC. The remaining portion consists of 4.5% gallocatechin gallate, 1.0% catechin gallate, and some other catechins from green tea. Sunphenon EGCg consists of 95.1% EGCG and 2.8% ECG. Sunphenon EGC consists of 81.4% EGC and 7% EC. Green tea extract consists of 11.5% EGCG, 7.6% EGC, 2.5% ECG, 2.1% EC and 5.6% caffeine.

Memory acquisition test

A step-through passive avoidance task was carried out using 11-month-old mice as described previously [11,12]. In brief, when a mouse entered the dark chamber from the light chamber, the door was closed and an electric foot-shock was delivered at 50 μA for 1 s (SGS-003, Muromachi Kikai Co., Ltd., Chuou-ku, Tokyo, Japan). Acquisition of the avoidance response was judged as successful if the mouse remained in the light chamber for 300 s. The trial was repeated until the mouse satisfied the acquisition criterion within five trials. The time that a mouse could not stay in the light chamber, *i.e.*, the time spent in the dark chamber in a 300-s trial, was recorded. This result from successive trials was summed for each mouse to give a measure of the time required for learning not to enter the light chamber (*i.e.*, "learning time").

Measurements of cerebral weight and level of synaptophysin

Mice were sacrificed when 12 months old under ether anesthesia and their brains were removed immediately. The wet weight of the cerebrum was measured for the observation of cerebral atrophy. The cerebral cortex was separated from the cerebrum for the measurement of synaptophysin. The brain samples were immediately frozen in liquid N_2 and stored at –80°C until measurements were made.

The cerebral cortex of a 12-month-old mouse was homogenized in 30 volumes of 50 mM phosphate buffer containing 0.1 mM ethylendiamine tetraacetic acid (pH 7.0) as described previously [16]. The protein content was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA). The homogenate was heated at 80°C for 3 min in the same volume of sample buffer containing 2% sodium-dodecyl sulfate (SDS), 2% mercaptoethanol, 20% glycerol and 2 mM phenylmethylsulfonyl fluoride. The sample containing 2.0 µg proteins was separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P™, Millipore Corp., MA, USA). The membrane was processed with the anti-synaptophysin mouse monoclonal antibody (SYP (D-4), Santa Cruz Biochemistry, Inc., CA, USA). The complex of antibody and synaptophysin was visualized by using horseradish-peroxidase-conjugated second antibody and an ECL™ detection system (GE Healthcare Bio-Science Corp., NJ, USA). Band density was measured by densitometry (Scion Image software, Scion Corp., MD, USA). Tubulin was detected with anti-β tubulin monoclonal antibody (Affinity BioReagents, CO, USA) as a control.

Statistical analyses

Data are expressed as mean ± SE. The effect of GT-catechin intake was determined by one-way analysis of variance followed by the Bonferroni *t*-test for multiple comparisons.

Results

Learning ability

The time for learning not to enter the dark room was measured at 11 months of age using a step-through passive avoidance task. A shorter learning time implies higher learning ability. The learning times of mice that ingested GT-catechin, EGCG and green tea extract were significantly shorter than age-matched control mice ($F(4, 70) = 24.751$, $p = 8.6 \times 10^{-13}$; one-way ANOVA) (***Fig. 2***). The learning time of mice that ingested EGCG was as similar to that of mice that ingested GT-catechin. On the other hand, mice that ingested EGC showed as long learning time as control mice. These results suggested that the active component in green tea catechins is EGCG.

Next, the effect on starting period of EGCG ingestion on learning ability was compared. The learning time was significantly shorter in mice that ingested EGCG for > 5 months, such as, 1-11, 3-11, 6-11, 3-9 and 1-6 months than age-matched control mice ($F(7, 36) = 3.454$, $p = 6.25 \times 10^{-3}$; one-way ANOVA) (***Fig. 3***). The learning ability of mice that ingested EGCG from 1-11 months was similar to that of mice that ingested EGCG from 3-11 and 6-11 months of age. The difference in starting age of EGCG ingestion had little effect

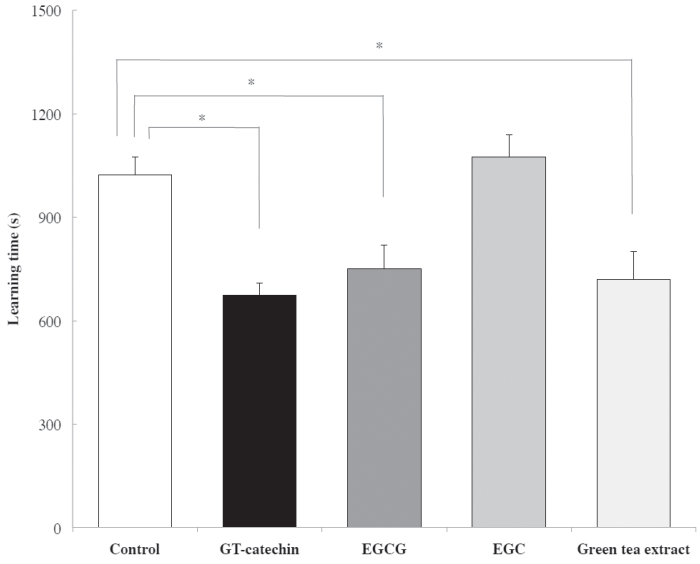

Fig. 2. **Learning ability of mice that ingested green tea catechins.**
SAMP10 mice started to take GT-catechin (0.2 mg/ml), EGCG (0.06 mg/ml), EGC (0.03 mg/ml) or green tea extract (0.66 mg/ml) in drinking water when 6 months old. Learning ability was measured using a step-through passive avoidance task when 11 months old. A shorter learning time implies a higher learning ability. Control mice drank tap water. Each bar represents mean ± SE (n=12-17, * $p < 0.05$).

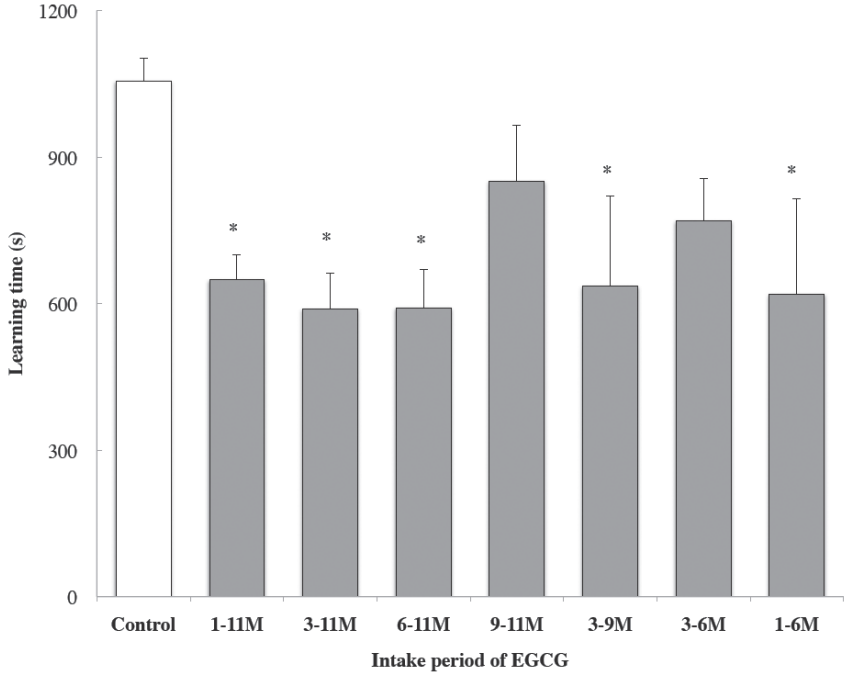

Fig. 3. **Learning ability of mice that ingested EGCG for various periods of time.**
SAMP10 mice ingested either EGCG (0.06 mg/ml) or tap water from 1 to 11 months of age. Each bar represents mean ± SE (n=4-10, * $p < 0.05$).

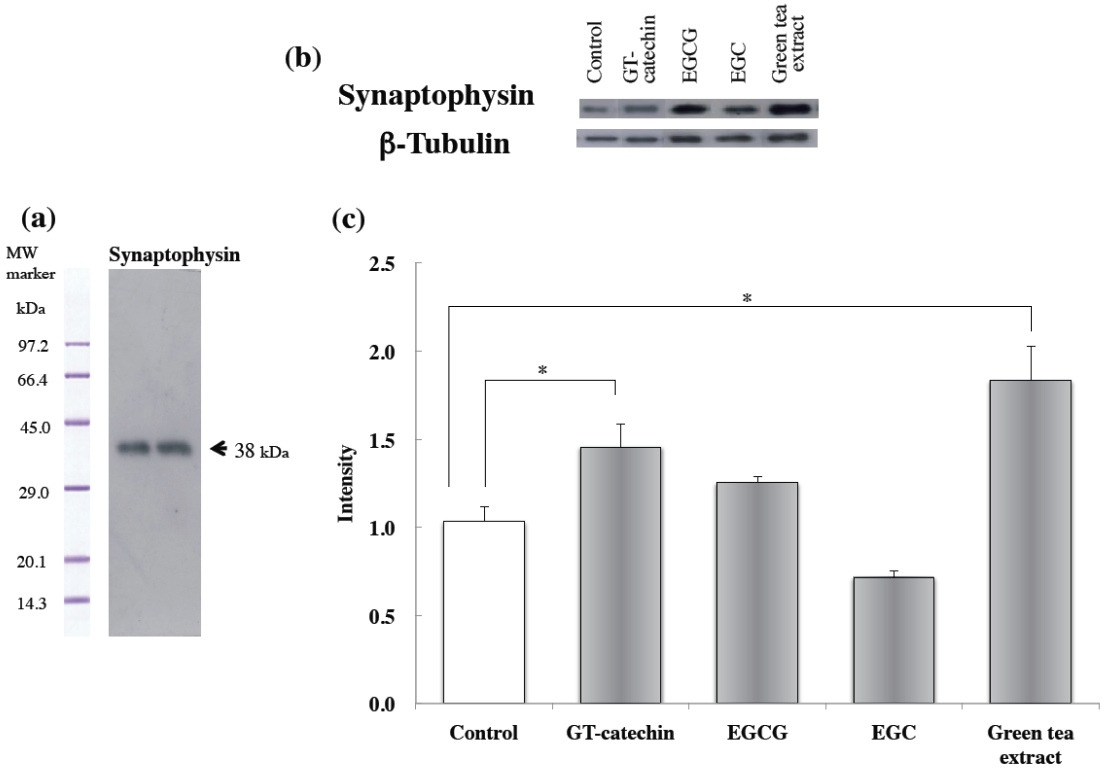

Fig. 4. **Expression of synaptophysin in brain of aged SAMP10.**
A typical band of synaptophysin; a band of 38 kDa was detected by western blot analysis in the cerebral cortex of mice (a). The levels of synaptophysin and β-tubulin in the cerebral cortex of mice that ingested GT-catechin (0.2 mg/ml), EGCG (0.06 mg/ml), EGC (0.03 mg/ml) or green tea extract (0.66 mg/ml) in drinking water from 6 to 12 months of age were detected. Control mice drank water. Tubulin (53 kDa) served as the control (b). The density of synaptophysin of mice that ingested green tea catechins for 6 months was measured by densitometry (c). Each bar represents the mean ± SE (n = 3-9, * $p < 0.05$).

on learning ability. In addition, both the mice that ingested EGCG from 1-6 months of age (5 months) and the mice that ingested EGCG from 3-9 months of age (6 months) exhibited a significantly shorter learning time than control mice. These results suggest that the effective period of EGCG (> 5 months) was much more important than the starting age of ingestion. A shorter ingestion period of 2 and 3 months (9-11, and 3-6 months of age) tended to suppress the decrease in learning ability (***Fig. 3***).

Cerebral atrophy

Cerebral weight was measured as a marker of brain senescence when mice were 12 months old. Cerebral weights were compared in SAMP10 mice that ingested GT-catechin, EGCG, EGC and green tea extract for 6 months (***Table 1***). The cerebral weight in mice that ingested GT-catechins was significantly higher, while that in mice that ingested EGCG, EGC and green tea extract tended to be higher than control mice (F (4, 69) = 2.154, p = 0.0833; one-way ANOVA).

Table 1 Cerebral weight in mice ingested green tea catechin

Catechin	Cerebral weight (g)			
Control	0.360	±	0.003	
GT-catechin	0.374	±	0.003	*
EGCG	0.372	±	0.005	
EGC	0.372	±	0.005	
Green tea extract	0.370	±	0.005	

Mice ingested each catechin from 6- to 12-month-old. Each value represent mean ± SE (n=12-17, * $p < 0.05$ vs. control).

Level of synaptophysin

The level of synaptophysin, a marker of presynapse, was significantly higher in mice that drank GT-catechin or green tea extract, and tended to be higher in mice that ingested EGCG (F (4, 18) = 10.35, p =1.57 × 10^{-4}; one-way ANOVA) (***Fig. 4***). On the other hand, the level of synaptophysin in mice that ingested EGC did not increase. The level of tubulin, a control protein, was not different between these mice.

Discussion

Active component in green tea catechin

The learning ability using a step-through passive avoidance task indicated that the ability of mice that ingested EGCG was similar to that of mice that ingested GT-catechin (***Fig. 2***). On the other hand, learning ability was not improved in mice that ingested EGC. The observed functional activity of green tea catechins (GT-catechin = EGCG >> EGC) indicated that EGCG was an active component of GT-catechins for preventing brain dysfunction.

When we measured the learning time of SAMR1 as a positive control mouse, the times were 524 ± 53 s at 11 months of age and 679 ± 80 s at 14 months of age, respectively [11]. The result suggests that the learning ability of SAMP10 mice that ingested GT-catechin or EGCG was similar that of SAMR1.

As the concentrations of EGCG and EGC used in this experiment were 0.131 and 0.098 mM, respectively, the difference of concentrations was only a small or negligible reason for the difference of functional activity. To discuss the mechanism to inhibit the brain dysfunction by catechins, the relation between structure of catechin and brain function was considered. Green tea catechins are generally regarded as antioxidants because they all have multiple hydroxyl substituents on the A, B, C and/or D ring (***Fig. 1***), and the reducing activities of gallocatechin (EGCG and EGC) were similarly strong [17]. On the other hand, the difference between EGCG and EGC might be caused, in part, by the high affinity of EGCG for a lipid bilayer stemming from extensive hydrogen bonding with the lipid head group [18,19]. Therefore, a gallate group in EGCG (***Fig. 1***, ring D) might be important for the prevention of cognitive dysfunction.

Next, the effect of catechin administration on oxidative stress was also considered. As the generation of superoxide anion was higher in SAMP10 than age-matched SAMR1 [9], the level of carbonyl protein, a marker of oxidative damage, was higher in SAMP10 than age-matched SAMR1 [10]. Oxidative damage in some important proteins seems to be critical for nerve cells. For example, glutathione peroxidase, a major antioxidative enzyme, exhibited low activity in aged SAMP10, but the decreased activity was significantly improved by the ingestion of GT-catechin [10]. The decreased activity of antioxidative enzyme with aging seems to be a reason for the increased oxidative damage; however, further investigation is needed for resolve the mechanism of GT-catechin in brain.

On the other hand, the ability of green tea extract was investigated at a concentration of 0.66 mg/ml, which was as same content of total catechins as found in GT-catechin. The learning ability of mice that ingested green tea extract was similar to that of GT-catechin (green tea extract = GT-catechin), suggesting that caffeine and other soluble components in green tea do not, at least, inhibit the effect of catechins. This data means that the ingestion of green tea is as effective as the ingestion of GT-catechin for prevention of brain aging.

Effective intake period of EGCG for prevention of brain dysfunction

How long should green tea catechins be ingested? When should the ingestion of green tea catechins start? The results indicate that an ingestion period > 5 months (1-11, 3-11, 6-11, 3-9, and 1-6) was significantly effective while ingestion periods of 2 and 3 months (9-11 and 3-6) were slightly effective (***Fig. 3***). We predicted that mice that ingested EGCG only when young (*i.e.*, 1-6 months) might not fully suppress cognitive dysfunction if the accumulation of damage might start from adulthood or middle age. However, the ingestion of EGCG for 1-6 months was as effective as ingestion for 3-9 and 6-11 months, suggesting that oxidative damage accumulated from a young age.

As the average longevity of SAMP10 mice is about 17 months, 8-9 months is considered to be the middle of the life span. On the other hand, body weight increased until about 4 months; thereafter, body weight was maintained to a plateau level (data not shown), suggesting that an age above 4 months was a mature adult. Therefore, in SAMP10, we considered

that 6 and 9 months of age to be adulthood and middle age, respectively. An intake period above 5 months accounts for about 1/3 of the lifespan of these mice, suggesting that the intake of catechin ought to start at adulthood or middle age at the latest.

Synaptic plasticity

Cerebral atrophy was significantly suppressed in mice that ingested GT-catechin from 6- to 12-months of age (***Table 1***). The progression of atrophy was slightly suppressed in mice that ingested EGCG, EGC and green tea extract, but a significant difference was not observed among these groups. Although neuronal loss is minimal in most cortical regions of the normal aging brain, gene expression profiling studies of aging mice, monkey and human brains have shown significant changes in the expression of synaptic genes [20-24]. Then the levels of synaptophysin, a marker of presynapse, were compared. The level of synaptophysin in the anterior cortex of aged SAMP10 was about 50-70% of age-matched SAMR1, and the loss of synapse has been observed in aged SAMP10 as a reason for cognitive dysfunction [13]. The level in mice that ingested GT-catechin was significantly higher than that in control mice (***Fig. 4***). The level in mice that ingested EGCG tended to be higher than that in control mice, but no effect was observed in mice that ingested EGC. The level in EGCG-treated mice was slightly lower than that in GT-catechin-treated mice, suggesting that EGCG is an active component in synaptic plasticity while other catechins such as ECG, but not EGC, might additionally suppress synaptic loss. The level of synaptophysin in mice that ingested green tea extract tended to be higher than that in mice that ingested GT-catechin. As caffeine is found in green tea extract, it might additionally suppress synaptic loss. Caffeine acts on plastic change in structure and function from synapse to cortical network levels [25], and abrogates both memory impairment and synapto-toxicity in hippocampus of animal models such as Alzheimer's disease [26] and in aged rats [27]. Therefore, both the intake of catechin, mainly EGCG, and caffeine is thought to be better for the prevention of synaptic loss.

Although more study is needed for understanding the mechanism of GT-catechin in brain, the continued ingestion of GT-catechin or green tea, at least from adulthood or middle age, is effective for suppression of age-related cognitive dysfunction.

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