Original Article

Tocotrienol improves learning and memory deficit of aged rats

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To define whether tocotrienol (T-3) improves cognitive deficit during aging, effect of T-3 on learning and memory functions of aged rats was assessed. It was found that T-3 markedly counteracts the decline in learning and memory function in aged rats. Quantitative analysis of T-3 content in the rat brain showed that the aged rats fed T-3 mixture-supplemented diet revealed the transport of α- and γ-T-3 to the brain. In contrast, normal young rats fed the same diet did not exhibit brain localization. Furthermore, the T-3 inhibited age-related decreases in the expression of certain blood brain barrier (BBB) proteins, including caludin-5, occludin and junctional adhesion molecule (JAM). It was found that the activation of the cellular proto-oncogene c-Src and extracellular signal-regulated protein kinase (ERK), in the mitogen-activated protein kinase (MAPK) cell signaling pathway for neuronal cell death, was markedly inhibited by T-3. These results may reveal that aging induces partial BBB disruption caused by oxidative stress, thereby enabling the transport of T-3 through the BBB to the central nervous system, whereupon neuronal protection may be mediated by inhibition of c-Src and/or ERK activation, resulting in an improvement in age-related cognitive deficits.

***Key Words*: cognitive deficit, aging, tocotrienol, BBB, c-Src**

The vitamin E family is composed eight members, including α-, β-, γ-, δ-tocopherols and α-, β-, γ-, δ-tocotrienols. It is known that vitamin E has potent antioxidant effects as well as non-antioxidant properties. Among the vitamin E family, the roles of α-tocopherol (α-Toc) in living tissues have been extensively studied for several decades. Many reports have demonstrated that α-Toc potently protects against oxidative stress and/or prevents oxidative damage in tissues.[1] It is well recognized that oxidative stress occurs in living tissues during periods of imbalance between reactive oxygen species (ROS) generation and antioxidant detoxification. ROS that overwhelm the antioxidative mechanisms induce oxidative damage to many organs. In the nervous system, it has been reported that the antioxidant properties of α-Toc protect neurons by inhibiting oxidative damage to nerve terminals,[2–6] thereby maintaining homeostasis in neurotransmission. Furthermore, α-Toc prevents cognitive deficits in aged rats and young rats subjected to oxidative stress,[7–9] and delays institutionalization and the onset of severe dementia in Alzheimer's disease (AD).[10] Although the non-antioxidant effect of α-Toc has been widely studied,[11] its neuroprotective effects remain unclear.

Although it has been shown that tocotrienol (T-3), which is a tocopherol homologue, also has antioxidant properties,[12] recent reports have shown that T-3 has potent functions that are independent of its antioxidant properties, such as a cholesterol-lowering effect,[13] suppression of cancer,[14,15] anti-angiogenic and anti-inflammatory effects and a neuroprotective property.[15–17] In particular, Sen *et al.*[18] studied the non-antioxidant properties of T-3 in neuroprotection, including the inhibitory effects on glutamate-, homocysteic acid-, and oxidized glutathione (GSSG)-induced neuronal cell death.[19–22] However, most of these studies were carried out in *in vitro* systems using neuronal cell culture. Presently, *in vivo* studies of the neuroprotective effects of T-3 have not been undertaken.

Although it is well known that extracellular signal-regulated protein kinase (ERK), which is member of the mitogen-activated protein kinase superfamily (MAPK), is involved in cell survival, it has recently been suggested that the activation of ERK contributes to death in certain cells, including neurons.[23] Since the activation of ERK is regulated by the cellular proto-oncogene c-Src activation, which is upstream in the MAPK pathway, it is important to assess whether T-3 is involved in the blockade of ERK and c-Src activation *in vivo*.

The aim of this study is to assess whether oral supplementation with T-3 protects against neuronal death in the aged rat brain, thereby improving cognitive deficits, and to define the neuroprotective mechanism by T-3.

Materials and Methods

Animals. All animal experiments were performed with the approval of the Animal Protection and Ethics Committee of the Shibaura Institute of Technology. Young male Wistar rats (12 weeks old) were subjected to oxidative stress (100% oxygen) at 20°C for 48 h in an oxygen chamber, followed by feeding with T-3s-mixed diet [250 mg/100 g diet, kindly supplied by Eisai Food & Chemical Co., Ltd. (Tokyo, Japan)] for 3 weeks. Aged male Wistar rats (23 months old) were fed *ad libitum* with d-α-Toc-supplemented diet (253 mg/100 g diet), or the same T-3s-mixed diets for 3 weeks. The composition of T-3s mixture is: d-α-T-3, 31.5%; d-β-T-3, 4.2%; d-γ-T-3, 45.7% and d-δ-T-3, 17.2%. Young control, young control subjected to oxidative stress and aged control rats were fed *ad libitum* with a standard diet (α-tocopherol content: 3–5 mg/100 g diet).

Chemicals. Dithiothreitol, leupeptin, isoluminol and microperoxidase were purchased from Sigma Chemical Co. (St. Louis, MO); PIPES [piperazine-1,4-bis(2-ethnesulfonic acid)] and CHAPS {3-[(3-cholamidopropyl)dimethyl-ammonio] propane-sulfonic acid} from Dojindo Laboratories (Kumamoto, Japan); pepstatin-A, PMSF (phenylmethylsulfonyl fluoride), sodium tetraborate decahydrate and BHT (3,5-di-tert-butyl p-hydroxytoluene) from Wako Pure Chem. Co., Ltd. (Osaka, Japan); aprotinin from Takara Bio Inc. (Otsu, Japan); Can Get Signal solution 1 and 2 from

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TOYOBO Co., Ltd. (Osaka, Japan); ECL Prime Western Blotting Detection Reagent from GE Healthcare Japan Co., Ltd. (Tokyo, Japan); and β-galactosidase from Calbiochem (San Diego, CA). All other chemicals were of the highest grade available. Antibodies: Anti-c-Src (B-12), p-ERK1/2 (Thr 202/Tyr 204) and junctional adhesion molecule-A (JAM-A) (H-80) were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany); anti-occludin was from ProSci Incorporated (Poway, CA); anti-claudin-5 (S201) pAb was from Bioworld Technology, Inc. (Louis Park, MN). The IgG fraction of a polyclonal rabbit antiserum to β-galactosidase was obtained from Nordic Immunological Laboratories, Inc. (Tilburg, Netherlands). Horseradish peroxidase (HRP)-conjugated goat IgG fraction against mouse IgG and HRP-conjugated donkey anti-rabbit IgG were purchased from Promega Corporation (Madison, WI).

Behavioral testing. After 3 weeks of feeding with T-3 supplemented diet, aged rats fed T-3 were tested for their learning ability and memory function using a Morris water maze apparatus.[24] The bottom of the pool was divided into quadrants using white lines, and a transparent platform was submerged 2 cm below the surface of the water at the center of one of the quadrants; the water was maintained at 21 ± 1°C. For pre-training, the rats were allowed to swim freely in the pool for 120 s without the platform. Daily training consisted of one trial in which the rats swam from the start point to a fixed goal; this was conducted on 15 consecutive days. The time to reach the goal and the swimming distance from the start point to the platform were measured. The swimming distance was measured by tracing the tracks of swimming. The rates of decreases in swimming time and distance from the first trial values were used as measures of learning ability.

After all the groups had learned the task through their own abilities, they were rested in a normal atmosphere at 21 ± 1°C for 48 h without further trials. Subsequently, their memory functions to the place of the platform were assessed using the same water maze apparatus.

Quantitative analyses of T-3 in the brain. After assessment of memory function, all rats were sacrificed by decapitation. The brains were immediately removed and homogenized in an ice-cold phosphate buffered saline (pH 7.4, PBS). An aliquot of each brain homogenate was mixed with, 6% BHT solution in ethanol (2 ml), 35% KOH solution (1 ml) and 5,7,8-penta methyl chromanol (PMC, 240 pmol) as an internal standard. The mixture was saponified at 100°C for 45 min. After cooling, a 1% NaCl solution and a mixture of hexane-ethyl acetate (9:1, by mol) were added. Each extract was evaporated under nitrogen gas, and methanol (200 μl) was added to the residue. The solution was analyzed by HPLC at 10°C with electrochemical detection using a Develosil C-30-DG column (Nomura Chemicals, Tokyo, Japan).

Measurement of the expression of BBB proteins, c-Src and p-ERK. Aliquots of brain homogenate (protein content: 10 μg for analysis of c-Src, 40 μg for p-ERK, 20 μg for claudin-5 and 50 μg for occludin and JAM) were homogenized three times in an ice bath for 5 s using an ultrasonic homogenizer. The homogenate was dissolved in 5 μl of CHAPS cell extract buffer (100 mM PIPES, 4 mM EDTA, 0.2% (w/v) CHAPS, 110 mM DTT, 40 μg/ml leupeptin, 20 μg/ml pepstatin-A, 20 μg/ml aprotinin, 2 mM PMSF, pH 6.8) and mixed with a β-galactosidase solution (1 μl, 500 μg/ml in PBS). After the mixture was incubated for 20 min in an ice bath, 125 mM Tris-HCl buffer (pH 6.8), containing 4% sodium dodecyl sulphate (SDS), 20% glycerol, 0.01% (w/v) bromophenol blue and 0.55% (w/v) mercaptoethanol, was added. The mixture was fractionated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) together with a protein molecular weight marker (NIPPON Genetics Co., Ltd., Tokyo, Japan). Separated proteins were transferred onto Immobilon transfer membranes (Millipore, Bedford, MA). The membranes were washed twice with a 25 mM Tris buffer, containing 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween-20 (TBS-T, pH 7.4). After blocking non-specific binding sites for 1 h with a blocking solution [TBS-T with 2% bovine serum albumin (BSA), pH 7.4], blots were incubated with anti-c-Src (1:1,000), anti-p-ERK (1:1,000), anti-claudin-5 (1:750), anti-occludin (1:500), anti-JAM (1:500) and anti-β-galactosidase antibodies (1:1,000) in Can Get Signal solution 1 at 4°C for 12 h. Blots were washed with TBS-T, and incubated with HRP-linked anti-mouse secondary antibody (1:2,000) in Can Get Signal solution 2 for synapsin-I antibody. HRP-linked anti-rabbit secondary antibody was used for all other antibodies.

The HRP-labeled antibodies were detected with the enhanced chemiluminescence detection system using a luminoanalyzer (Las-3000, FUJI film imaging Co., Ltd., Tokyo, Japan). β-Galactosidase was used as an internal standard. The chemiluminescence of each sample was measured for three times to evaluate the loading efficiency.

Analyses of the lipid peroxides and oxidized proteins in the brain. Thiobarbituric acid reactive substances (TBARS) levels were measured as previously reported by Ohkawa *et al.*[25] The content of TBARS is express as nmol equivalents of malondialdehyde per mg protein in the samples. Lipid hydroperoxides (LOOH), formed by the peroxidation of unsaturated fatty acids, were analyzed as follows; one drop of 0.05% BHT, 500 μl of PBS, 2 ml of a mixture of chloroform and methanol (2:1) and 200 μl of 10% NaCl solution were added to 100 μl of the brain homogenate. The reaction mixture was centrifuged at 1,400 × *g* for 10 min at 4°C. After centrifugation, the resultant organic phase was dried under nitrogen gas. The residue was dissolved in 200 μl of methanol and added to 150 μl microperoxidase-luminol (1:100) chemiluminescence reagent. The reaction mixture was measured using a luminescencer PSN AB-2200 (ATTO Corporation, Tokyo, Japan), LOOH was calculated using a calibration curve obtained by measuring 8–128 pmol cumen hydroperoxidase solution and cumulative luminescence. Protein carbonyl, as an index of protein oxidation, was determined according to Levine *et al.*[26] Briefly, each homogenate in PBS (200 μl) was treated with 800 μl of 10 mM 2,4-dinitrophenyl hydrazine (DNPH) in 2 N HCl and incubated at 37°C for 1 h. The mixture was treated with 20% trichloroacetic acid (TCA) to precipitate the protein, and kept in an ice bath for 10 min. After centrifuging at 10,000 × *g* at 4°C for 10 min, the precipitate obtained was washed several times with a solution of ethyl alcohol and ethyl acetate (1/1, v/v) to remove the un-reacted DNPA. The precipitate was mixed with 1 ml of 6 M guanidine HCl (pH 2.3) to obtain the protein solution. Samples were analyzed using a spectrophotometer (Shimadzu UV-1200, Kyoto, Japan) at 365 nm.

Analyses of antioxidative enzymes in the brain. The activities of antioxidative enzymes; superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GSHPx) were analyzed using aliquots of the brain homogenates in accordance with previous methods.[27–29]

Statistical analysis. Results are presented as means ± SE. Comparisons between multiple groups were made by ANOVA, followed by a Tukey's test. A p value less than 0.05 was considered to be statistically significant.

Results

Improvement of age-related cognitive deficits by T-3 dietary supplementation. To evaluate the effect of T-3 on age-related cognitive impairment, learning and memory functions were assessed using a Morris water maze test. As shown in Fig. 1a, normal aged rats needed 15 trials to recognize the position of the platform, which is consistent with our previous reports.[8–9] Furthermore, since the rate of swimming time (from start point to the platform) of the aged rats did not change after 15 trials, their maximum learning ability was calculated by approximately 60% (Fig. 1a). Conversely, when aged rats were fed the α-Toc-

supplemented diet for 3 weeks before the start of the trial, their learning ability was markedly enhanced. Interestingly, it was found that T-3, the homologue of α-Toc, improved their learning ability more significantly than α-Toc. Similarly, young rats subjected to oxidative stress showed the decline of their learning ability. Such a decrease in their learning ability was also enhanced markedly by the T-3 supplementation.

After 15 trials, when all rats had learned the location of the platform, the animals were rested for 48 h under normal conditions without further behavioral testing. Subsequently, their memory function was tested. Normal aged rats and young rats subjected to oxidative stress showed a marked decline in memory retention (Fig. 1b). In contrast, the memory function of the α-Toc-supplemented aged rats was markedly improved, although its efficacy decreased after 8 trials. However, although the tendency for memory retention in aged rats fed the T-3-supplemented diet was similar to the α-Toc supplemented aged rats, efficacy in the T-3-supplemented group was largely retained throughout the 19 trials. The memory loss of young rats subjected to oxidative stress was similarly improved by the T-3 supplementation.

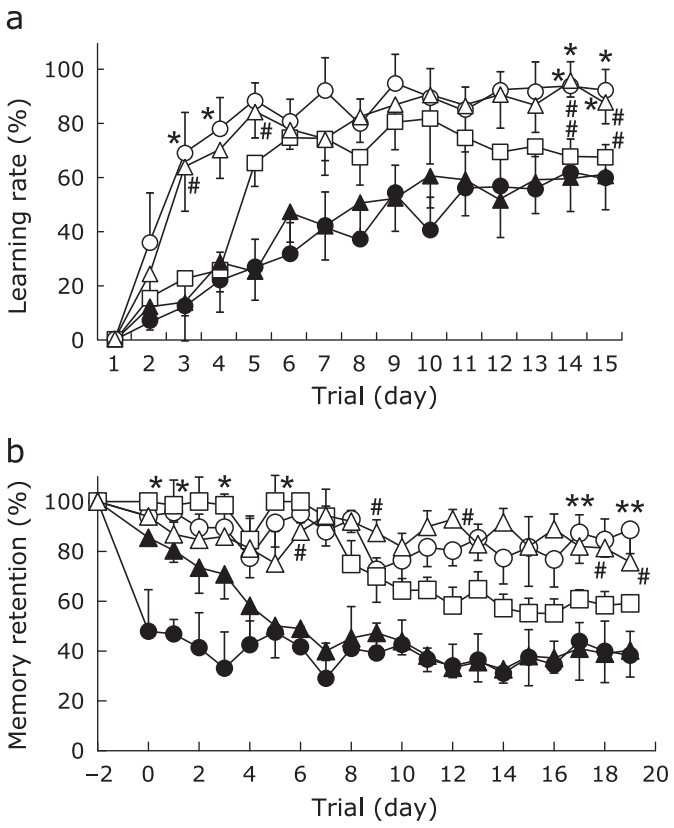

Fig. 1. Effect of T-3 on learning and memory deficits in aged rats. (a) Cognitive performance in learning trials of aged rats (●), α-Toc supplemented aged rats (□), T-3 supplemented aged rats (○), young rats subjected to hyperoxia (▲), and T3 supplemented young rats subjected to hyperoxia (△) *p<0.01, **p<0.05 vs aged rats fed α-Toc supplemented diet, $^{\#}p$<0.01, $^{\#\#}p$<0.05 vs young rats subjected to hyperoxia; n = 9 for each group. Trials were carried out daily for 15 days. (b) Memory retention of aged rats (●), α-Toc supplemented aged rats (□), T-3 supplemented aged rats (○), young rats subjected to hyperoxia (▲), and T3 supplemented young rats subjected to hyperoxia (△). *p<0.01 vs aged control, **p<0.05 vs aged rats fed α-Toc supplemented diet, and $^{\#}p$<0.01 vs young rats subjected to hyperoxia; n = 9 for each group. Each trial was carried daily for 19 days.

Content of T-3 in the brain of aged rats with T-3 supplementation. Since it was found that T-3 has potent effects on cognitive improvement, we assessed whether T-3 is present in the brain. T-3 content was undetected in the brains of normal aged rats and young rats without T-3 dietary supplementation. In contrast, it was found that α- and γ-T-3 molecules, which are presented in the T-3s mixed diet, were existed at pmol/mg protein levels in the brains of aged rats and young rats subjected to oxidative stress which received T-3 dietary supplementation. Interestingly, the content of γ-T-3 was higher than that of α-T-3, which is inconsistent with the composition in the T-3s mixed diet. However, in this analysis β- and δ-T-3 were not detected in the brain (Table 1).

T-3-mediated inhibition of changes in BBB status by oxidative stress and aging. Since α- and γ-T-3 were found in the brain of T-3 supplemented aged rats and young rats subjected to oxidative stress, it is evident that these molecules passed through the BBB, which tightly regulates molecular transport into the nervous system, to elicit neuroprotection. In order to confirm this hypothesis, we analyzed the status of BBB protein components, namely, the expression levels of claudin-5, occludin and JAM-A. As shown in Fig. 2a–c, the expression of these proteins decreased markedly in the brain of normal aged rats and rats subjected to oxidative stress. T-3 supplementation in young rats subjected to oxidative stress produced a tendency to prevent the oxidative stress-induced disruption of these proteins. Additionally, the reduced levels of these proteins in aged rats were not inhibited by T-3 supplementation.

T-3-induced changes in the levels of oxidized components and antioxidative enzymes in the brain. To determine whether the T-3-induced improvements in cognitive deficits in aged rats is mediated by antioxidant activity or non-antioxidant activity, changes in the levels of TBARS, LOOH and protein carbonyls in the brain were analyzed. Furthermore, the status of antioxidative enzymes, SOD, Cat and GSHPx, was also assessed. As shown in Fig. 3a and b, T-3 did not statistically inhibit an increase in these oxidized denature components with age. Furthermore, the activity of antioxidative enzymes was not changed by T-3 supplementation.

Inhibitory effect of T-3 on oxidative stress- and aging-induced activation of c-Src and ERK in the brain. As shown in Fig. 4a and b, it was found that oxidative stress and aging induced the activation of c-Src and ERK, and that T-3 inhibits this activation. Although the expression of c-Src and p-ERK in the brain of aged rats and young rats subjected to oxidative stress was greatly increased, expression levels were maintained at levels

Table 1. Analyses of T-3 homolog translocated to the brain

	α-T-3	β-T-3	γ-T-3	δ-T3
Young control	nd	nd	nd	nd
Young control supplemented T-3 mix	nd	nd	nd	nd
Young subjected to hyperoxia and supplemented T-3 mix	7.26 ± 2.43	nd	38.51 ± 7.26	nd
Aged control	nd	nd	nd	nd
Aged supplemented T-3 mix	1.23 ± 1.19	nd	27.80 ± 1.23	nd

Values are mean ± SE, n = 9, and expressed as pmol/mg protein in the brain.

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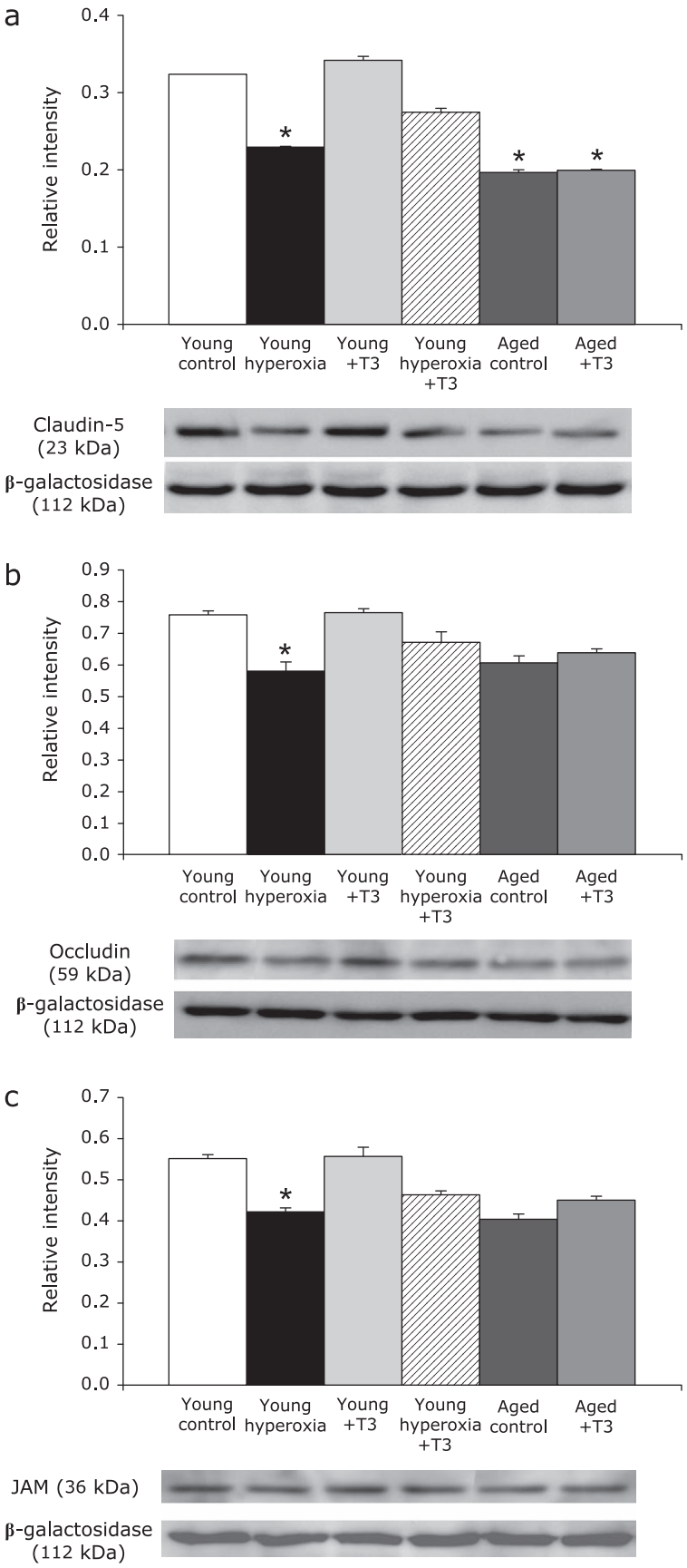

Fig. 2. Effect of T-3 on the disruption of BBB component proteins. (a) The expression of claudin-5 in aged and young rats. *p<0.02 vs young control. (b) The expression of occludin in aged and young rats. *p<0.03 vs young control. (c) The expression of JAM in aged and young rats. *p<0.03 vs young control; n = 9 for each group.

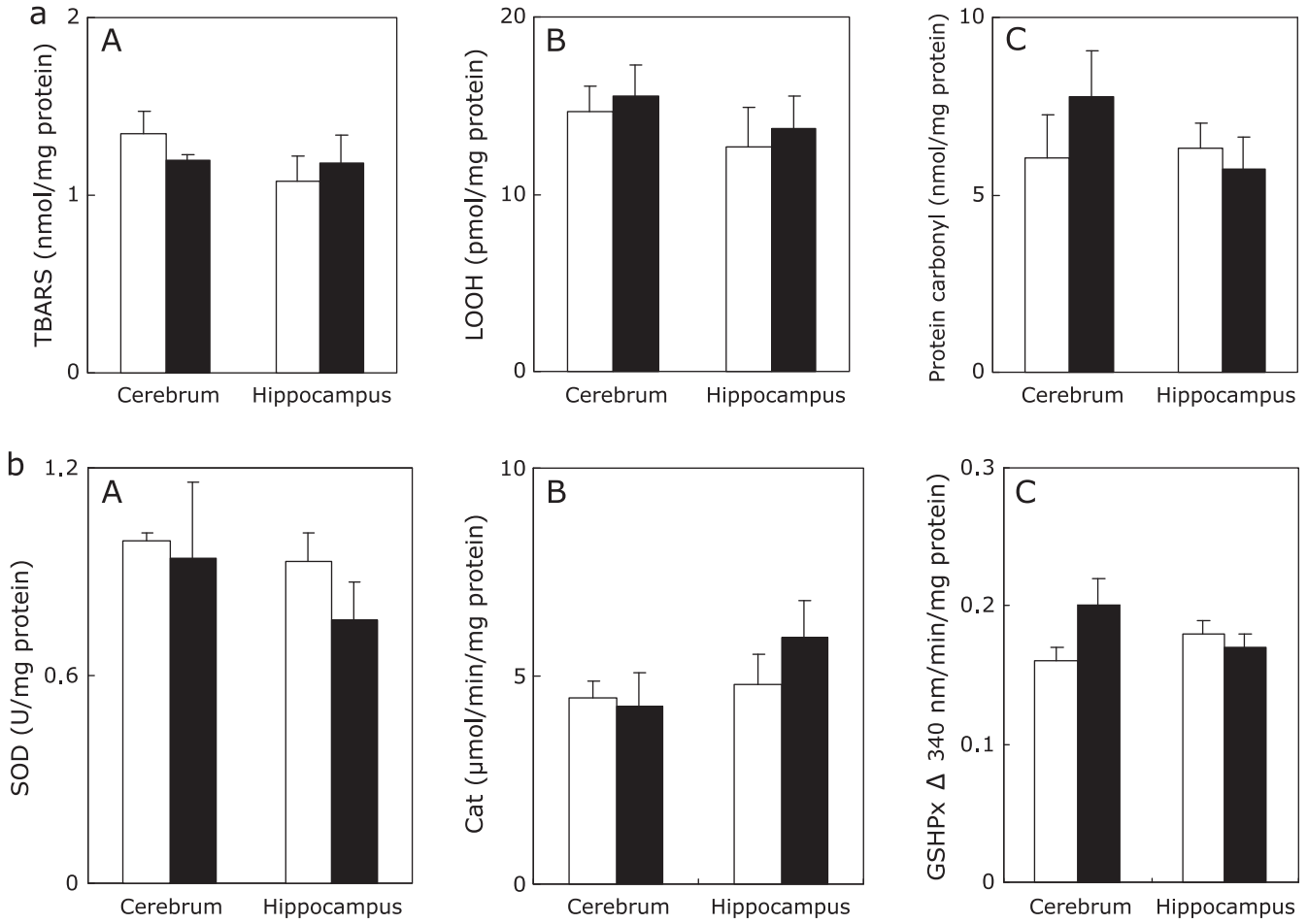

Fig. 3. Levels of denatured components and antioxidant enzymes in the cerebrum and hippocampus of aged rats. (a) Levels of TBARS (A), LOOH (B) and protein carbonyls (C). Open columns, aged control; closed columns, T-3 supplemented aged rats. (b) The activities of SOD (A), Cat (B) and GSHPx (C). Open columns, aged control; closed columns, T-3 supplemented aged rats; *n* = 9 for each group.

comparable to those of young control rats when both groups were fed a T-3-supplemented diet (Fig. 4a and b).

Discussion

There is a theory that, during aging, chronic oxidative stress acts over long periods of time to produce ROS in living tissues. This theory proposes that most changes during aging are caused by free radical reactions and the formation of lipid peroxides, which lead to age-related damage and eventually to various aging processes and phenomena.[30] The brain is more susceptible to oxidative stress than other organs due to the high content of polyunsaturated-lipids in the neural parenchyma, high levels of oxygen consumption (accounting for one-fifth of the total systemic consumption), low catalase activity and moderate SOD and GSHPx activities.[31] Furthermore, since neurotoxic iron, which induces ROS, accumulates during aging in the rat brain,[32] the levels of peroxidized substances, such as lipid peroxides, oxidized proteins and modified DNA, increase in the brain with oxidative stress.[3,33–36] In addition, abnormalities observed in neurological disorders in the aged are similar to those observed in vitamin E (antioxidant)-deficient individuals.[37,38] Consequently, neurodegeneration during brain aging has been speculated to be mediated by ROS-induced peroxidative damage through chronic oxidative stress. Considering these phenomena, it is reasonable to infer that the antioxidant properties of vitamin E prevent neuronal dysfunction caused by oxidative stress. In fact, it has been suggested that α-Toc prevents cognitive deficits in young rats subjected to oxidative stress.[7] However, the brain of aged rats is thought to be more oxidatively damaged than that of normal young rats. Consequently, it can be presumed that the antioxidant properties of α-Toc may be ineffective in improving cognitive deficits in aged rats, even when they fed a vitamin E-supplemented diet. Conversely, it is reasonable to speculate that α-Toc and/or T-3 could improve cognitive impairment during brain aging through effects not related to antioxidation.

As mentioned earlier, T-3 possesses higher antioxidant efficacy than α-Toc,[12] as well as potent, antioxidation independent, neuroprotective activity that is not often exhibited by tocopherols.[18] In this study, we assessed the efficacy of T-3 on counteracting learning and memory deficits in aged rats as well as young rats subjected to oxidative stress. Aged rats and young rats subjected to oxidative stress showed very weak learning function. In contrast, when they were fed by T-3 for 3 weeks before the start of the trial, their learning ability was markedly enhanced (Fig. 1a). Interestingly, their maximum faculty in space cognition was extremely elevated compared to that of aged rats fed the α-Toc diet. Thus, it is evident that T-3 improves the oxidatively impaired learning ability in aged rats.

After 15 learning trials, aged rats and young rats subjected to oxidative stress were rested for 48 h without trial in a normal atmosphere, and their memory retention of the platform placement

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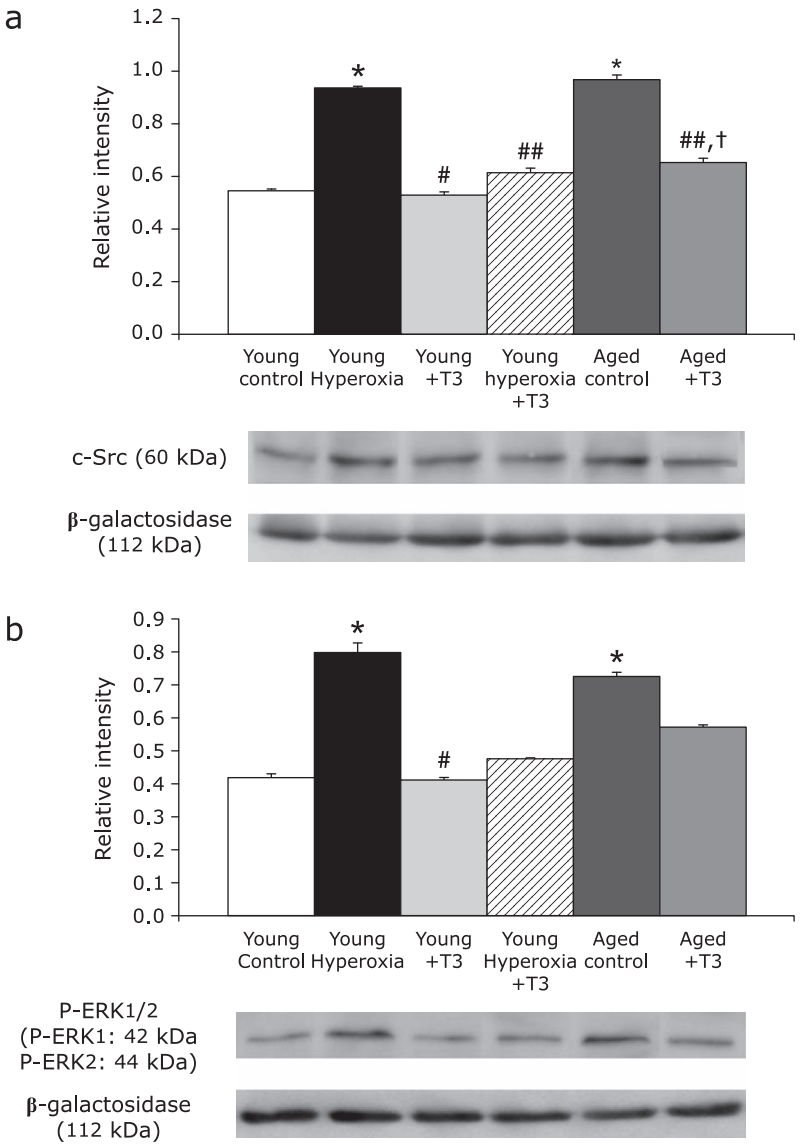

Fig. 4. Inhibitory effect of T-3 on oxidative stress-induced activation of c-Src and ERK in the rat brain. (a) Expression of c-Src in aged and young rats. **p*<0.01 vs young control, #*p*<0.02, ##*p*<0.05 vs young rats subjected to hyperoxia, †*p*<0.05 vs aged control. (b) The expression of p-ERK1/2 in aged and young rats. **p*<0.03 vs young control, #*p*<0.03 vs young rats subjected to hyperoxia; *n* = 9 for each group.

was assessed. The memory retention of the normal aged rats declined ≈50% for the first trial. Aged rats fed the T-3-supplemented diet showed a marked improvement in the memory deficit observed in the aged control rats. Although α-Toc also enhanced memory function, its efficacy was decreased after 8 trials. In contrast, T-3 fed rats retained their memory for 19 trials (Fig. 1b). These results suggest that T-3 improves cognitive deficits caused by oxidative stress in the aged, and maintains function for longer periods of time than α-Toc. The memory of young rats subjected to oxidative stress gradually declined, and reached to the level of the aged rats after 5 trials. Similarly, T-3-supplementation improved markedly their memory loss (Fig. 1b). Although it is commonly accepted that T-3 does not reach to the brain,[39] the existence of T-3 in the brain has been reported in human epilepsy and stroke patients, as well as pregnant rats and their fetuses.[40,41] In this study, although T-3 was not determined in the brain of normal young rats and aged rats fed the standard diet, transport of T-3 to the brains in young rats subjected to oxidative stress and aged rats was observed by pmol/mg protein levels after T-3 supplementation. On the contrary, previous reports showed that α-Toc exists in the brain of these animals at the higher levels such as nmol or μmol levels,[2,3,39] so that T-3 may not be transported easily to the brain under normal condition. Interestingly, the chemical structure of T-3 has only three double bonds in the side chain of α-Toc. However, it is impossible at the present to elucidate this reason.

Considering the damage to the capillaries caused by oxidative stress, that is, abnormally swollen astrocytes and endothelial cells,[2] the results obtained here suggest that T-3 can reach to the brain of aged rats due to oxidative damage to arteries during aging. In fact, recent study revealed that ROS generated by oxidative stress directly induce the degradation of tight junctions (TJs), leading to BBB disruption.[42–44] In order to reach the brain, and to improve cognitive deficits in aged rats, T-3 molecules need to pass through the BBB, which is a complex cellular gate that regulates the transport of molecules into the central nervous system. Since the occurrence of BBB alterations during human aging and dementia, including AD, have been suggested,[45,46] it follows that

the BBB structure in the aged rat brain may be partially disrupted during aging, thereby enabling T-3 transport to the brain observed in this study. This contention is consistent with previous reports on epilepsy and stroke, as well as on rat fetuses, which demonstrated the transport of T-3 to the brain.[40,41] Since epilepsy and stroke are associated with blood vessel dysfunction in the brain, the BBB may be partially disrupted in these conditions. Furthermore, formation of the blood vessel occurs during the fetal period, indicating that the fetal BBB is immature. Consequently, this implies that T-3 transport to the fetal brain occurs due to incomplete BBB formation. Although Patel *et al.*[40] found T-3 transport in the brain of pregnant rats, the mechanism in pregnancy may be different from this hypothesis.

In this study, the expression of BBB constituent proteins (claudin-5, occuldin-1 and JAM) was decreased in the brain of aged rats and rats subjected to oxidative stress (Fig. 2a–c). Consequently, it implies that T-3 is transported to the brain through the partially disrupted BBB in the aged rats. Since loss of these BBB components in the brain of aged rats and rats subjected to oxidative stress was not inhibited by T-3, it is evident that T-3 did not inhibit loss of the BBB components caused by oxidative stress and aging. Therefore, the ability of T-3 to improve cognitive deficits may not be due to its antioxidant property. To confirm this notion, we assessed the effect of T-3 on the inhibition of peroxidized components and changes in antioxidative enzymes in the brain. As shown in Fig. 3a and b, the levels of lipid peroxides and oxidized proteins did not change, even when aged rats were supplemented with T-3. Furthermore, activities of antioxidative enzymes were not influenced by T-3. However, it is impossible at present to explain the reason of no influence of T-3.

These results may support the contention that T-3 improves cognitive deficits in aged rats due to non-antioxidant effects. In this study, since the influence of T-3 on the denatured components and activity of antioxidative enzymes in the brain of young rats was not analyzed, further assessment is necessarily needed.

The neuroprotective mechanism involved in improving cognitive deficits after transport of T-3 into neuronal cells in aged rats was investigated. It has been suggested that α-T-3 inhibits apoptotic cell death by blocking the phosphorylation of c-Src and ERK in the MAPK signaling pathway.[19] Although it is well recognized that the activation of ERK promotes cell survival, recent studies suggest that ERK activation also contributes to apoptotic cell death under certain conditions, such as oxidative stress.[23] It has been suggested that ERK activation-induced apoptosis is caused by caspase-3 activation through tumor necrosis factor-α (TNF-α) activation and/or cytochrome c release from mitochondria.[23] In fact, when young rats were subjected to hyperoxia, the expression of c-Src and ERK in the brain were markedly enhanced. Aged rats also showed enhanced activation of c-Src and ERK, presumably due to long-term exposure to oxidative stress (Fig. 4a and b). Based on these observations, it is reasonable that neuronal cell death caused by oxidative stress may be involved in the activation of c-Src and ERK.[8] Since we did not demonstrated in this study whether c-Src/ERK activation and its inhibition by T-3 are performed in nerve cells, an intensive study, such as the immunohistochemical examination, is required on the matter.

As shown in Fig. 4a and b, neuroprotection was likely due to T-3 significantly inhibiting the activation of c-Src and ERK, thereby blocking the MAPK signaling pathway for apoptotic cell death. Based on the previous findings that delayed-type neuronal apoptosis appeared in the brain of young rats at 7 days after hyperoxia,[7] it will be expected that the activation of c-Src and ERK caused by oxidative stress may accord with the appearance of such delayed-type neuronal apoptosis. However, the analyses of c-Src and ERK were carried out after assessment of memory function of animals, so that it is impossible in the present study to clarify the accordance of both phenomena.

It is still unclear whether the target of T-3 in this inhibition is c-Src or ERK *in vivo*, although previous report proposed that both components in cultured cells were blocked by T-3.[19] To solve this question, further studies, such as an investigation using the specific inhibitor of ERK activation, are necessarily needed.

In conclusion, the results obtained in this study are the first to demonstrate that T-3 can reach to the brain through the oxidatively damaged BBB in the aged rats, and, then, markedly improves age-related deficits in learning and memory. This fact is supported by the finding that T-3 improves similarly the cognitive deficit of young rats subjected to oxidative stress. When considering the partial disruption of the BBB observed during aging, it is understandable that the aged have low tolerance for many medicines. Furthermore, it implies that after reaching neuronal cells, T-3 inhibits the activation of cell death-promoting protein ERK, which results in neuroprotection and the potential for improvements in cognitive deficits in the aged. This notion is supported by the observation that oxidative stress and aging induce the hippocampal cell death in the rat brain, and that α-Toc inhibits these phenomena.[8]

The results obtained in this study may, to a certain extent, explain the fact that vitamin E delays institutionalization and the onset of severe dementia.[10] This study did not elucidate the reason for γ-T-3 levels being greater than α-T-3 levels in the brain. Consequently, it is not evident which T-3 subtype contributes to the improvement of cognitive deficits in aged rats, and further studies are necessary to clarify this phenomenon.

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Abbreviations

AD	Alzheimer's disease
α-Toc	α-tocopherol
BBB	blood brain barrier
Cat	catalase
c-Src	cellular proto-oncogene
ERK	extracellular signal-regulated protein kinase
GSHPx	glutathione peroxidase
GSSG	oxidized glutathione
JAM	junctional adhesion molecule
LOOH	lipid hydroperoxide
MAPK	mitogen-activated protein kinase
ROS	reactive oxygen species
SOD	superoxidedismutase
T-3	tocotrienol
TBARS	thiobarbitric acid reactive substances
TNF-α	tumor necrosis factor-α

Conflict of Interest

No potential conflicts of interest were disclosed.

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