Radial maze–learning ability. Both 2 and 5 mo after starting the PE administration, the rats' learning ability was tested by an assessment of their behavior in an 8-arm radial maze (Toyo Sangyo) as described (9). Briefly, the rats were trained to acquire a reward (food pellet) at the end of each of the 4 arms of an 8-arm radial maze. The performance involved 2 parameters of memory function: reference memory error (RME), i.e., entry into unbaited arms; and working memory error (WME), i.e., repeated entry into arms that had already been visited in the same trial. Each rat was given 2 trials, 6 d/wk, for a total of 5 wk.

Tissue preparation. After completing the maze task, the rats were anesthetized with sodium pentobarbital (50 mg/kg BW, i.p.), and their blood was collected; the cerebral cortex and hippocampus were then separated as described (4,5). A portion of the frontal cortex (100 mg) was immediately homogenized on ice in 1.0 mL of ice-cold 0.32 mol/L sucrose buffer (pH 7.4) containing 2 mmol/L EDTA, 0.5 mg/L leupeptin, 0.5 mg/L pepstatin, 0.5 mg/L aprotinin, and 0.2 mmol/L phenylmethylsulfonyl fluoride using a Polytron homogenizer (PCU 2–110; Kinematica). The residual tissues were stored at −80°C after flash-freezing in liquid N_2 until use. The homogenates were immediately subjected to the assays described below or stored at −80°C after liquid N_2 flash and bath until use.

Measurements of antioxidative status. The LPO concentration was assessed by the TBARS assay of Ohkawa et al. (10), as described (4,5), with the concentration measured in nanomoles malondialdehyde/mg protein. Malondialdehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane.

Plasma total antioxidant activity was measured by the ferric reducing antioxidation power (FRAP) assay of Benzic and Strain (11) with slight modification. The working FRAP reagent was prepared by mixing 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tripyridyl-*s*-triazine (TP) in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution. After mixing 3 mL of the working FRAP reagent with 400 μL plasma or standard solution in a test tube, a second reading was taken at 600 nm. A blank reading with only the FRAP reagent was subtracted from the absorbance of the FRAP reagent with a sample to measure the actual FRAP value of each tube.

The levels of ROS were determined as described (4,5). Briefly, 50 μL of freshly prepared tissue homogenate was mixed with 4.85 mL of 0.1 mol/L potassium phosphate buffer (pH 7.4) and incubated with 2′,7′-dichlorofluorescein diacetate (Molecular Probes) in methanol at a final concentration of 5 μmol/L for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500 × *g* for 10 min at 4°C. The pellet was mixed on a vortex at 0°C in 5 mL of 0.1 mol/L potassium phosphate buffer (pH 7.4) and incubated for 60 min at 37°C. Fluorescence was measured with a Hitachi 850 spectrofluorometer at wavelengths of 488 nm for excitation and 525 nm for emission. The cuvette holder was maintained at 37°C. ROS were quantified from a dichlorofluorescein standard curve in methanol. The protein concentration was estimated by the method of Lowry et al. (12).

Statistical analysis. Results are expressed as means ± SE. Behavioral data were analyzed by a 2-factor (group and block) randomized block factorial ANOVA; all other variables were analyzed for intergroup differences by 1-way ANOVA. ANOVA was followed by Fisher's Protected Least Significant Difference test for post hoc comparisons. Correlation was determined by simple regression analysis. The statistical programs used were GB-STAT 6.5.4 (Dynamic Microsystems) and StatView 4.01 (MindVision Software, Abacus Concepts). A level of $P < 0.05$ was considered significant.

RESULTS

PE intake and body weight. Daily water intake did not differ among the control [27.7 ± 1.7 mL/(rat·d)], 0.1% PE [26.0 ± 1.4 mL/(rat·d)], and 0.5% PE [26.2 ± 1.0 mL/(rat·d)] groups. PE intakes were 26.0 ± 1.4 mg/(rat·d) in the 0.1% PE group and 131 ± 7.0 in the 0.5% PE group). Final body weights did not differ among the groups and were 496 ± 8 g in the control group, 503 ± 10 g in the 0.1% PE group, and 508 ± 11 g in the 0.5% PE group.

Radial-maze learning ability. After 2 mo of PE administration, the scores of RME and WME in block 10 of the radial maze tasks undergone by the 0.5% PE rats were not lower than those of the control and the 0.1% PE rats. Therefore, we reestimated the learning ability (over a period of 6 wk) 20 wk after starting the administration of PE.

The effect of PE administration for 26 wk on reference (Fig. 1*A*) and working (Fig. 1*B*) memory-related learning ability is expressed as the mean number of RME and WME for each group, with the data averaged over blocks of 6 trials (Fig. 1). Randomized 2-factor (block and group) ANOVA, for analyzing the effect of PE (0.1 and 0.5%), revealed significant main effects of both blocks of trials ($P < 0.0001$) and groups ($P < 0.0001$) on the number of RME (Fig. 1*A*), but without a significant block × group interaction. Similarly, a significant main effect of both blocks of trials ($P < 0.0001$) and groups ($P = 0.0002$) was observed on the number of WME (Fig. 1*B*), but with a significant block × group interaction ($P < 0.0001$). Subtest analysis (**Table 1**) of the number of RME showed the effect of 0.1% PE group on control group (blocks of trials and groups, without a significant block × group interaction); the effect of 0.5% PE group on control group (blocks of trials and groups, without a significant block × group interaction); and the effect of the PE dose on PE-administered rats (blocks of trials and groups, without a significant block × group interaction), demonstrating that rats administered 0.1 and 0.5% PE had a lower RME score than the control rats (Fig.1*A*). Similarly, subtest analysis (Table 1) of the number of WME showed the effect of 0.1% PE group on control group (blocks of trials and groups, with a significant block × group interaction); the effect of 0.5% PE group on control group (blocks of trials and groups, with a significant block × group interaction); and the effect of the PE dose on PE-administered rats (blocks of trials, but not groups), without a significant block × group interaction, demonstrating that rats administered 0.1% and 0.5% PE had a lower WME score than the control rats (Fig. 1*B*). These analyses suggested that long-term administration of PE improved reference and working memory–related learning ability of rats.

Oxidative status of rat plasma and brains. Plasma TBARS concentrations were dose dependently decreased in the groups administered PE compared with the control group ($P = 0.0002$, Table 2). The plasma FRAP concentration was higher in the 0.5% PE group than in the control group ($P = 0.007$) (Table 2).

TBARS levels in the hippocampus were reduced in the 0.1 and 0.5% groups, compared with the control group ($P = 0.002$)

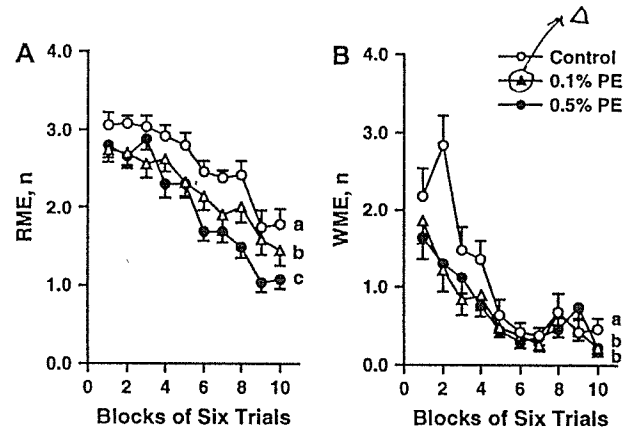

FIGURE 1 Reference (*A*) and working (*B*) memory–related learning ability in the radial maze task of rats administered 0 (control, $n = 8$), 0.1% PE ($n = 7$), or 0.5% PE ($n = 9$) for 26 wk. Values are means ± SEM in each block of 6 trials. Groups without a common letter differ, $P < 0.05$.

TABLE 1

Results of the 2-factor ANOVA and PLSD test conducted on RME and WME data obtained in rats administered 0 (control, n = 8), 0.1% PE (n = 7), or 0.5% PE (n = 9) for 26 wk[1]

	Reference memory error			Working memory error		
Group	Block	Group	Block × Group interaction	Block	Group	Block × Group interaction
0 vs. 0.1% PE	< 0.0001 ($F_{9, 423}$ = 17.55)	0.0003 ($F_{1, 47}$ = 15.18)	NS ($F_{9, 423}$ = 0.99)	< 0.0001 ($F_{9, 423}$ = 23.31)	0.0036 ($F_{1, 47}$ = 9.38)	0.003 ($F_{9, 423}$ = 2.83)
0 vs. 0.5% PE	< 0.0001 ($F_{9, 477}$ = 34.69)	< 0.0001 ($F_{1, 53}$ = 42.91)	NS ($F_{9, 477}$ = 0.15)	< 0.0001 ($F_{9, 477}$ = 24.58)	0.0005 ($F_{1, 53}$ = 13.56)	0.0002 ($F_{9, 477}$ = 3.61)
0.1% PE vs. 0.5% PE	< 0.0001 ($F_{9, 477}$ = 35.05)	0.0254 ($F_{1, 53}$ = 5.29)	NS ($F_{9, 477}$ = 1.86)	< 0.0001 ($F_{9, 477}$ = 25.83)	NS ($F_{1, 53}$ = ~~17.58~~)	NS ($F_{9, 477}$ = 0.76)

[1] Data are presented in Figure 1. NS, not significant, $P > 0.05$.

(Table 3). Similarly, the levels of ROS in the hippocampus were reduced in the 0.1 and 0.5% groups, compared with the control group ($P = 0.021$). Cortex TBARS and ROS levels did not differ among the rat groups. These results indicate that PE has antioxidative effects on oxidative status in rat plasma and the hippocampus.

Regression analysis revealed a significant positive correlation ($r = 0.520$, $P = 0.032$) between the hippocampal TBARS levels and the number of RME in block 10 of the radial maze task in control and 0.5% PE-administered rats (Table 4). There was a significant negative correlation between the plasma FRAP levels and the number of RME in block 10 of the radial maze task in control rats and those administered 0.5% PE ($r = -0.570$, $P = 0.017$) (Table 4). Similarly, the number of WME in block 10 of the radial maze task in control rats and those administered 0.5% PE correlated positively with plasma TBARS levels ($r = 0.622$, $P = 0.008$). The hippocampal TBARS levels and the number of WME tended to be positively correlated ($r = 0.480$, $P = 0.051$; Table 4).

DISCUSSION

The present study demonstrated that long-term administration of green tea catechins (PE) improves the performance in radial maze tasks and that the level of LPO in the hippocampus correlates significantly with the RME score. Thus, green tea catechins may be involved in protecting against neuronal degenerative stress and in the accumulation of LPO and ROS.

Green tea catechins comprise EGCG, EGC, ECG, and EC and protect the brain, liver, and kidney from lipid peroxidation injury (13). The relative antioxidant activity among tea catechins is EGCG = ECG > EGC > EC (14) Catechins have a protective effect against age-related neurological diseases associated with ROS (15). In this study, long-term (26 wk) administration of PE decreased the plasma and hippocampal oxidative status. In the process of aging, LPO and ROS accumulate and are constantly involved in some of the pathophysiologic effects associated with oxidative stress in cells and tissues. An increase in the production of LPO exacerbates the neurodegenerative process by deteriorating cellular enzymes (1). Antioxidative enzymes are activated by green tea catechin intake (16), and the antioxidative potency of human plasma increases with continual ingestion of green tea (17). These antioxidative defense systems might also prevent oxidative damage in the brain. Long-term intake of green tea catechins may be important because cells are constantly exposed to oxidative stress.

Aging leads to a decline in spatial memory–related learning ability. Oxidative damage to the brain is associated with age-related cognitive dysfunction (18), and some antioxidants are effective in improving such dysfunction; examples include the effects of a garlic extract on aged SAMP10 mice, a model of brain senescence with cerebral atrophy and cognitive dysfunction (19), and of vitamin E on rats with oxidative stress (20). Catechins are more effective radical scavengers than vitamins E and C (21,22). Long-term administration of green tea catechins to SAMP10 mice also suppressed cognitive dysfunction, as demonstrated by the duration of learning needed to acquire an avoidance response and by the assessment of working memory in the Y-maze (23). Chronic administration of catechins for 3.5 mo improved learning memory in maze behavior of both adult and old mice, although the mechanism of the improvement has not been clarified (24). In this study, to estimate the effects of the administration of green tea catechins on the learning ability of rats, PE was administered for 26 wk starting at 5 wk of age. Therefore, the point in time at which the effect of green tea catechins on the improvement of learning ability becomes apparent may differ among animal species.

The hippocampus and the cerebral cortex are the key structures of memory formation. Because the hippocampus is especially indispensable in the integration of spatial information, a decline in learning ability may be induced by the deterioration of hippocampal function. In this study, both a decrease in TBARS levels in the hippocampus and an increase in FRAP levels in the plasma were related to the acquisition of higher reference memory-related learning ability; in addition, a decrease in plasma TBARS levels was related to the acquisition of higher working memory-related learning ability (Table 4). A decrease in hippocampal LPO levels suggests an improvement in spatial cognitive learning memory in aged rats (3). Furthermore, an increase in the antioxidative effects of docosahexaenoic acid on the hippocampus prevents impairment of

TABLE 2

Plasma oxidative status of rats administered 0, 0.1% PE, or 0.5% PE for 26 wk[1]

		TBARS	FRAP
	n	*μmol/L*	
0%	8	4.02 ± 0.18[a]	223.7 ± 10.8[b]
0.1% PE	7	3.51 ± 0.12[b]	257.2 ± 17.7[ab]
0.5% PE	9	3.00 ± 0.12[c]	270.9 ± 11.4[a]

[1] Values are means ± SEM. Means in a column without a common letter differ, $P < 0.05$.

TABLE 3

Oxidative status of cerebral cortex and hippocampus in rats administered 0, 0.1%, or 0.5% PE for 26 wk[1]

		Cerebral cortex		Hippocampus	
		TBARS	ROS	TBARS	ROS
	n	*nmol/mg protein*	*pmol/(min·mg protein)*	*nmol/mg protein*	*pmol/(min·mg protein)*
0%	8	1.452 ± 0.101	0.206 ± 0.027	0.626 ± 0.087[a]	0.126 ± 0.030[a]
0.1% PE	7	1.231 ± 0.102	0.195 ± 0.031	0.371 ± 0.036[b]	0.055 ± 0.017[b]
0.5% PE	9	1.265 ± 0.083	0.186 ± 0.035	0.332 ± 0.029[b]	0.049 ± 0.015[b]

[1] Values are means ± SEM. Means in a column without a common letter differ, $P < 0.05$.

reference-memory learning ability in a rat model of Alzheimer's disease, in which an accompanying increase in the level of hippocampal LPO was demonstrated (4). Intraperitoneal injection of EGCG markedly protects against hippocampal neuronal damage after transient global ischemia in gerbils (25). Taken together, these findings suggest that PE-induced improvement in spatial cognitive learning ability is due to the antioxidative effect of PE. In this study, PE administration decreased levels of both TBARS and ROS in the hippocampus but not in the cerebral cortex (Table 3), suggesting that chronic administration of green tea catechins reduces prooxidant levels and oxidative stress in the hippocampus but not in the cerebral cortex. It is difficult to explain why these variables were not affected in the cerebral cortex. The level of protein carbonyl, one of the markers of oxidative stress, increased in the hippocampus but not in the cerebral cortex of 16-mo-old rats compared with 4-mo-old controls (26). In addition, chronic administration of docosahexaenoic acid decreased the levels of TBARS in the hippocampus but not in the cerebral cortex of aged rats (3). These results suggest that the hippocampus is more susceptible to the effects of ROS. It is thus likely that green tea catechins exert a stronger antioxidative effect in the hippocampus than in the cerebral cortex of rats.

The metabolism of green tea catechins has been studied in various animals and in human subjects. Tea polyphenols can be retained in the brain and can exert neuroprotective effects simply by their ingestion (27). When EGCG, the most abundant component in green tea catechin PE, is administered orally, it is detected as EGCG or as its conjugates, or both, and peaks 1–2 h postdose in the rat systemic circulation (28). EGCG administered orally is rapidly absorbed and distributed into the plasma, liver, and brain (8). EC metabolites (epicatechin glucuronide and 3′-*O*-methylated epicatechin glucuronide) form after oral ingestion of EC by rats and gain entry into the brain (29). Furthermore, labeled EGCG orally administered to rats has demonstrated wide distribution of radioactivity in their organs, including the brain (30). Green tea catechins administered orally are thus absorbed by and retained in the brain, and their antioxidative and neuroprotective effects could ultimately result in beneficial effects on an age-related decline in spatial cognition. Additionally, green tea catechins might be a prophylactic means of preventing neurodegenerative diseases such as Alzheimer's disease which is associated with oxidative damage and neurotoxicity. There may be questions concerning whether the amount of intake of green tea catechins used in this study could be proportionately represented to benefit human health. A green tea polyphenol extract (100 mg EGCG) ingested 3 times a day for 7 d significantly decreased plasma FRAP levels in humans (31). In this study, an increase in plasma FRAP levels was related to the acquisition of higher reference memory–related learning ability (Table 4). Therefore, long-term administration of an amount of PE that could induce antioxidative effects may protect against age-related declines in memory and learning ability in humans.

In the process of aging, LPO accumulates and induces disorders of cellular functions (32). Aging also leads to a decline in spatial memory–related learning ability (33). Changes in the ability may be associated with the degeneration of cholinergic neurons in the hippocampus (34) because these neurons are the key structure for spatial memory learning. Interruption of hippocampal pathways causes crucial memory deficits in the radial arm maze task (35). Therefore, the effects of long-term administration of PE on memory and oxidative stress may be even greater in aging rats. Further studies are required to clarify the protective effect of green tea catechins on aging-related memory decline.

TABLE 4

Correlation coefficients between learning ability and oxidative stress of plasma and hippocampus of rats administered 0, 0.1% PE, or 0.5% PE for 26 wk[1]

	Plasma		Hippocampus	
	TBARS	FRAP	TBARS	ROS
RME	NS	−0.570	+0.520	NS
P-value	—	0.017	0.032	—
WME	+ 0.622	NS	+0.480	NS
P-value	0.008	—	0.051	—

[1] The number of RME and WME in block 10 shown in Figure 1 was used as an indicator of learning ability. Differences were considered significant when $P < 0.05$. NS, not significant, $P > 0.05$.

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DOCOSAHEXAENOIC ACID PROMOTES NEUROGENESIS *IN VITRO* AND *IN VIVO*

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Abstract—Docosahexaenoic acid (22:6n-3), one of the main structural lipids in the mammalian brain, plays crucial roles in the development and function of brain neurons. We examined the effect of docosahexaenoic acid on neuronal differentiation of neural stem cells *in vitro* and *in vivo*. Neural stem cells obtained from 15.5-day-old rat embryos were propagated as neurospheres and cultured under differential conditions with or without docosahexaenoic acid for 4 and 7 days. Docosahexaenoic acid significantly increased the number of Tuj1-positive neurons compared with the control on both culture days, and the newborn neurons in the docosahexaenoic acid group were morphologically more mature than in the control. Docosahexaenoic acid significantly decreased the incorporation ratio of 5-bromo-2′-deoxyuridine, the mitotic division marker, during the first 24 h period; it also significantly decreased the number of pyknotic cells on day 7. Thus, docosahexaenoic acid promotes the differentiation of neural stem cells into neurons by promoting cell cycle exit and suppressing cell death. Furthermore, dietary administration of docosahexaenoic acid significantly increased the number of 5-bromo-2′-deoxyuridine(+)/NeuN(+) newborn neurons in the granule cell layer of the dentate gyrus in adult rats. These results demonstrate that docosahexaenoic acid effectively promotes neurogenesis both *in vitro* and *in vivo*, suggesting that it has the new property of modulating hippocampal function regulated by neurogenesis.

Key words: docosahexaenoic acid, neural stem cells, neuronal differentiation, neurogenesis, adult rats.

Neural stem cells (NSCs) are defined as immature cells with self-renewing and multipotent abilities. In the mammalian brain, most neurons are generated by NSCs in the later embryonic period. In two specific brain regions, the dentate gyrus (DG) of the hippocampus and the olfactory bulb (OB), however, new neurons are generated by NSCs throughout life (Eriksson et al., 1998; Bedard and Parent, 2004). In the DG, newborn neurons behave like functional granule neurons with electrophysiologic properties (van Praag et al., 2002) and extend axonal projections to the hippocampal CA3 regions. Also, the levels of hippocampal neurogenesis correlate with the performance of memory tasks (Shors et al., 2001; Drapeau et al., 2003). These findings indicate that adult hippocampal neurogenesis is associated with the formation and function of memory and learning ability.

Docosahexaenoic acid (DHA), one of the predominant polyunsaturated fatty acids in the brain, is essential for normal neural development, and deficiency of DHA induces loss of discriminative learning ability (Green and Yavin, 1998). Chronic administration of DHA enhances long-term memory in young and aged rats (Gamoh et al., 1999, 2001), and dietary DHA has preventive and ameliorative effects on the impairment of spatial cognition learning ability in amyloid-β-infused rats (Hashimoto et al., 2002, 2005). These findings suggest that DHA plays crucial roles in the development and function of the CNS throughout life. Interestingly, a reduction in DHA concentrations in the brain impairs the performance of spatial learning tasks regulated by the OB (Greiner et al., 1999), where neurogenesis occurs in adulthood. These findings imply some relation between DHA and adult neurogenesis, although it is still unclear whether DHA affects the potential of NSCs for differentiating into neurons. This study examines the effect of DHA on neural differentiation.

EXPERIMENTAL PROCEDURES

Cell culture and differentiation into neural lineage

NSCs were cultured by the neurosphere method as described (Reynolds and Weiss, 1992). Rat embryos on embryonic day 15.5 (E15.5, with the morning of plug detection designated as E0.5) were dissected out into PBSG (0.6% glucose in PBS (−) containing 1 U/mL penicillin/streptomycin), and the telencephalons were isolated. Tissues were mechanically disrupted into single cells by repeated trituration in a serum-free conditioned medium (N2 medium) comprising DMEM/F12 1:1, 0.6% glucose, sodium bicarbonate, 5 mM HEPES, 100 μ/mL human transferrin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, and 25 μg/mL insulin. The dissociated cells were plated onto cell culture dishes at a density of 1×10^5 cells/mL in N2 medium with basic fibroblast growth factor (bFGF; 20 ng/mL, R&D Systems, Minneapolis, USA) and heparin. All experimental procedures were carried out in accordance with the *Guidelines for Animal Experimentation* of the Japanese Association for Laboratory Animal Science.

The differentiation into neurons was induced by bFGF withdrawal from the culture medium. Dispersed cells were plated at a density of 2×10^5 cells/well onto poly-L-ornithine (15 μg/mL) -coated 24-well plates with or without DHA (10 μM) in a complex with 0.1% fatty acid-free bovine serum albumin and cultured for 4 and 7 days. The culture medium was changed on days 2 and 4. Differentiation was assayed with four independent cell lines obtained from four pregnant rats.

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Abbreviations: bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2′-deoxyuridine; DG, dentate gyrus; DHA, docosahexaenoic acid; E, embryonic day; LTP, long-term potentiation; NSCs, neural stem cells; OB, olfactory bulb; PI, propidium iodide; TBS, Tris-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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Immunohistochemistry and data analysis

For fluorescent staining, cells were fixed with 4% paraformaldehyde for 15 min and washed for 10 min×three with 0.1 M Tris-buffered saline, pH 7.5 (TBS). After blocking with 3% goat serum in TBS containing 0.3% Triton X-100 for 1 h, the cells were incubated with primary antibodies at 4 °C overnight. The primary antibodies used were mouse anti-Tuj1 (1:1000, R&D Systems), mouse anti-MAP2ab (1:1000, Chemicon, Temecula, USA), mouse anti-nestin (1:2000, Chemicon), and rabbit anti-GFAP (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, USA). After washing in TBS, the FITC- or Cy5-conjugated secondary antibody (1:500, Southern Biotechnology, Birmingham, USA and Chemicon) was added, and the cells were incubated for 1 h. To estimate their total number, the cells were treated with propidium iodide (PI; 2 μg/mL, Dojin Kagaku, Mashiki, Japan) for 30 min at room temperature. Fluorescent signals were then visualized by the confocal laser microscope system (CLSM FV300, Olympus, Tokyo, Japan) and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) or NIH image version 1.63. The number of Tuj1 immunoreactive cells was counted in each of seven random fields per well. Data are indicated as the means±S.E.M. Statistical significance was evaluated by Student's *t*-test; a level of $P<0.05$ was considered significant.

Proliferation study

The proliferative activity of NSCs was investigated by treating cells with 10 μM of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, USA), a thymidine analog incorporated into genetic material during the S phase of mitotic division, for 24 h with or without DHA. Cells were treated with 2 M HCl for 10 min at 37 °C to denature DNA and with 0.1 M borate buffer ($Na_2B_4O_7$, pH 8.5) for 5 min. Cells were treated with mouse anti-BrdU antibody (1:1000, Chemicon) then with FITC-conjugated secondary antibody. Seven random fields were captured in each well, and BrdU-incorporated cells were counted. The ratio of BrdU/total cells was calculated, and statistical significance was evaluated by Student's *t*-test.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL staining was carried out with Red *In Situ* Apoptosis Detection Kit (Chemicon) according to the manufacturer's protocol. Cells cultured for 7 days under differential conditions with or without DHA were fixed with 4% paraformaldehyde, and incubated for 1 h at 37 °C with digoxigenin-dUTP in the presence of TdT. TUNEL labeled cells were visualized by Rhodamine-conjugated anti-digoxigenin antibody and observed under a confocal laser microscope.

DHA administration and BrdU injection into animals

Eighteen-month-old male Wistar rats fed a fish-oil deficient diet (F-1R; Funabashi Farm Co., Funabashi, Japan) over three generations were used in this study. To estimate the effects of DHA on hippocampal neurogenesis, the rats were orally fed ethyl-ester 4,7,10,13,16,19-docosahexaenoate (Harima Chemicals, Tokyo, Japan) emulsified in 5% gum arabic solution at 300 mg/kg/day; controls were fed a similar volume of only the 5% gum arabic solution. DHA or vehicle supplementation was continued for seven weeks. Two weeks after the start of DHA administration, the rats received one daily i.p. injection of 50 mg/kg BrdU dissolved in PBS (−) for 5 days.

Tissue preparation and immunohistochemistry

Four weeks after the last BrdU injection, the rats were perfused transcardially with PBS (−) and fixed with 4% paraformaldehyde. The brain was removed and post-fixed for 24 h in paraformaldehyde, and replaced by the 25% sucrose. Coronal sections (40 μm) were cut on a microtome and subjected to immunohistochemical analysis.

BrdU-labeled nuclei were visualized as described (Liu et al., 1998). Free-floating brain sections were treated with 50% formamide–2× SSC (0.3 M NaCl and 0.03 M sodium citrate) for 2 h at 65 °C and incubated for 30 min at 37 °C in 2 M HCl. The sections were treated with 0.1 M boric acid (pH 8.5) and incubated for 24 h in rat anti-BrdU antibody (Oxford Biotechnology Ltd., Oxfordshire, UK) with 3% goat serum in TBS containing 0.3% Triton X-100. After washing with TBS, Cy5-conjugated secondary antibodies were added, and the brain sections were incubated for 1 h at 25 °C. To estimate the number of newborn neurons, the brain sections were treated with mouse anti-NeuN antibody (Chemicon) then with FITC-conjugated secondary antibody. Finally, fluorescent signals were visualized under a confocal laser microscope as described above.

Stereology

Every sixth section (240 μm apart) was selected throughout the rostrocaudal extent of the granule cell layer in the complete DG, and BrdU–NeuN-colocalized cells were counted in the granule cell layer and the subgranular zone (the inner region lining the granule cell layer). To avoid over- or undersampling of BrdU–NeuN positive nuclei because of size differences, every sixth adjacent section was stained with 2 μg/mL PI. The reference volume of the granule cell layer was determined by summing the granule cell areas for each section multiplied by the distance between sections sampled. Total BrdU–NeuN positive cells were expressed as the mean±S.E.M for each hemisphere; statistical significance was analyzed by Student's *t*-test. Differences of $P<0.05$ were considered significant.

RESULTS

Effects of DHA on neuronal differentiation of NSCs *in vitro*

Cells isolated from E15.5 embryos generated neurospheres in the presence of bFGF, and numerous cells were immunoreactive to nestin (data not shown). Under differential conditions, some of the cells were immunoreactive to neural markers Tuj1, MAP2ab or to mature astrocyte marker GFAP (data not shown) and consequently identified as NSCs.

To evaluate the effect of DHA on NSCs, the cells were cultured for 4 and 7 days with or without 10 μM DHA (Fig. 1). DHA is effective in a narrow range (i.e. 2–10 μM); higher concentrations induce neuronal cell death (Insua et al., 2003). On day 4, the ratio of Tuj1 (+) neurons/total cells increased 1.47-fold in the presence of DHA (Fig. 1C; control: 9.3±0.4%, $n=5$; DHA: 13.0±0.7%, $n=5$; $P=0.0004$). Also on day 7, the ratio of Tuj1(+)/total cells increased 1.46-fold in the presence of DHA (Fig. 1C; control: 11.5±0.8%, $n=6$; DHA: 16.8±0.9%, $n=6$; $P=0.0003$). No difference in the total number of cells was observed between the control and the DHA-treated group on both culture days (day 4: control 2198±192, DHA; 2155±248 cells/well, $P=0.950$; day 7: control 2496±244, DHA 2450±248 cells/well, $P=0.986$).

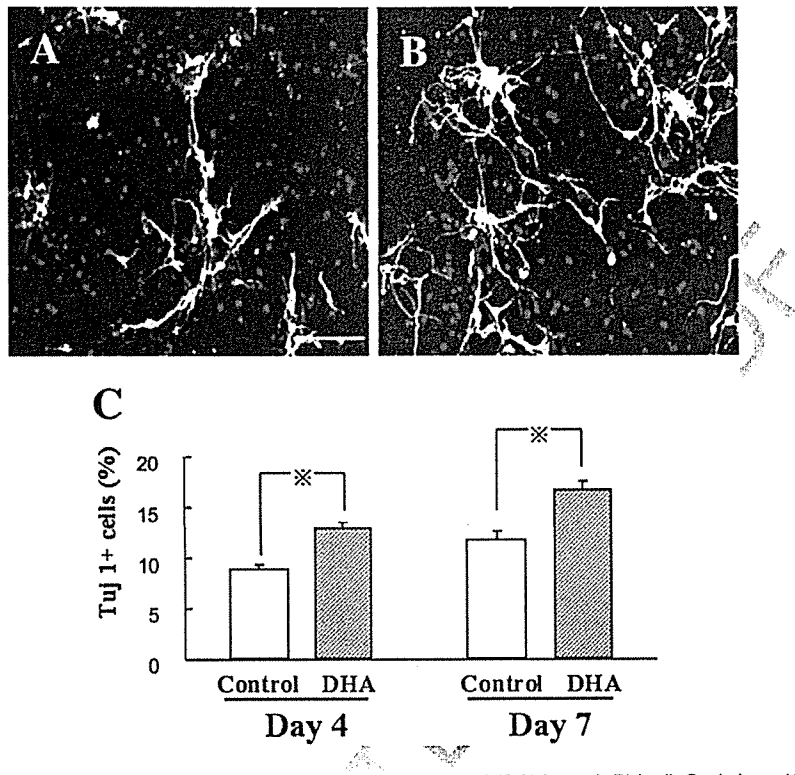

Fig. 1. (A) Confocal images of Tuj1 immunostaining in control and DHA groups on day 7, Tuj1 (green), PI (red). Scale bar=100 μm. (B) Quantification of Tuj1 immunoreactive cells in control and DHA groups. Data are shown as the means±S.E.M. obtained from five to six independent cultures. Seven random fields were counted in each culture. ※$P<0.0005$.

Influence of DHA on the proliferative activity of NSCs *in vitro*

BrdU was added to the NSC culture during the first 24 h of the differentiation period, and BrdU incorporated into the cells was visualized immunohistochemically. The number of BrdU-immunoreactive cells decreased significantly in the presence of DHA (Fig. 2A; control: 23.6±2.2%, n=6; DHA: 16.3±1.0%, n=6; P=0.02). No difference in the number of total cells was observed between the control and the DHA-treated group (control: 1807±159, DHA: 1640±86 cells/well, P=0.125), indicating that DHA inhibits the proliferation of NSCs in the early stages of differentiation.

The preventive effects of DHA on cell death *in vitro*

The nuclei of differentiated NSCs were stained with PI, and the pyknotic cells were quantified. The pyknotic cells

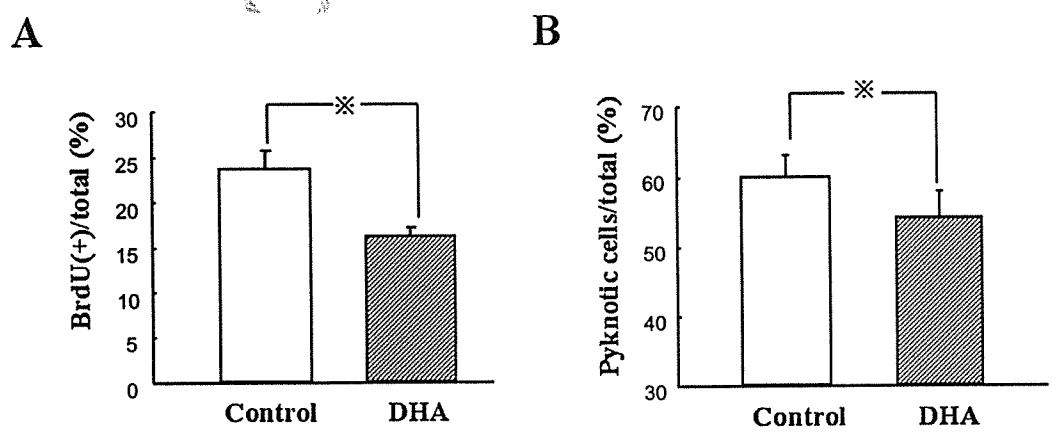

Fig. 2. (A) Quantification of BrdU immunoreactive cells in control and DHA groups during a first 24 h period. Data are shown as the means±S.E.M. obtained from six independent cultures. Seven random fields were counted in each culture. ※$P<0.05$. (B) Quantitative analysis of pyknotic cells in control and DHA groups on day 7. Data are shown as the means±S.E.M. obtained from six independent cultures. Seven random fields were counted in each culture ※$P<0.01$.

showed characteristic features of dense nuclei and shrunken cell-bodies, whereas the surviving cells had large cell-bodies, non-fragmented and weak PI-stained images (data not shown). Stained by TUNEL, the labeling of specifically the 3′-hydroxyl terminal of DNA strands broken by apoptosis, pyknotic cells was strongly positive for TUNEL, whereas the cells with unshrunken cell-bodies were negative (data not shown). Thus, cells with pyknotic PI images were regarded as apoptotic.

The quantitative analysis of pyknotic cells revealed that DHA supplementation significantly decreased the ratio of pyknotic cells/total cells on day 7 (Fig. 2B; control: 60.0±3.2%, n=6; DHA: 54.3±3.7%, n=6; P=0.01). No difference in the number of total cells was observed between the control and the DHA-treated group (control: 2496±244, DHA: 2500±248 cells/well, P=0.996), indicating that DHA prevents cell death of differentiating NSCs.

Effects of DHA on neurite growth of neurons differentiated from NSCs *in vitro*

To examine whether DHA accelerates the maturation of neurons differentiated from NSCs, we compared the morphological appearance of neurons between the control and the DHA group on day 7. In the control, most neurons had bipolar neurites and few branches characteristic of immature neurons (Fig. 3A, arrows), whereas in the DHA group, each neuron had characteristic features of more mature and longer neurons, multipolar neurites and more branches (Fig. 3B, arrows), suggesting that DHA accelerates neurite growth and promotes the maturation of neurons differentiated from NSCs.

Effects of DHA on hippocampal neurogenesis in adult rats

To examine whether neurogenesis in the DG of adult rats can be activated by DHA administration, the dividing cells were labeled with BrdU in the DG, and the newborn neurons were visualized by using specific antibodies of BrdU and neuron-specific marker NeuN as described (Liu et al., 1998; Cameron and Mackay, 1999).

Four weeks after the last BrdU injection, most BrdU-labeled cells distributed throughout the entire granule cell layer and their nuclei were morphologically large and round, characteristics of mature granule neurons (Kuhn et al., 1996). Furthermore, most BrdU (+) cells also expressed NeuN (Fig. 4A, B and C) in the control and the DHA groups, suggesting that most of the cells that incorporated BrdU in the granule cell layer differentiated into mature neurons, which is in agreement with previous reports (van Praag et al., 1999; Yagita et al., 2001).

The number of newborn neurons (BrdU/NeuN double immunoreactive cells) in the entire granule cell layer of DG increased significantly, 1.6-fold in DHA-administrated rats (Fig. 4D-a; control: 538±60.2, DHA: 878±72.3, six hemispheres from three animals, P=0.005). This difference was not due to the difference in the size of the granule cell layer, especially that the reference volume of the analyzed area did not differ between the control and the DHA groups (Fig. 4D-b; control: 0.919±0.0515 mm^3, DHA: 0.920±0.0547 mm^3, P=0.993), suggesting that the dietary administration of DHA promotes hippocampal neurogenesis in adult rats.

DISCUSSION

NSCs are identified in many regions of the embryonic CNS (cerebral cortex, hippocampus, spinal cord, and cerebellum; Temple 2001), where they generate the neural and glial cell lineage during the developmental period. The levels of DHA in the rat brain increase in the period between E14 and E17, which coincides with the peak of neurogenesis (Green et al., 1999). Therefore, NSCs isolated from E15.5 rats, used in this study, are fitting for examining the effects of DHA on developmental neurogenesis *in vitro*. DHA supplementation significantly increased the number of Tuj1 immunoreactive cells *in vitro* (Fig. 1); the increase was not due to any difference in the neural progenitors of the control and DHA groups, especially that most NSCs, isolated and purified from the E15.5 rat embryonic brain, expressed nestin (>90%, data not shown), but few expressed Tuj1 (<1%, data not shown).

Several mechanisms may be involved in the induction of neural differentiation by DHA. One suggests that DHA promotes the proliferative activity of neural stem/progenitor cells and increases the number of newborn neurons. To

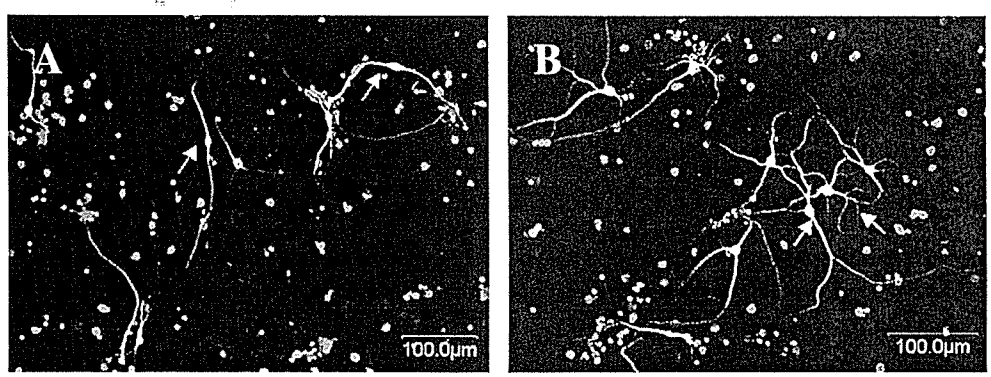

Fig. 3. NSCs were cultured for 7 days in the absence (A) or the presence (B) of DHA and stained with Tuj1 (white) and PI (gray). Morphological features of neurons are observed in both groups. In control, each neuron shows immature morphology (arrows in A). In contrast, neurons in the DHA group show more mature morphology than controls (arrows in B).

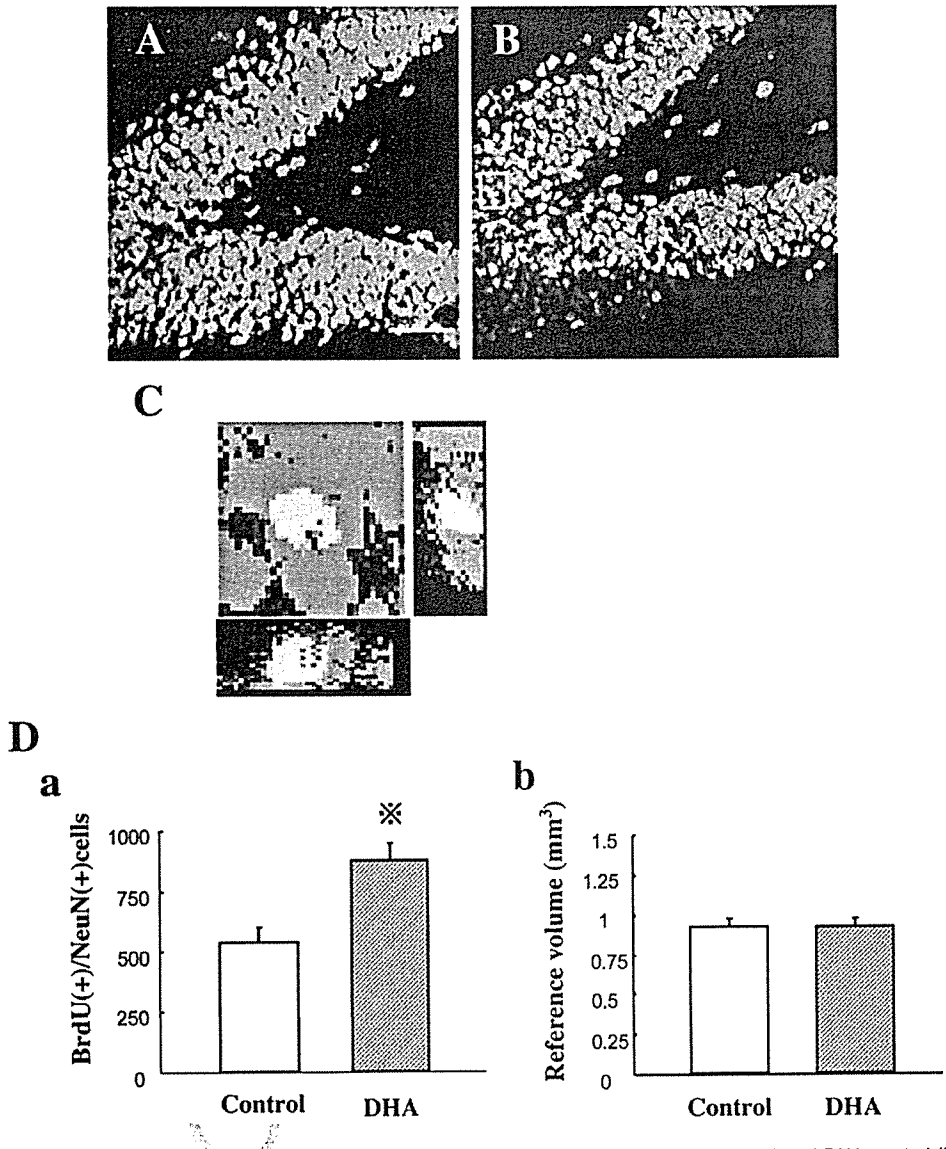

Fig. 4. Neuronal identity of newly divided cells in the adult DG. (A, B) Confocal images of DG in vehicle- (A) and DHA-treated (B) rats. BrdU (red), NeuN (blue). Scale bar=50 μm. (C) BrdU(+)/NeuN(+) newborn neuron in the white box in B. (D) Quantitative analysis of the number of newborn neurons (a) and reference volume (b) in the entire granule cell layer of the DG in the control and the DHA groups. Data are shown as the means±S.E.M. obtained from six hemispheres in three animals, respectively. ※$P<0.005$.

elucidate this possibility, our examination of proliferative activity of NSCs in the presence of DHA revealed that DHA significantly decreased BrdU incorporation into NSCs during the first 24 h (Fig. 2A), which is consistent with a report demonstrating that DHA promotes cell cycle exit and decreases the expression of nestin in retinal neural progenitor cells (Insua et al., 2003); it also reduces mitosis in tumor cells by inhibiting S phase entry (Chen and Istfan, 2001). Thus, the negative effect that DHA exerts on the proliferative activity of cells is not likely to increase the number of NSCs/neural progenitor cells. In general, however, NSCs proceed to mitotic division during the undifferentiated phase; when they transit into the differentiation phase, they stop dividing and begin differentiating into neurons. Therefore, DHA may promote the cell cycle exit of NSCs and, consequently, accelerate their transition from the undifferentiated to the differentiated phase into neurons.

In this study, DHA significantly decreased the number of pyknotic cells in the culture of differentiated NSCs on day 7 (Fig. 2B). Chronic administration of DHA decreases the levels of lipid peroxide in the cerebral cortex and hippocampus (Gamoh et al., 1999; Hashimoto et al., 2002, 2005), and prevents an increase in neuronal apoptotic products induced by the infusion of amyloid-β into the rat cerebral ventricle (Hashimoto et al., 2002). Membrane lipid peroxidation increases the production of 4-hydroxy-2,3-nonenal, which induces apoptotic cell death in cortical

neurons through caspase activation (Camandola et al., 2000). DHA also prevents the apoptosis of retinal photoreceptors by regulating pro- and anti-apototic molecules Bax and Bcl-2 (Rotstein et al., 2003). Furthermore, DHA activates Akt signaling, which inactivates pro-apoptotic factors such as BAD, caspase 9, FKHRL1 and blocks cell death induced by these mediators (Namikawa et al., 2000; Akbar et al., 2005). Thus, DHA may prevent apoptotic cell death and increase the survivability of newborn neurons.

Interestingly, the neurons in the DHA group demonstrated more mature morphology than the controls (Fig. 3). Several studies have described the beneficial effects of DHA on neural maturation: It stimulates phospholipid synthesis and promotes neurite growth induced by NGF in PC12 cells (Ikemoto et al., 1997, 1999); it also promotes neurite growth in the culture of embryonic hippocampal neurons *in vitro*, and reduced DHA in the embryonic hippocampus *in vivo* inhibits neurite growth in the same culture (Calderon and Kim, 2004). Thus, DHA may enhance neurite growth of newborn neurons differentiated from NSCs, suggesting that it plays important roles in the developmental neurogenesis of CNS.

NSCs in the adult hippocampus including those in rat and humans generate new neurons in adulthood (Schinder and Gage, 2004). The above results raise the question: Does DHA promote hippocampal neurogenesis in adult animals? To elucidate this issue, we administrated dietary DHA to adult rats and examined the effect on active hippocampal neurogenesis. Indeed, dietary administration of DHA significantly increased the number of newborn neurons (Fig. 4D), demonstrating that DHA promotes neurogenesis not only in cultured embryonic NSCs but also in the hippocampus of adult rats, and suggesting that DHA also modulates the generation of new neurons in the adult brain.

The physiological function of adult hippocampal neurogenesis remains unclear. Recent growing evidence strongly suggests, however, that newborn neurons participate in the formation of learning and memory (Schinder and Gage, 2004). Newly generated neurons are functionally integrated into hippocampal circuits and can survive for several months (Song et al., 2002). They have the unique membrane properties that facilitate synaptic plasticity, and it is suggested that their increase causes the enhancement in long-term potentiation (LTP: Schmidt-Hieber et al., 2004; Schinder and Gage, 2004). The mouse housed with a running wheel demonstrates increases of neurogenesis in the hippocampus, improved learning ability in the Morris water maze and selectively enhanced LTP in the DG (van Praag et al., 1999). Moreover, a significant relation between the number of newborn neurons and cognitive learning performance in the Morris water maze task has been demonstrated (Drapeau et al., 2003; Kempermann et al., 1997). Conversely, blocking NSCs proliferation ir the hippocampus with antimitosis drugs or by X-ray irradiation impairs hippocampal-dependent learning tasks (Shors et al., 2001; Madsen et al., 2003). Based on these evidences, it is highly probable that the endogenous neurogenesis modulates learning and memory functions.

DHA is crucial for inducing of LTP in the CA1 region of rat hippocampus slices and for enhancing the potassium chloride-evoked release of acetylcholine in the rat hippocampus (Fujita et al., 2001; Aid et al., 2005). Moreover, dietary administration of DHA increases the levels of DHA in the hippocampus and improves learning and memory performance in the eight-arm radial maze task (Gamoh et al., 1999, 2001). In addition to these findings, the data in the present study suggest that DHA increases newborn neurons and imparts beneficial properties to the function of learning and memory. We therefore hypothesized that DHA modulates both the generation of new functional neurons and the already existing neural function, as well as improves the hippocampal function of learning and behavior.

If endogenous NSCs could be activated by specific manipulations, they may contribute to the generation of new neurons and repair injured CNS function. The beneficial effects of DHA on transient forebrain ischemia and Alzheimer's disease (Okada et al., 1996; Calon et al., 2004; Hashimoto et al., 2005) focus mainly on the protective effect of DHA on cell-death damage in existing neurons and on accelerating their function. In addition to these, the present study proposes that DHA has a new property that offers an appropriate neurogenic environment and helps in the recovery of injured CNS function. To restore injured CNS function, newborn neurons need to be integrated into existing networks in order to establish the new connections and synaptic plasticity, and to ameliorate the pathological conditions at all levels in the whole animal (Schinder and Gage, 2004). Although, whether DHA induces new neurons to meet these criteria remains unclear in this study, further investigations aimed at clarifying these issues would provide insights into the significance of DHA in neuronal replacement therapy.

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