Title

DOCOSAHEXAENOIC ACID PROMOTES NEURONAL DIFFERENTIATION BY REGULATING BASIC HELIX–LOOP–HELIX TRANSCRIPTION FACTORS AND CELL CYCLE IN NEURAL STEM CELLS

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DOCOSAHEXAENOIC ACID PROMOTES NEURONAL DIFFERENTIATION BY REGULATING BASIC HELIX–LOOP–HELIX TRANSCRIPTION FACTORS AND CELL CYCLE IN NEURAL STEM CELLS

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Abstract—Recent studies have suggested that docosahexaenoic acid (DHA) enhances neuronal differentiation of neural stem cells (NSCs) isolated from rat embryonic day 14.5. However the underlying mechanism remains largely unknown. One hypothesis supported by DHA controls the expression level of basic helix–loop–helix (bHLH) transcription factors, such as hairy and enhancer of split 1 (Hes1), Mash1, neurogenin1, and NeuroD; another is that previous studies in retinal progenitor cells DHA affects the cell cycle. In this study, we show that treatment with DHA under differentiation conditions without basic fibroblast growth factor, (1) increases Tuj-1 and MAP2 positive cells in NSCs, (2) that the expression level of Hes1 mRNA and protein decreased significantly from day 1 to day 4, on the other hand, the NeuroD mRNA expression level increased from day 1 to day 4 after treatment with DHA and (3) decreased the percentage of S-phase cells, which correlated with prolonged expression of cyclin-dependent kinase inhibitor $p27^{kip1}$, suggesting that DHA enhances neuronal differentiation of NSCs, in part, by controlling the bHLH transcription factors and promoting cell cycle exit. We therefore speculate that DHA is one of the essential key molecules for neuronal differentiation of NSCs.

Key words: bHLH, DHA, Hes, n-3 fatty acid, neurogenesis.

Docosahexaenoic acid (DHA), an essential n-3 polyunsaturated fatty acid, found abundantly in phospholipid bilayer at synapses and in retinal photoreceptor outer segment membranes, is necessary for normal brain development and vision (Innis, 2007; Uauy et al., 2001). Several studies have demonstrated that dietary administration of DHA improves spatial learning ability in young and aged rats (Gamoh et al., 1999, 2001), and protects against and/or ameliorates the decline of memory and learning ability in Alzheimer's disease (AD) model rats (Hashimoto et al., 2002, 2005), and transgenic AD model mice (Lim et al., 2005). It is believed that DHA protects neural cells from oxidative stress (Hashimoto et al., 2002, 2005; Hossain et al., 1998, 1999), however, the exact mechanism of the beneficial effect of DHA is not conclusively explained. New neurons generated from multipotent neural progenitor cells by proliferation, migration, and differentiation into specific neuronal phenotypes (Reynolds and Weiss, 1996; Gage, 2000), play a critical role in learning and memory processing (Becker, 2005; Schinder and Gage, 2004). Recently, we have shown that DHA promotes neurogenesis both *in vitro* and *in vivo* (Kawakita et al., 2006). However, the molecular mechanisms of DHA-induced neurogenesis remain explored. In the present study, we hypothesize that DHA induced amelioration of memory and learning ability (Hashimoto et al., 2002, 2005), at least partially, could be ascribed to the ability of DHA to enhance differentiation of neural stem cells (NSCs) in the brain.

Basic helix–loop–helix (bHLH) transcription factors expressing cells maintained as NSCs during embryogenesis (Ohtsuka et al., 2001); and bHLH transcription factors are important regulators of the cell cycle, specifically the proliferation and differentiation of NSCs (Kageyama et al., 2005). Activator-type bHLH transcription factors such as neurogenin, Mash1 and NeuroD enhance neuronal differentiation; on the other hand, repressor-type bHLH transcription factors such as hairy and enhancer of split 1 (Hes1) and Hes5 are essential for the maintenance and proliferation of NSCs (Kageyama et al., 2008). A crosstalk between these two types of bHLH transcription factors allows some NSCs to undergo differentiation and maintain as NSCs.

Regulation of the cell cycle plays an important role in cell proliferation, differentiation, and apoptosis of NSCs. It is shown that neuronal differentiation is highly coordinated by various factors, such as transcription factors, trophic factors, and those regulating the cell cycle (Cremisi et al., 2003; Ohnuma et al., 2001). For differentiation, cells are arrested at the G1/S-phase and enter the G0-phase without passing the cell cycle restriction point. Deferoxamine, a G1/S-phase blocker, promotes neuronal differentiation of NSCs (Kim et al., 2006). Kawakita et al. (2006) demonstrated that DHA reduces the incorporation of 5-bromo-2′-deoxyuridine, an S-phase cell division marker, into the

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Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; bFGF, basic fibroblast growth factor; bHLH, basic helix–loop–helix; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; CDK, cyclin-dependent kinase; DHA, docosahexaenoic acid; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Hes1, hairy and enhancer of split 1; MAP2, microtubule-associated protein 2; MTT, methyl thiazol tetrazolium; NSCs, neural stem cells; PI, propidium iodide; PKC, protein kinase C; PPAR, peroxisome proliferator–activated receptor; RXR, retinoid X, receptor; TBS, Tris-buffered solution; Tuj-1, neuron-specific class III beta-tubulin.

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NSCs during their differentiation, suggesting that DHA affects the cell cycle of NSCs. We investigate the point(s) at which DHA affects the cell cycle.

With all the foregoing evidence at hand, we investigated the effects of DHA on the expression level of bHLH transcription factors and the cell cycle during the differentiation of NSCs. Finally, our objective was to evaluate the mechanism as to how DHA induces neurogenesis by affecting the bHLH transcription factors and the cell cycle.

EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the "Guidelines for Animal Experimentation" of the Center for Integrated Research in Science, Shimane University and were approved by the "Animal Care and Use Committee" of the same institution the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Science" of the Physiological Society of Japan and "Guide for the care and use of laboratory animals" of NIH. A minimum number of animals were used for the collection of embryonic NSCs from anesthetized rats. Protocols were designed in order to minimize pain and suffering during the procedures.

Embryonic NSC culture

NSCs were cultured by the neurosphere method as described previously (Reynolds and Weiss, 1992). Briefly, rat forebrain cortices were isolated from embryonic day 14.5 rats. The cortices were mechanically disrupted into single cells by repeated pipetting in a serum free conditioned medium (N2 medium) containing Dulbecco's Modified Eagle Medium/Ham's F12 1:1, 0.6% glucose, sodium bicarbonate, 2 mM *L*-glutamine, 5 mM Hepes, 100 μg/mL human apo-transferrin (Sigma-Aldrich, St. Louis, MO, USA), 20 nM progesterone (Sigma-Aldrich), 30 nM sodium selenite (Sigma-Aldrich), 60 μM putrescine (Sigma-Aldrich), and 25 μg/mL insulin (Sigma-Aldrich). The dissociated cells were cultured in dishes at a density of 1×10^5 cells/mL in N2 medium with 20 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN, USA) and 2 mg/mL heparin (Sigma-Aldrich) in a humidified 5% CO_2/95% air incubator at 37 °C. Within 3–5 days the cells grew as free floating neurospheres which were then collected by centrifugation and passaged after mechanical dissociation by pipetting.

NSC differentiation

For differentiation, the neurospheres (passage 2) were mechanically dissociated and 2×10^5 cells were plated onto poly-*L*-ornithine (15 μg/mL, Sigma-Aldrich)-coated 24-well plates in N2 medium without bFGF and heparin. The cultures were then treated with DHA (0, 0.01, 0.1, 1.0, 10, 20, and 50 μM, Sigma-Aldrich) which was dissolved in N2 medium containing 1.0% fatty acid-free bovine serum albumin (BSA, Sigma-Aldrich) at a final concentration of 0.01%. BSA (0.01%) was used as the vehicle control in this experiment and the culture medium was changed every other day.

Cell viability assay

NSCs were seeded onto poly-*L*-ornithine-coated 24- or 96-well plates at a density of 2×10^5 or 2×10^4 cells/well in N2 medium with or without DHA. The methylthiazoltetrazolium assay (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Dojindo Laboratories, Kumamoto, Japan) was conducted to measure cell viability. The cells were incubated with 0.25 mg/mL of MTT at 37 °C for 4 h, the reaction was terminated by the addition of 20% sodium dodecyl sulfate/50% dimethylformamide, and then the cells were gently shaken at room temperature for 12 h. The amount of MTT formazan product was determined using a microplate reader, and the absorbance was measured at 550 nm. The data are expressed as percentages of the control group.

Immunofluorescence staining

Cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with 0.1 M Tris-buffered solution (pH 7.5, TBS), blocked with 3% normal goat serum (Dako Cytomation, Carpinteria, CA, USA) in TBS containing 0.3% Triton X-100 at room temperature for 60 min, and incubated with primary antibodies at 4 °C overnight. The primary antibodies were mouse anti-nestin (1:100, Millipore Corporate Headquarters, Billerica, MA, USA), rabbit anti-prominin (1:100, Abgent, San Diego, CA, USA), mouse anti–neuron-specific class III beta-tubulin (Tuj-1, 1:1000, R&D Systems, Inc.), mouse anti-microtubule-associated protein 2 (MAP2, 1:500, Millipore Corporate Headquarters), and rabbit anti–glial fibrillary acidic protein (GFAP, 1:1000, Sigma). The cells were washed with TBS and incubated with Alexa Fluor 488–conjugated secondary antibody (1:1000, Invitrogen Corp., Carlsbad, CA, USA) at room temperature for 60 min. To visualize nuclei, the cells were counterstained with 2 μg/mL propidium iodide (PI, Dojindo Laboratories). Finally, the cells were mounted with 80% glycerol, visualized under a fluorescent laser microscope (CLMS FV300, Olympus Corp., Tokyo, Japan) and then processed using Image J software (NIH, Bethesda, MD, USA). The number of Tuj-1, MAP2, GFAP, nestin, and prominin positive cells and total cells was counted in each of seven random fields per well.

Western blot analysis

Cultured dishes were washed with phosphate-buffered saline (pH 7.6) and lysis buffer (25 mM Hepes (pH 7.7), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail (Roche Diagnostics, GmbH, Mannheim, Germany)) was added to each dish. Cells were homogenized and incubated on ice for 60 min. The supernatant was harvested by centrifugation at $10{,}000\times g$ at 4 °C for 30 min, and the protein concentration of the supernatant was assayed using the BCA protein assay kit (Pierce, Rockford, IL, USA). Ten micrograms of each sample was separated on a 10% polyacrylamide gel, and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). For Western blot analysis, the membrane was blocked with 5% block agent (GE Healthcare, UK Ltd., Buckinghamshire, UK) in TBS containing 0.1% Tween 20 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and incubated with primary antibodies at 4 °C overnight. The primary antibodies were mouse anti-Tuj-1 (1:1000), mouse anti-MAP2 (1:500), rabbit anti-GFAP (1:1000), rabbit anti-Hes1 (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-$p27^{kip1}$ (1:500, BD Biosciences, San Jose, CA, USA), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, Research Diagnostics, Inc., Flanders, NJ, USA). The membrane was washed with TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase–conjugated secondary antibodies (1:5000, GE Healthcare, UK Ltd.) at room temperature for 60 min. The detection was performed by using an ECL plus Western blotting detection reagents (GE Healthcare, UK Ltd.). Band relative densities were determined and normalized with GAPDH.

Real-time PCR

The NSCs were allowed to differentiate for 6, 12, 24, and 96 h in differentiation medium in the presence of DHA. Total RNA was isolated using Isogen (Wako Pure Chemical Industries, Ltd.), and then cDNA was synthesized with the Quantitect reverse transcription kit (QIAGEN, GmbH, Hilden, Germany) and amplified by the

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Table 1. List of primers for real-time PCR

Gene	Forward sequence	Reverse sequence
Hes1	TTCCTCCCATTGGCTGAAAG	CCAGCTCCAGATCCAGTGTGAT
NeuroD	AAGACGCATGAAGGCCAATG	GCCAAGCGCAGTGTCTCTATCT
Neurogenin1	CCTCGGCTTCAGAAGACTTCA	GAGCCAGTCACAAAGGAGGTTT
MAP2	GTTTACATTGTTCAGGACCTCATGG	TCGGTAAGAAAGCCAGTGTGGT
GAPDH	ATCTTCTTGTGCAGTGCCAGC	CCTTGACTGTGCCGTTGAACT

ABI prism 7000 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA). Real-time PCR was carried out with the Quantitect SYBR Green PCR kit (QIAGEN). The primer sequences are listed in Table 1. The specificity of PCR products was confirmed by both melting curve analysis and agarose gel electrophoresis (data not shown). In initial experiment, we determined amplification efficiencies of all genes. All amplification efficiencies were comparable (data not shown). The PCR conditions were as follows: initial activation at 95 °C for 15 min, then 40 cycles of amplification cycles of denaturation at 94 °C for 15 s, annealing at 63 °C for 30 s, and extension at 72 °C for 30 s. The relative changes in gene expression levels were determined by the $2^{-\Delta\Delta Ct}$ method described in User Bulletin #2 of the ABI prism 7000 sequence detection system.

Cell cycle analysis

The cells were analyzed for DNA content by staining with PI. The differentiated cells were collected, washed with phosphate-buffered saline, and centrifuged, and the cell pellets were fixed in cold 70% ethanol at 4 °C. The cells were then centrifuged, washed with phosphate-buffered saline, treated with RNase (2 mg/mL) at 37 °C for 30 min, washed with phosphate-buffered saline, and stained with 50 μg/mL of PI. PI fluorescence was measured by flow cytometer (Epics Elite, Beckman Coulter, Inc., Fullerton, CA, USA), and the percentage of cells existing within the various phase (G1/G0, S, G2/M) in the cell cycle was estimated by multicycle for Windows (Beckman Coulter, Inc.). Data from a minimum of 10^4 cells per sample were collected.

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

BrdU (10 μM, Sigma-Aldrich) was added to the medium for 24 h with or without DHA. Cells were fixed with 4% paraformaldehyde and treated with 2 M HCl at 37 °C for 10 min and then with 0.1 M borate buffer (pH 8.5) for 10 min. After blocking with 3% normal goat serum in TBS containing 0.3% Triton X-100 at room temperature for 60 min, the cells were incubated with rat anti-BrdU antibody (1:10, AbD Serotec, Oxford, UK) at 4 °C for overnight. The cells were washed with TBS and incubated with Alexa Fluor 488−conjugated secondary antibody (1:1000, Invitrogen Corp.) at room temperature for 60 min. To visualize nuclei, the cells were counterstained with 2 μg/mL of PI. Finally, the cells were mounted with 80% glycerol and visualized under a fluorescent laser microscope (CLMS FV300, Olympus Corp.) and processed using Image J software. The number of BrdU positive cells and total cells was counted in each of seven random fields per well.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA). The results are expressed as the means± standard error (SE). One-way ANOVA followed by Dunnett's test was compared with the control group. A $P<0.05$ was considered statistically significant.

RESULTS

Effect of DHA on neuronal differentiation and cell viability of NSCs

Six hours after culturing in N2 medium with bFGF, the plated cells were nestin-positive cells (NSC marker; 96.3%±3.7%, $n=7$), prominin-1 (CD133)-positive cells (NSC marker; 98.24%±0.2%, $n=4$), and few Tuj-1- and GFAP-positive cells (less than 3%) (Fig. 1A).

To examine changes in neuronal differentiation treatment of NSCs with varying concentrations (0, 0.01, 0.1, 1.0, and 10 μM) of DHA for 7 days (Fig. 1B) revealed that the number of Tuj-1 (neuron marker) positive cells increased significantly with 0.01–1.0 μM concentration of DHA, suggesting that DHA enhances neuronal differentiation of NSCs in a dose-dependent manner. With 10 μM of DHA, however, the percentage of Tuj-1-positive cells decreased to the control level.

Furthermore, cell viability was analyzed using MTT reduction assay. The NSCs were exposed to 0, 0.1, 1.0, 10, 20, and 50 μM concentrations of DHA for 7 days. As shown in Fig. 1C, cell viability showed no change in the 0.1 and 1.0 μM DHA treated NSCs; it decreased significantly, however, at concentrations of DHA over 10 μM. Therefore, DHA at a concentration of 1.0 μM was used in this experiment.

Effect of DHA on neuronal differentiation of NSCs

On day 7 after differentiation, we used confocal microscopy to detect the expression of Tuj-1, MAP2 (neuron marker), and GFAP (astrocyte maker) (Fig. 2A). The number of Tuj-1 and MAP2 positive cells increased by treatment with DHA (Fig. 2B). The effect of DHA on the astroglial differentiation of NSCs was examined by staining the cells with GFAP. On day 7 after differentiation, the number of GFAP positive cells decreased slightly, but not significantly, by treatment with DHA. Furthermore, Western blot analysis revealed that the Tuj-1 and MAP2 protein levels increased significantly in NSCs treated with DHA for 4 days (about 50% and 75%, respectively), while no difference was observed in GFAP protein levels (Fig. 2C).

Effect of DHA on mRNA expression of bHLH transcription factors

DHA treatment for 24 and 96 h significantly decreased the mRNA expression level of Hes1 (Fig. 3A) and concurrently increased the mRNA level of NeuroD (Fig. 3C). The neu-

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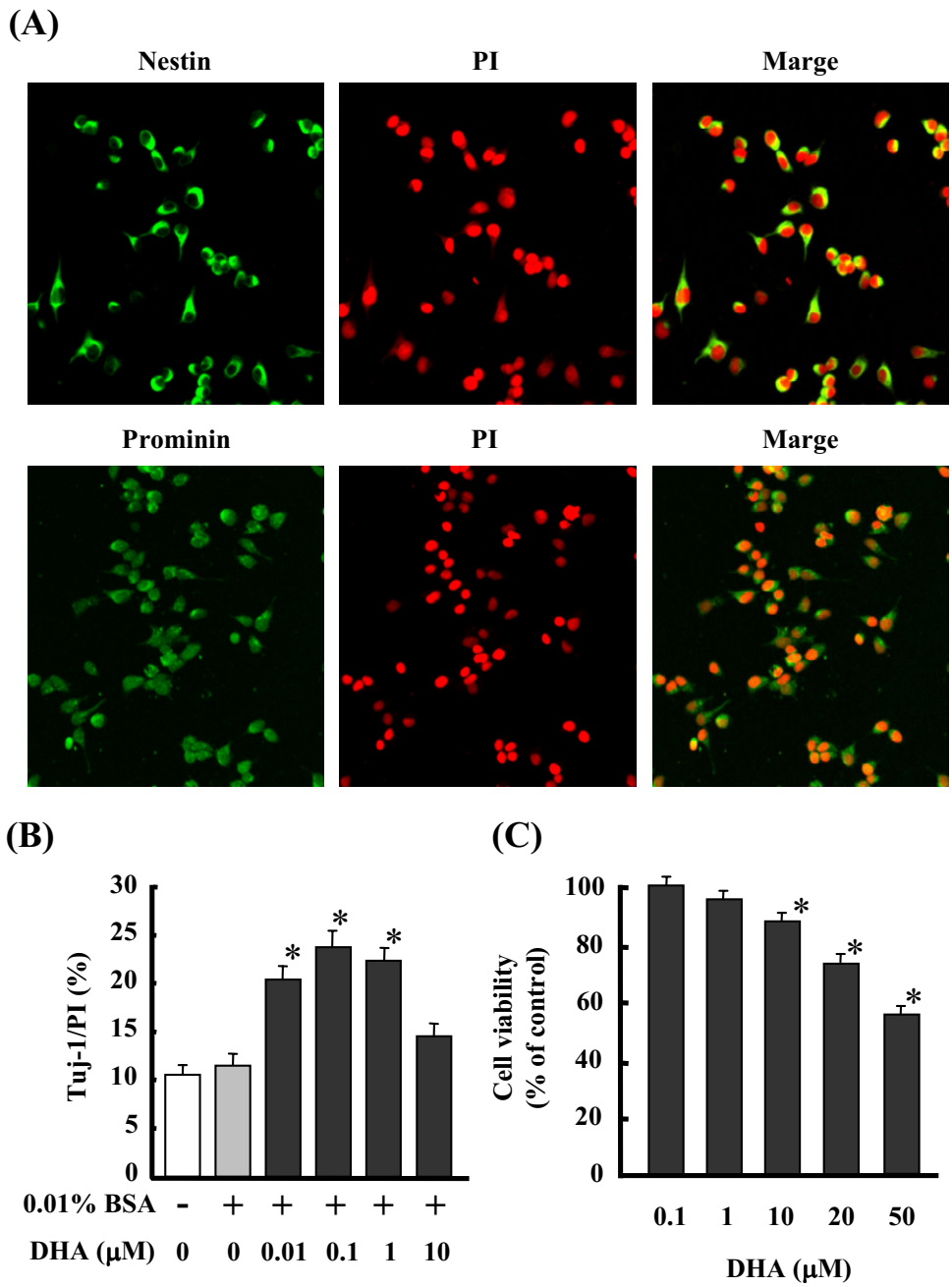

Fig. 1. Immunofluorescence images of NSCs and DHA enhances neuronal differentiation and modulates cell viability of NSCs. (A) The expression of nestin and prominin was detected in the NSCs after 6 h cultured. (B) Dose-dependent changes in the number of Tuj-1 (neuron marker) positive cells. NSCs were treated with 0, 0.01, 0.1, 1.0, and 10 μM DHA for 7 days. The percentage of Tuj-1 positive cells was counted. The values are presented as the means±SE (n=4). (C) Viability of NSCs was measured by MTT assay. NSCs were treated with 0, 0.1, 1.0, 10, 20, and 50 μM DHA for 7 days. Data are expressed as percentages of the control group (n=3–7). * The values are significantly different from the control group (P<0.05). BSA at 0.01% was used as a vehicle control.

rogenin1 mRNA level increased significantly in the DHA-treated NSCs at 12 h (Fig. 3B); however, the expression level of Mash1 did not change during this period (data not shown). Since Hes1 and NeuroD bind directly to the promoter region of the MAP2 gene and compete for binding to the MAP2 promoter region (Bhat et al., 2006), our investigation of the level of MAP2 expression revealed that treatment with DHA for 24 and 96 h significantly increased its mRNA expression level (Fig. 3D), reflecting the expression levels of Hes1 and NeuroD, and coinciding closely with the duration of the in crease in the number of Tuj-1 and MAP2 positive cells. We also measured the expression of Hes1 protein. Western blot analysis revealed that the amount of Hes1 protein was decreased in the DHA treated NSCs for 24 and 96 h (about 50% and 30%, respectively) (Fig. 3E).

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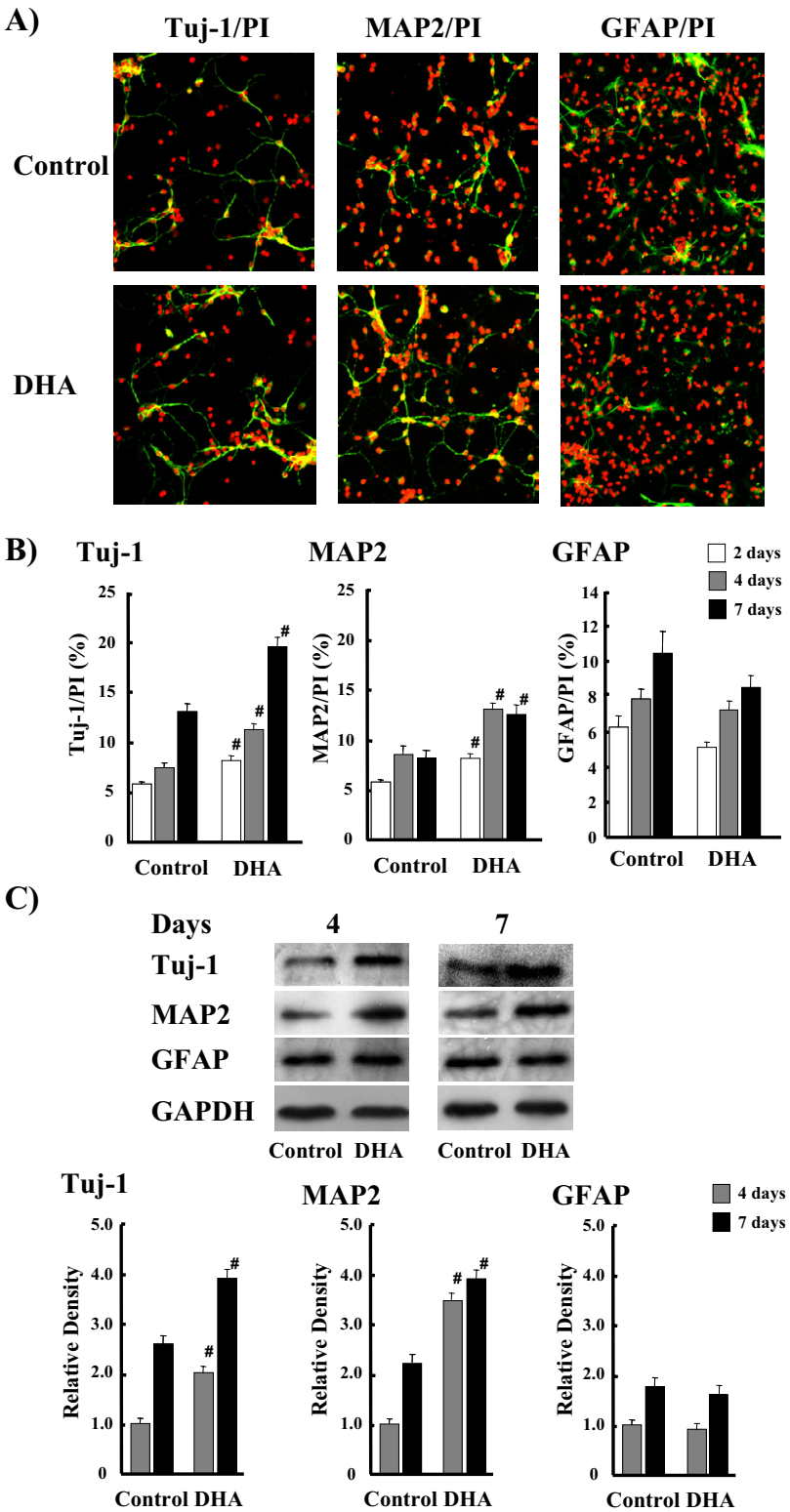

Fig. 2. Treatment of NSCs with DHA increased the number of neurons after differentiation. (A) Immunofluorescence images of Tuj-1 and MAP2 (neuron marker) or GFAP (astrocyte marker) and PI (nuclei) in control (0.01% BSA treated) and DHA (1 μM) groups on day 7. (B) Quantification of Tuj-1, MAP2, and GFAP positive cells in the control and DHA groups. The values are presented as the means±SE (n=3–7). (C) Detection of Tuj-1, MAP2, and GFAP protein level alternations in NSCs treated with control or DHA for 4 and 7 days. Cells were collected for applying Western blot analysis (upper); quantification of Tuj-1, MAP2, and GFAP protein is shown in the lower graph. The intensity of Tuj-1, MAP2, and GFAP protein in each group was normalized to that of GAPDH and the value of the control group (24 h) taken as 1.0 (n=3). Statistical analysis was carried out by Student's t-test. # The values are significantly different from the control group (P<0.05).

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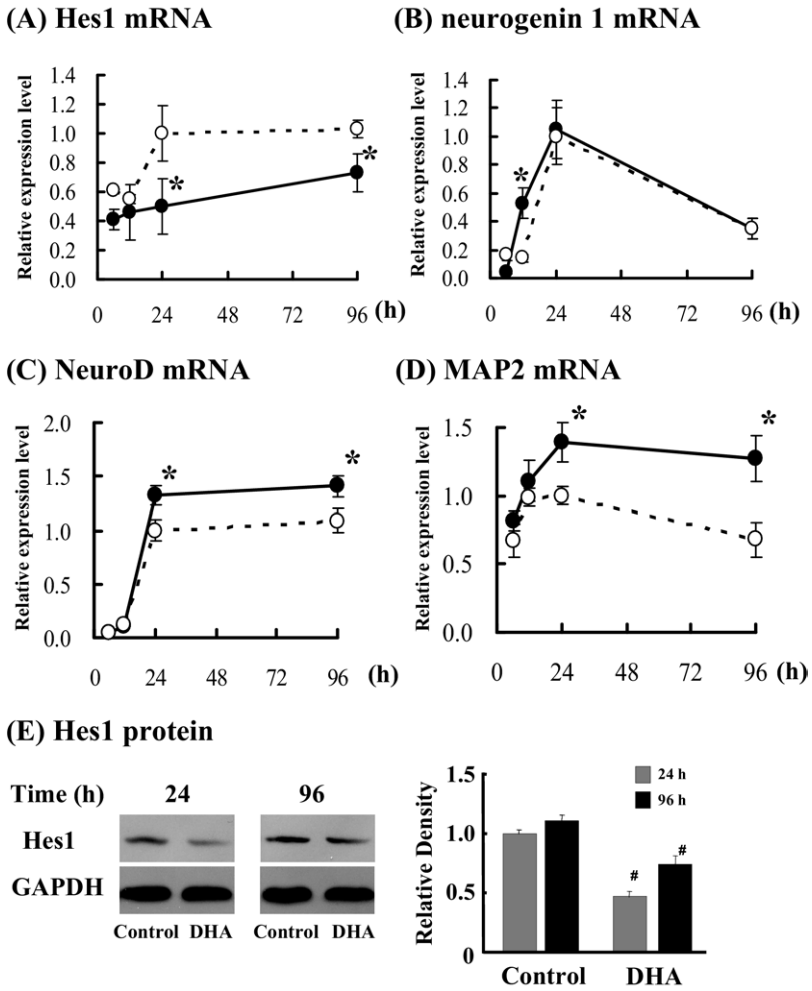

Fig. 3. Effects of DHA on the changes in mRNA expression levels of bHLH transcription factors. NSCs were cultured with 0.01% BSA (control, open circle) or DHA (1 μM, closed circle) in the differentiation medium for the periods of time indicated. Total RNA was prepared from each culture, and cDNA was synthesized, and subjected to real-time PCR using specific primers for Hes1 (A), neurogenin1 (B), NeuroD (C), and MAP2 (D). GAPDH was used as an internal control. The values are expressed as the means±SE of the fold increase in the ratio of each gene/GAPDH, with the value of the control group (24 h) taken as 1.0. Statistical analysis was carried out by ANOVA followed by Student's *t*-test. * The values are significantly different from control group ($P<0.05$). (E) Detection of Hes1 protein level alternations in NSCs treated with control or DHA for 24 and 96 h. Cells were collected for applying Western blot analysis (upper); quantification of Hes1 protein is shown in the lower graph. The intensity of Hes1 protein in each group was normalized to that of GAPDH and data are expressed as percentages of the control group ($n=3$). Statistical analysis was carried out by Student's *t*-test. * The values are significantly different from the control group ($P<0.05$).

Effect of DHA on cell cycle in NSCs

Cell cycle analysis at 1 and 4 days after DHA treatment revealed an increase in the percentage of G0/G1-phase cells (control 56.6%, DHA 62.6%) and a decrease in the percentage of S-phase cells (control 31.6%, DHA 28.5%, Fig. 4A). The relative percentage of G0/G1-phase cells 4 days after differentiation was higher than 1 day after. Of the cells treated with DHA, 89.3% were in the G0/G1-phase, 1.8% in the S-phase, and 9.0% in the G2/M-phase. In the control group, 88.6% of the cells were in the G0/G1-phase, 8.1% in the S-phase, and 3.3% in the G2/M-phase (Fig. 4A). To detect proliferating cells in the S-phase, BrdU incorporated in the cells was measured. The number of BrdU-positive cells decreased significantly in cells treated with DHA (about 5%; Fig. 4B), reflecting the cell cycle analysis and indicating that DHA promotes G1/S arrest and entry into the G0-phase. Investigation of the expression of $p27^{kip1}$, a cyclin-dependent kinase (CDK) inhibitor, by Western blot analysis revealed that the amount of $p27^{kip1}$ protein increased in the DHA-treated NSCs on day 1 and day 4 (about 50% and 45%, respectively; Fig. 4C).

DISCUSSION

Treatment of NSCs with DHA increased Tuj-1 positive cells in a dose-dependent manner but did not increase the percentage of GFAP positive cells, suggesting that DHA promotes the neural differentiation of the NSCs. Differentiation of NSCs is regulated by both activator-type and repressor-type bHLH transcription factors. Chae et al.

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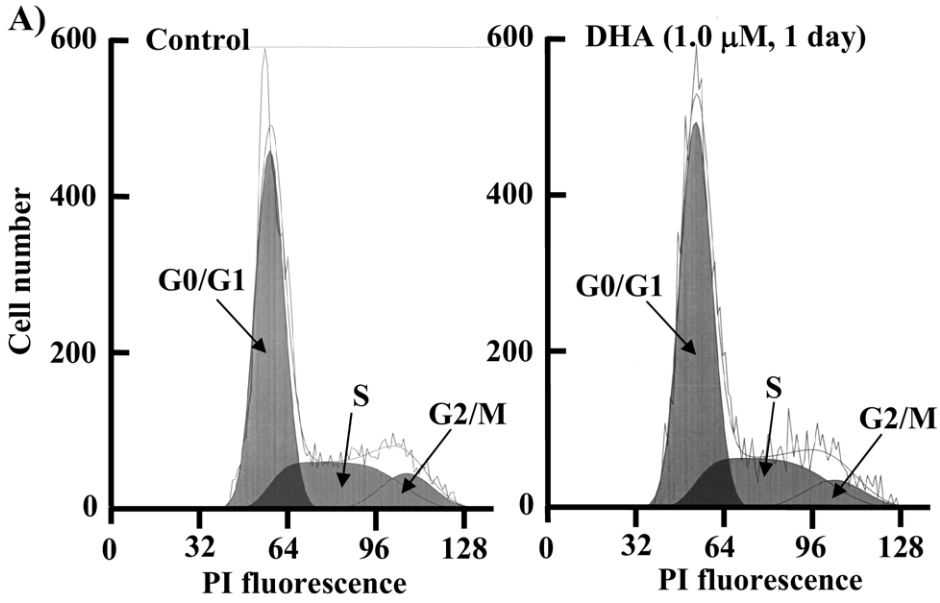

Phase	G0/G1	S	G2/M
	(% of total cells)		
Day 1			
Control	56.6 ± 2.1	31.6 ± 0.2	11.8 ± 1.5
DHA	62.6 ± 1.8*	28.5 ± 0.5*	8.9 ± 0.8
Day 4			
Control	88.6 ± 3.2	8.1 ± 0.6	3.3 ± 0.3
DHA	89.3 ± 4.1	1.8 ± 0.2*	9.0 ± 2.1*

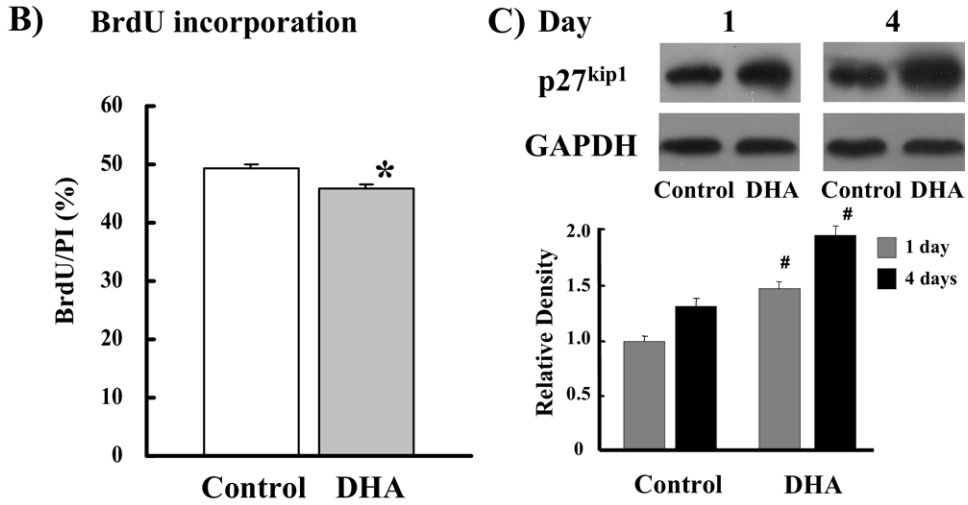

Fig. 4. Cell cycle distribution in the NSCs after treatment with DHA. NSCs were cultured with or without DHA for 1 and 4 days. The cells were collected, washed, and fixed in 70% ethanol, and then treated with RNase. After PI labeling, PI fluorescence was measured with a flow cytometer. (A) Percentage of cells distributed in each phase of the cell cycle on day 1 and day 4 after treatment with DHA. The experiment was repeated three times and the mean and standard error for each cell phase are indicated in the table. (B) BrdU incorporation into the NSCs. NSCs were cultured for 1 day with or without DHA containing BrdU. The cells were fixed and stained with anti-BrdU antibody and PI, and then BrdU positive cells were counted. (C) Detection of CDK inhibitor $p27^{kip1}$ protein level alternations in NSCs treated with control or DHA for 1 and 4 days. Cells were collected for applying Western blot analysis (upper); quantification of $p27^{kip1}$ protein is shown in the lower graph. The intensity of $p27^{kip1}$ protein in each group was normalized to that of GAPDH and data are expressed as percentages of the control group ($n=3$). The value of the control group (1 day) was taken as 1.0 ($n=3$). Statistical analysis was carried out by Student's t-test. * The values are significantly different from the control group ($P<0.05$).

(2004) reported that neurogenesis is promoted by activator-type bHLH transcription factors such as Mash1, neurogenin, and NeuroD. On the other hand, repressor-type bHLH transcription factors such as Hes1 and Hes5 maintain NSCs in the undifferentiated state, or delay neuronal differentiation (Ishibashi et al., 1994; Ohtsuka et al., 1999). These repressor-type transcription factors not only repress activator-type bHLH transcription factors' gene expression by binding directly to the promoter region, but also inhibit the transcriptional activity of Mash1 and NeuroD resulting in suppressed neurogenesis (Sasai et al., 1992; Bhat et al., 2006). In this study, treatment with DHA decreased the mRNA and protein expression level of Hes1 and increased the mRNA expression levels of neurogenin1 and NeuroD (Fig. 3). Contrary to our expectations, the mRNA expression level of Mash1 did not changed by treatment with DHA (data not shown). NeuroD expression is directly upregulated by Mash1, and Mash1 expression level increases before upregulating the NeuroD expression level (Miyachi et al., 1999). It is assumed that the transcriptional activity of Mash1 is activated by reduced Hes1 inhibition, resulting in the induction of NeuroD expression. In addition, MAP2, a neuron specific protein, promoter is activated by NeuroD and repressed by Hes1 and its gene is regulated by the relative levels of these factors, predominantly by the level of Hes1 (Bhat et al., 2006). In our study, treatment with DHA increased the mRNA and protein level of MAP2, corresponding to the expression patterns of Hes1 and NeuroD mRNA. These data suggest that DHA stimulates neuronal differentiation by altering the balance of bHLH transcription factors. Further studies are needed to establish the mechanisms of Hes1 repression by DHA in the NSCs.

Endogenous DHA may be involved in the differentiation of NSCs in the brain. The levels of DHA in the rat brain increase from embryonic day 14 to day 17 (Green and Yavin, 1998) when the expression level of Hes1 decreases gradually (Tokunaga et al., 2004). Our results demonstrated that treatment with DHA decreased the level of Hes1 expression, and increased the number of Tuj-1 positive cells, suggesting that the expression of Hes1 downregulates in the presence of physiological concentration of DHA, and cell fate is switched from the proliferation state to the differentiation state.

Hes1 directly controls cell proliferation through the transcriptional repression of (CDK–cyclin complex inhibitor, $p27^{kip1}$ (Murata et al., 2005). $p27^{kip1}$ Has been shown to arrests cell cycle through inhibition of CDK2 and cyclinE complex and play an essential role during neuronal differentiation. Regulation of the cell cycle plays an important role in differentiation of NSCs. For differentiation, cells achieve G1/S-phase arrest and enter G0-phase without passing the cell cycle restriction point. An important regulator of cell cycle is like $p27^{kip1}$. Treatment of NSCs with deferoxamine, a G1/S-phase blocker, increases the number of Tuj-1 positive cells by increasing the expression of $p27^{kip1}$, which in turn enhances the activation of NeuroD promoter (Kim et al., 2006). In this context, DHA induces cell cycle exit and concomitantly increases neuronal differentiation of photoreceptor progenitors that also are regulated by $p27^{kip1}$ (Insua et al., 2003). In the present experimental paradigm, DHA reduced BrdU incorporation during the first 24 h (Fig. 4B); however, the exact mechanisms are not clearly known. Treatment of NSCs with DHA for 24 h significantly decreased the proportion of S-phase cells (by 10% as compared with that of the control) whereas it significantly increased the proportion of G0/G1-phase cells. These significant alternations of S- and G2/M-phase cells were accompanied by an increase in the expression level of $p27^{kip1}$. Thus DHA decreases the proliferation of S-phase cells, as shown by reduced BrdU incorporation, indicating that regulation of the cell cycle and the increased expression of $p27^{kip1}$ concurrently enhance neuronal differentiation.

In this study, it is not clear how DHA represses the expression of Hes1 and induce the expression of $p27^{kip1}$. In primary rat brain neurons, oleic acid induces differentiation of neurons associated with the induction of MAP2, growth-associated protein 43, and NeuroD expression levels through a protein kinase C (PKC)–dependent mechanism (Rodriguez-Rodriguez et al., 2004). In the present study, DHA increased MAP2 and NeuroD expression levels, suggesting that a PKC-dependent mechanism(s) is involved in DHA induced neurogenesis. Oleic acid also has been described as a ligand and activator for peroxisome proliferator–activated receptor α (PPARα). PPARα acts as a receptor for oleic acid in differentiating of neurons (Bento-Abreu et al., 2007). Although DHA is a ligand of PPARα, activation of PPARα is involved in astroglial differentiation of NSC (Cimini et al., 2008). PPARs activate the transcription of their target genes as heterodimers with retinoid X receptors (RXR), a nuclear receptor for DHA in the mouse brain (de Urquiza et al., 2000). RXR expression levels are low in undifferentiated NSCs; however, RXR is expressed in the differentiated NSC and primary hippocampal cells (Cimini et al., 2008; Calderon et al., 2007). DHA induces neurite outgrowth through RXR activation in neuro 2A cells (Calderon et al., 2007). Thus we hypothesize that PPARα and RXR are involved in neuronal differentiation from intermediate neuronal progenitor cells, and another mechanism other than PPAR and RXR is involved in the differentiation of neuronal intermediate cells from NSCs by DHA. This hypothesis assumes the differential effect of DHA on NSCs and intermediate neuronal progenitor cells (Fig. 5B). In this study, we found that DHA promotes neuronal differentiation of NSCs, at least in part, through Hes1 repression and $p27^{kip1}$ induction. At present, however, it is not clear how DHA repress the Hes1 gene expression.

Treatment with 10 μM DHA did not affect ether Tuj-1 or GFAP positive cells. DHA at high doses such as 20 and 50 μM was associated with lower survival rate. Our results were consistent with those of Kim et al. (2000). Treatment of rat pheochromocytoma PC12 and mouse neuroblastoma neuro 2A cells with low concentrations of DHA does not affect their DNA fragmentation; high concentrations of DHA, however, significantly increase their

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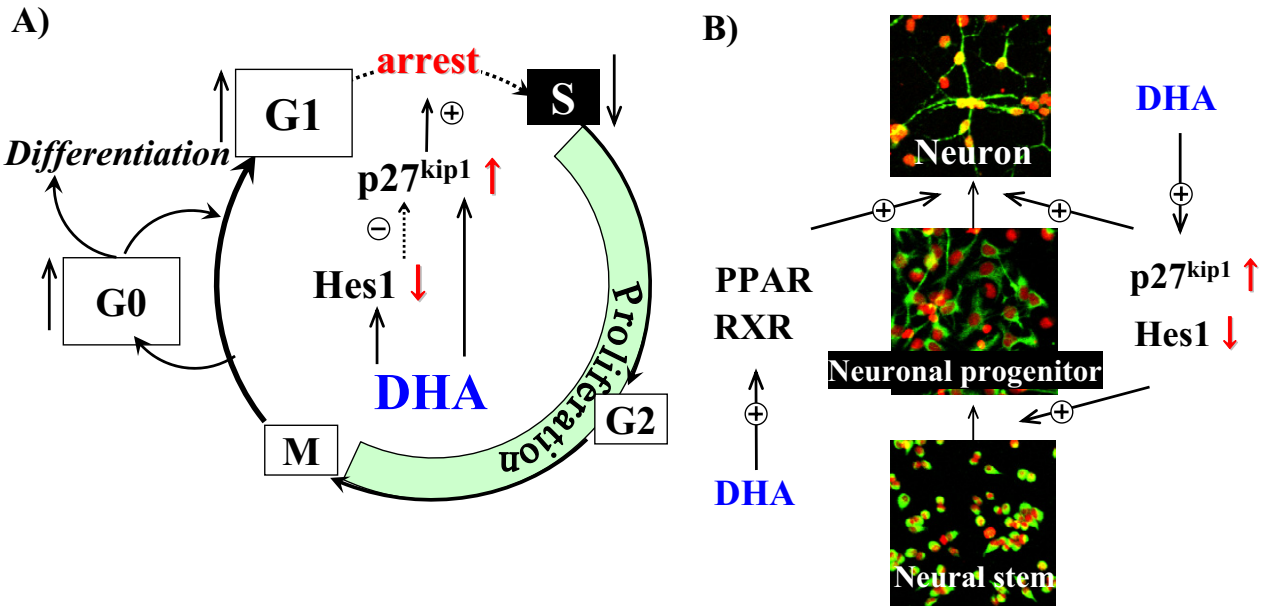

Fig. 5. Diagram showing our results (A) and hypothesis (B) of DHA-induced neuronal differentiation of NSCs.

DNA fragmentation (Kim et al., 2000), implying that high concentrations of DHA may induce apoptosis and lead to neuronal death. Therefore, the effect of DHA at 1.0 μM of concentration was used to study its effect on NSC differentiation.

CONCLUSION

In summary, DHA promotes the neural differentiation from NSCs by suppressing Hes1 repressor, which in turn activates $p27^{kip1}$ to arrest cell cycle (Fig. 5A). Therefore, DHA may control the cell fate and using this characteristic of DHA may help in the recovery of injured neurons in neurodegenerative diseases including AD.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2009.02.057.

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