Title

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Cis-9,*trans*-11-conjugated linoleic acid promotes neuronal differentiation through regulation of Hes6 mRNA and cell cycle in cultured neural stem cells

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ABSTRACT

Conjugated linoleic acids (CLAs) are positional and geometrical isomers of linoleic acid (LA). *Cis*-9,*trans*-11-CLA (CLA), the main isomer of CLAs in foods derived from ruminants, has several beneficial effects for humans and animals; however, its effects on the central nervous system are largely unknown. In this study, we investigated the effects of LA and CLA on neuronal differentiation of neural stem cells (NSCs). NSCs cultured with or without LA and CLA were assessed by immunofluorescence staining, mRNA measurement of basic helix-loop-helix transcription factors and cyclin-dependent kinase inhibitors by real-time PCR, BrdU incorporation analysis and flow cytometry analysis. In NSCs treated with CLA, the number of Tuj-1-positive cells (neurons) and the mRNA expression levels of Hes6, MAP2, $p21^{cip1}$ and $p27^{kip1}$ increased, while the proportion of S-phase cells decreased; compared with the control, no change was demonstrated in NSCs treated with LA. These results suggest that CLA promotes neuronal differentiation by increasing, in part, the expression of Hes6 mRNA and by activating $p21^{cip1}$ and $p27^{kip1}$ to arrest cell cycle.

delete ", Fig. 1A"

Add (CLA, Fig. 1B)

1. Introduction

Conjugated linoleic acids (CLAs, Fig. 1A), a group of polyunsaturated fatty acids (PUFAs), are positional and geometrical isomers of linoleic acid (LA, Fig. 1B) [1]. *Cis*-9,*trans*-11-CLA (CLA) is produced directly by bacterial hydrogenation of LA in ruminants [2] or by delta-9 desaturation of vaccenic acid in most mammalian tissues [3]. CLA is the p[illegible] present in milk, dairy and ruminant m[illegible] tributes about 90% of total dietary intake of CLAs by humans [4]. CLAs have several beneficial effects, such as anti-adipogenic, anti-carcinogenic, anti-atherogenic, anti-diabetogenic, anti-inflammatoric [4] and anti-angiogenic [5] on animal models and/or cell cultures. CLA is actively incorporated into the brain and metabolized [6,7], suggesting some biological effects on the central nervous system. Although PUFAs such as docosahexaenoic acid (DHA) [8,9], eicosapentaenoic acid [10,11] and arachidonic acid [12] are known to have physiological functions in the central nervous system, the effects of CLA thereon have remained largely unknown.

Change to "Fig. 1A"

Neural stem cells (NSCs) are self-renewing and multipotent progenitor cells that differentiate into neuronal or glial cells [13–15]. Since the stages of neuronal development proceed in the order of proliferation of NSCs, neuronal differentiation and glial differentiation [16], and are maintained in cultured NSCs [16], we used cultured NSCs for investigating the effects of CLA on neuronal development *in vitro*.

Basic helix-loop-helix (bHLH) transcription factors play important roles in the proliferation and differentiation of NSCs [17]. Neuronal differentiation is promoted by activator-type bHLH factors such as Mash1 and NeuroD, whereas repressor-type bHLH factors, such as Hes1, inhibit neuronal differentiation and promote proliferation [17–19]. The balance of activity among these factors is thought to determine the cell fate.

Regulation of the cell cycle affects the proliferation, differentiation and apoptosis of NSCs. Cell differentiation is usually accompanied by irreversible cell cycle exit that is arrested at the G1/S-phase and enters the G0-phase without passing the cell cycle restriction point [20]. G1 regulatory molecules have been shown as exquisitely regulated during the differentiation process. Deferoxamine, a G1/S-phase cell cycle blocker, induces neuronal differentiation of NSCs [21]. Cyclin-dependent kinase (CDK) inhibitors $p21^{cip1}$ and $p27^{kip1}$ block cell cycle progression by inhibiting the activity of cyclin–CDK complexes, and regulate transition through G1-phase [22,23].

A number of studies on CLAs have used a mixture of various isomers containing *cis*-9,*trans*-11- and *trans*-10,*cis*-12 CLA as major compounds. Recently, studies using individual isomers indicate that the two isomers have very different health effects [4]. Therefore, purified *cis*-9,*trans*-11-CLA, the main isomer of CLAs present in foods, was used in our study.

Here, we investigated whether LA and CLA affect neuronal differentiation and modulate the expression level of bHLH transcription factors, CDK inhibitors and the cell cycle in NSCs.

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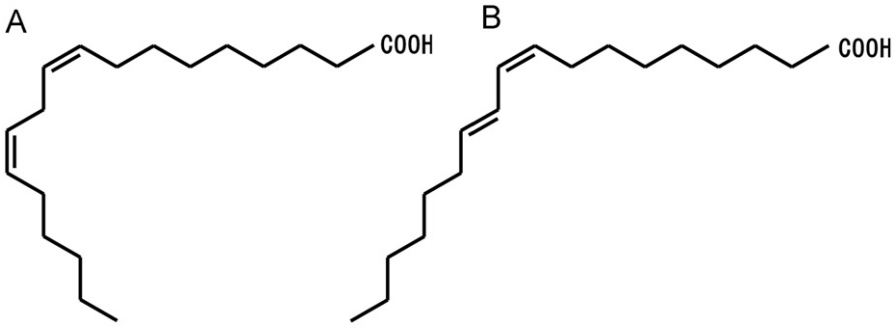

Fig. 1. Structures of linoleic acid (LA, A) and *cis*-9,*trans*-11-conjugated linoleic acid (CLA, B).

2. Materials and methods

All experiments were carried out in accordance with the "Guidelines for Animal Experimentation" of the Center for Integrated Research in Science, Shimane University, and approved by the "Animal Care and Use Committee" of the same institution under 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. The number of animals used and anesthetized for the collection of embryonic NSCs was kept to a minimum. Protocols were designed to minimize pain and suffering during the procedures.

2.1. Embryonic NSC culture

NSCs were cultured by the neurosphere method as described [24,25]. Briefly, forebrain cortices isolated from 14.5-day embryonic rats 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, 0.1% sodium bicarbonate, 2 mM of *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 (5% CO_2/95% air) humidified incubator at 37 °C. The cells grown as free floating neurospheres within 3–5 days were collected by centrifugation, dissociated by mechanical pipetting and passaged.

2.2. NSC differentiation

For differentiation, the neurospheres (passage 2) were mechanically dissociated and seeded onto poly-*L*-ornithine (15 μg/mL, Sigma-Aldrich)-coated 24-well plates at a density of 2×10^5 cells/well in N2 medium without bFGF or heparin. The cultures were then treated with LA (1.0 and 10 μM, Cayman, MI, USA) or CLA (0, 0.001, 0.01, 0.1, 1.0 and 10 μM, Cayman) and 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 control, and the culture medium was changed every other day.

2.3. Immunofluorescence staining

Cultured cells were fixed with 4% paraformaldehyde at room temperature for 30 min, 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 antibody was mouse anti-neuron-specific class III beta-tubulin (Tuj-1, 1:1000, R&D Systems, Inc.). 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 processed using the Image J software (NIH, Bethesda, MD, USA). The number of Tuj-1-positive cells and total cells was counted in each of the seven random fields per well.

2.4. Cell viability assay

Change from "4.5" to "4,5"

NSCs were seeded onto poly-*L*-ornithine-coated 96-well plates at a density of 2×10^4 cells/well in N2 medium with or without fatty acids (LA or CLA). 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 with a microplate reader, and the absorbance was measured at 550 nm. The data are expressed as percentages of the control group.

Change from "2.5" to "2,5"

2.5. Real-time PCR

The NSCs were allowed to differentiate for 6, 12, 24 and 96 h in differentiation medium in the presence of LA or CLA. Total RNA was isolated by Isogen (Wako Pure Chemical Industries, Ltd.), then cDNA was synthesized with the Quantitect reverse transcription kit (QIAGEN, GmbH, Hilden, Germany) and amplified by the 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 used are listed in Table 1. The specificity of PCR products was confirmed by both melting curve analysis and agarose gel electrophoresis (data not shown). The amplification efficiencies of all the genes determined in the initial experiment were all comparable (data not shown). The PCR conditions were as follows: initial activation at 95 °C for 15 min, then 40 amplification cycles of denaturation at 95 °C for [illegible] annealing at 58–63 °C for 30 s (see Table 1 ~~for temperatures used~~) and extension at 72 °C for 30 s. The relative changes in gene expression

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Table 1
Primers and reaction conditions of reverse transcription-polymerase chain reaction.

Genes	Primer sequence (5′–3′)	Size (bp)	Annealing temperature (°C)	Accession no.
Hes1	F: TTCCTCCCATTGGCTGAAAG R: CCAGCTCCAGATCCAGTGTGAT	129	60	NM024360
Hes6	F: TCCTTAGGCATCCTGACCAC R: TGGGCTATCTCCACCTCATC	149	63	BC087597
Mash1	F: GTTCGGCGGTCGAATACAT R: TGAATCTAAGTCCTGGTGCCC	411	63	X53725
NeuroD	F: AAGACGCATGAAGGCCAATG R: GCCAAGCGCAGTGTCTCTATCT	135	63	AF107728
MAP2	F: GTTTACATTGTTCAGGACCTCATGG R: TCGGTAAGAAAGCCAGTGTGGT	257	63	NM013066
$p21^{cip1}$	F: CAAAGTATGCCGTCGTCTGTTC R: CATGAGCGCATCGCAATC	70	63	BC100620
$p27^{kip1}$	F: GGCCAACAGAACAGAAGAAAATG R: GGGCGTCTGCTCCACAGT	67	58	NM031762
GAPDH	F: ATCTTCTTGTGCAGTGCCAGC R: CCTTGACTGTGCCGTTGAACT	216	63	AB017801

Hes, hairy and enhancer of split; Mash1, mammalian achaete–scute complex homolog 1; NeuroD, neurogenic differentiation; MAP2, microtubule-associated protein 2; $p21^{cip1}$ and $p27^{kip1}$, cyclin-dependent kinase inhibitors; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward primer; R, reverse primer.

levels were determined by the $2^{-\Delta\Delta Ct}$ method described in User Bulletin #2 of the ABI prism 7000 sequence detection system.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

BrdU (10 μM, Sigma-Aldrich) was added to the medium for 24 h with or without CLA. 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) at room temperature 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 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 with the use of Image J software. The number of BrdU positive cells and total cells was counted in each of the seven random fields per well.

2.7. Cell cycle analysis

After incubating the cells for 11 h, BrdU (10 μM) was added to the culture medium and the cells were allowed to incubate for another 1 h. Cell cycle was analyzed with a BrdU Flow Kit (Becton Dickinson and Company, SanDiego, CA, USA; BD). The cells were analyzed with a Becton Dickinson FACS Calibur cytometer equipped with a 15 mW, 488 nm, air-cooled argon ion laser for excitation of FITC (FL1), then 7-aminoactinomycin D (7-AAD, FL3) and FL1-H height signals were collected after logarithmic amplification, while both FSC-H and SSC-H height signals and the FL3-A area signal were collected after linear amplification. Samples were acquired and analyzed with the use of CELLQuest 3.3 software (Becton Dickinson), and the percentage of cells in G0/G1-, S- and G2/M-phases was determined.

2.8. 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. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Effects of LA and CLA on neuronal differentiation of NSCs

Fig. 2A shows fluorescence images of neuronal differentiated cells treated with CLA or BSA for 7 days. CLA dose-dependently increased the percentage of Tuj-1-positive cells by a maximum of 18.3% at 1.0 μM; the percentage was slightly lower at 10 μM CLA, but still higher than that in the control (Fig. 2B).

Tuj-1-positive cells cultured for 4 days with 1.0 or 10 μM of LA or CLA (Fig. 3A) showed that the number of cells treated with LA (1.0 and 10 μM) was not different from that of the control, whereas cells treated with CLA (1.0 and 10 μM) increased significantly by 134.5% and 121.7%, respectively, compared with those of the control (Fig. 3B). These data indicate that CLA, but not LA, enhances neuronal differentiation of NSCs.

As a concentration of 1.0 μM of LA and CLA did not affect cell viability (data not shown), it was used in subsequent experiments.

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3.2. Effects of L…n factors.

Compared with the control, a 96 h-treatment of NSCs with CLA significantly increased the mRNA expression level of Mash1

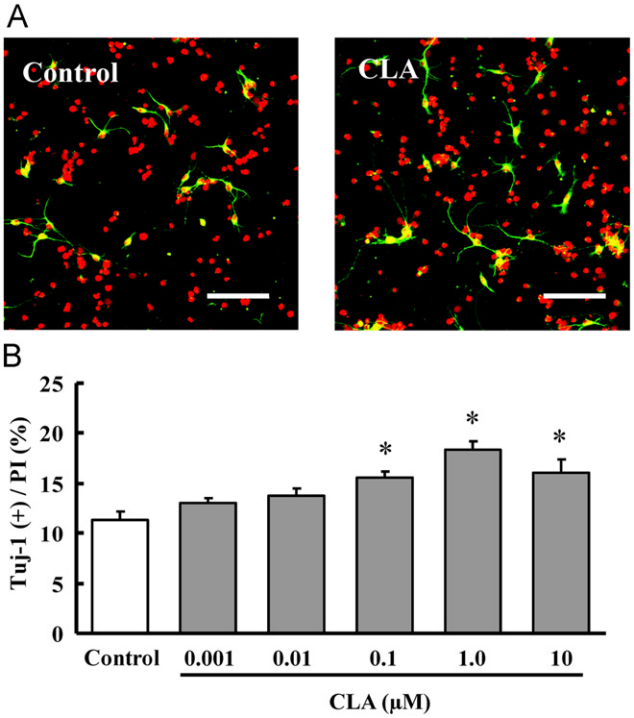

Fig. 2. CLA enhanced neuronal differentiation of NSCs in a dose-dependent manner. (A) Immunofluorescence images of Tuj-1 (neuron marker, green) and PI (nuclei, red) in control (0.01% BSA treated) and CLA (1.0 μM) groups on day 7. Scale bars: 100 μm. (B) NSCs were treated with 0, 0.001, 0.01, 0.1, 1.0 or 10 μM of CLA for 7 days. The number of neuronal differentiated cells is expressed as the proportion of Tuj-1-positive cells versus the total number of cells (PI-stained cells). The values are presented as the means ± SE ($n=6$). * The values are significantly different from the control group ($P < 0.05$). BSA at 0.01% was used as the control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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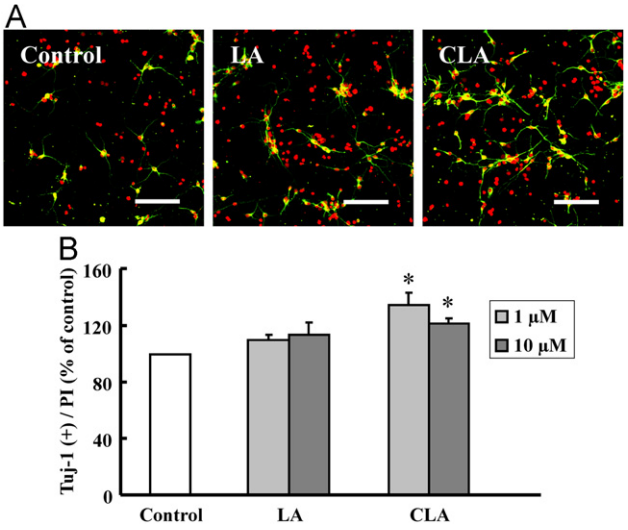

Fig. 3. Effects of LA and CLA on neuronal differentiation of NSCs. (A) Immunofluorescence images of Tuj-1 (neuron marker, green) and PI (nuclei, red) in control (0.01% BSA treated), LA (1.0 μM) and CLA (1.0 μM) groups on day 4. Scale bars: 100 μm. (B) NSCs were treated with LA or CLA (1.0 or 10 μM) for 4 days. The number of differentiating cells is expressed as the proportion of Tuj-1-positive cells versus the total number of cells (PI-stained cells). The control was set at 100%. The values are presented as the means ± SE ($n=3$). * The values are significantly different from the control group ($P<0.05$). BSA at 0.01% was used as the control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

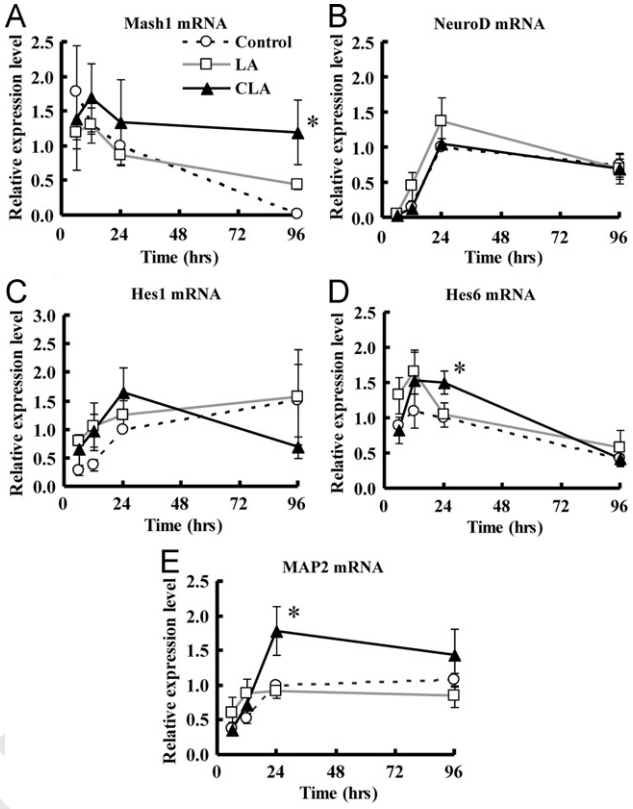

Fig. 4. Effects of LA and CLA on mRNA expression levels of bHLH transcription factors and MAP2. NSCs were cultured with 0.01% BSA (control, circle), LA (1.0 μM, square) or CLA (1.0 μM, triangle) in the culture medium for the periods of time indicated. Total RNA was prepared from each culture, and cDNA was synthesized and subjected to real-time PCR using specific primers for Mash1 (A), NeuroD (B), Hes1 (C), Hes6 (D) and MAP2 (E). 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 Dannett's test. * The values are significantly different from the control group ($P<0.05$).

(Fig. 4A). Similarly, a 24 h-treatment significantly increased the expression level of Hes6 and MAP2 by 50% and 80%, respectively (Fig. 4D and E). In contrast, the mRNA expression level of NeuroD and Hes1 demonstrated no significant difference (Fig. 4D and E). Treatment with LA did not affect the mRNA expression level of any of the bHLH transcription factors (Fig. 4).

3.3. Effects of LA and CLA on mRNA expression of $p21^{cip1}$ and $p27^{kip1}$, and on cell proliferation

The mRNA expression level of $p21^{cip1}$ and $p27^{kip1}$ in NSCs treated with CLA increased significantly by 247% and 80%, respectively, compared with that in the control. On the other hand, treatment with LA had no effect (Fig. 5A and B).

BrdU incorporation assay showed that the percentage of BrdU-positive cells of NSCs (53.7% in control) decreased significantly by treatment with 10 μM CLA (Fig. 5C and D), suggesting that CLA inhibits proliferation of NSCs.

3.4. Effects of LA and CLA on cell cycle regulation of NSCs

Representative scatter plot of FACS analysis in NSCs exposed to LA and CLA is shown in Fig. 6. The effects of LA and CLA on cell cycle distribution in NSCs are summarized in Table 2. Treatment with CLA significantly increased the percentage of G0/G1-phase and reduced that of S-phase of NSCs. On the other hand, treatment with LA did not affect cell cycle distribution in NSCs compared with the control. These data indicate that CLA promotes G1/S arrest and entry into the G0-phase.

4. Discussion

This study is, to our knowledge, the first to describe the effects of LA and CLA on neuronal differentiation of NSCs. Treatment of NSCs with CLA increased Tuj-1-positive cells in a dose-dependent manner, while LA had no effect, suggesting that CLA promotes neuronal differentiation of NSCs.

Cell fate such as proliferation and differentiation of NSCs is regulated by bHLH transcription factors [18,19]. Neuronal differentiation is promoted by activator-type bHLH transcription factors such as Mash1 and NeuroD [26,27], and inhibited by repressor-type bHLH transcription factors such as Hes1. Repressor-type bHLH transcription factors promote cell proliferation and retard neuronal differentiation of NSCs [28,29]. Mash1 forms a heterodimer with E47, another bHLH activator, and promotes neuronal differentiation of NSCs [30]. Hes1 represses Mash1 gene expression by forming a non-functional heterodimer with E47 and inhibits the transcriptional activity of Mash1 [30,31]. The expression of Hes1 is regulated by Notch signaling. Notch is a single transmembrane protein and cleaved by gamma-secretase, and releases intracellular domain (NICD). NICD moves into the nucleus and induces the expression of Hes1 that inhibits differentiation of NSCs. Hes6 promotes neural differentiation by inhibiting Hes1 function and promoting proteolytic degradation of Hes1 [32]. These reports indicate that Hes6 is dominant-negatively regulating the Notch pathway. In this study, the expression level of Hes6 was increased by treatment with CLA but did not affect the expression level of Hes1 (Fig. 4). It has reported that Hes6 suppressed Hes1 from inhibiting Mash1-E47 heterodimer and enables Mash1 and E47 to up-regulate transcription such as MAP2 in the presence of Hes1 [40]. CLA may inhibit Hes1 action by increasing the expression level of Hes6 and stimulates

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neuronal differentiation by altering the balance of bHLH transcription factors (Fig. 7).

Cell proliferation and differentiation of NSCs is affected by regulating the cell cycle [20]. Hes6 induces cell cycle arrest by enhancing $p21^{cip1}$ expression through the promotion of p53 activity [33]. $p21^{cip1}$ and $p27^{kip1}$, CDK inhibitors, negatively regulate cyclin/CDKs complex activity and promote cell cycle progression [22,23,34]. Cyclin D/CDK4 and cyclin D/CDK6 complexes act in G1-phase, and cyclin E/CDK2 complex operates in G1/S-phase of cell cycle [22,23,34]. Cell cycle arrest in G1-phase is induced by the inhibition of CDKs, which induces differentiation of cells [20]. Treatment of NSCs with deferoxamine increases the number of Tuj-1-positive cells by increasing the expression of $p27^{kip1}$ [21]. In this study, treatment of NSCs with CLA significantly increased the expression of Hes6 (Fig. 4D), and $p21^{cip1}$ and $p27^{kip1}$ mRNA (Fig. 5A and 5B). Furthermore, CLA depressed BrdU

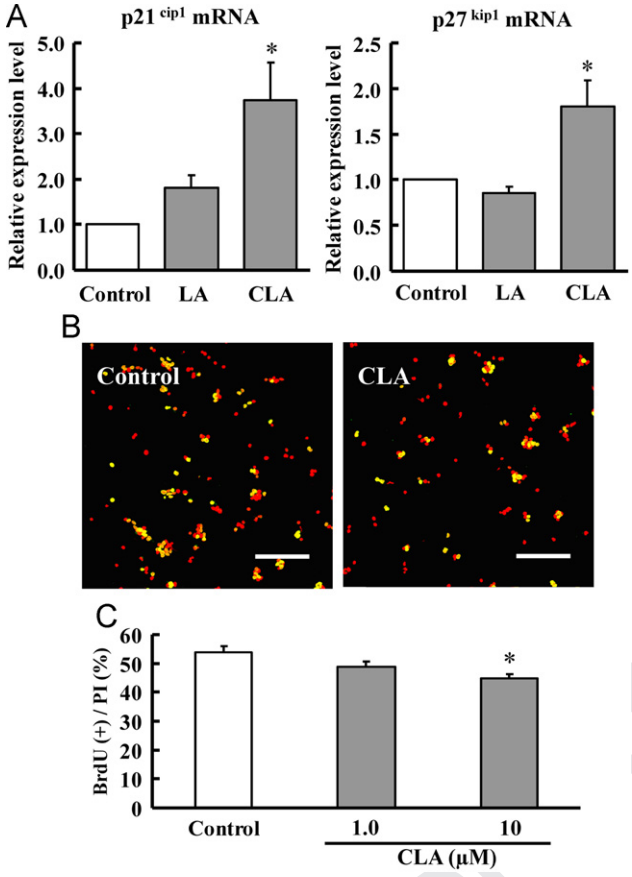

Fig. 5. Effects of LA and CLA on mRNA expression levels of $p21^{cip1}$ and $p27^{kip1}$. NSCs were cultured with 0.01% BSA, LA (1.0 μM) or CLA (1.0 μM) in the culture medium for 24 h. Total RNA was prepared from each culture, and cDNA was synthesized and subjected to real-time PCR using specific primers for $p21^{cip1}$ (A) and $p27^{kip1}$ (B). 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 control group taken as 1.0. (C) The effect of CLA on the proliferation of NSCs was determined by BrdU incorporated into the cells. Immunofluorescence images of BrdU (green) and PI (red) in control (0.01% BSA treated) and CLA (10 μM) groups. Scale bars: 100 μm. (D) NSCs were cultured for 24 h with or without CLA (1.0 or 10 μM) containing BrdU. The cells were fixed and stained with anti-BrdU antibody and PI, and then BrdU positive cells were counted. The values are presented as the means ± SE ($n=6$). Statistical analysis was carried out by Dannett's test. * The values are significantly different from the control group ($P<0.05$).

Table 2
Percentage of cells in the G0/G1-, S- and G2/M-phases of NSCs treated with or without LA and CLA.

Cell cycle phases	Control (%)	LA (%)	CLA (%)
G0/G1	80.87 ± 0.18	80.99 ± 0.29	88.07 ± 0.18*
S	15.49 ± 0.08	15.31 ± 0.39	7.41 ± 0.40*
G2/M	2.10 ± 0.15	2.24 ± 0.05	3.00 ± 0.19*

The values are expressed as the means ± SE ($n=3$). Statistical analysis was carried out by Dannett's test.

* The values are significantly different from the control group ($P<0.05$).

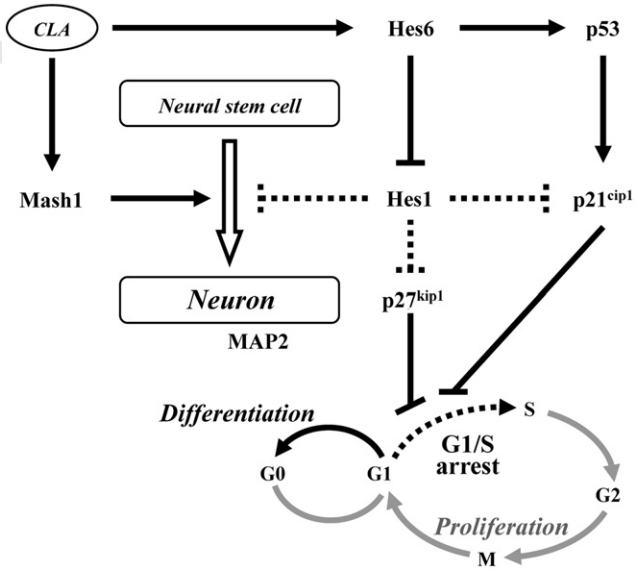

Fig. 7. Speculated mechanism of CLA in promoting neuronal differentiation of NSCs.

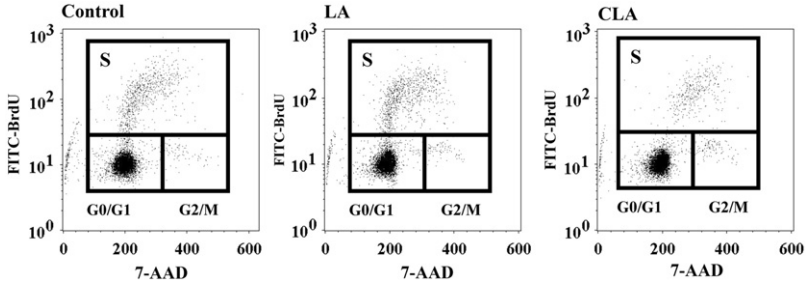

Fig. 6. Effects of LA and CLA on cell cycle distribution in NSCs. NSCs were treated with 0.01% BSA (control), LA (1.0 μM) or CLA (1.0 μM) for 12 h. BrdU was added to the final 1 h of culture. NSCs were then counterstained with FITC-conjugated anti-BrdU antibody and 7-AAD (for DNA staining), then analyzed by flow cytometer. Each gate indicated each phase of cell cycle.

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incorporation (Fig. 5C and 5D), significantly increased the proportion of G0/G1-phase cells and significantly decreased the proportion of S-phase cells by 52% as compared with the control (Fig. 6 and Table 2), indicating that G1/S-phase arrest was caused by treatment with CLA. These data are associated with the increase of Tuj-1-positive cells in NSCs treated with CLA.

Treatment of cancer cells with CLA inhibits cell proliferation through a p53-dependent mechanism [35]. p53 is also known to induce apoptosis of cells [36]. In this study, however, MTT assay showed that cell viability of NSCs was not influenced by LA or CLA at concentrations between 0.001 and 1.0 μM (data not shown). How CLA promotes the expression of Hes6 and induces the expression of both $p21^{cip1}$ and $p27^{kip1}$ is not clear. The treatment of NSCs with LA did not affect neuronal differentiation nor the expression of either $p21^{cip1}$ or $p27^{kip1}$. Therefore, the different neuronal effects of LA and CLA were attributed to the geometric isomerism of the fatty acids.

DHA (omega-3 PUFA) is well known for its beneficial effects on brain functions such as the enhancement of long-term memory in young and aged rats [37,38] and improvement of impaired memory and leaning ability in Alzheimer's disease model rats [8,39]. DHA promotes neuronal differentiation of NSCs by enhancing the expression of $p27^{kip1}$ and suppressing Hes1 [25]. In this study, CLA promoted neuronal differentiation of NSCs as effectively as DHA does, but increased Hes6 mRNA expression and did not suppress Hes1 mRNA expression. Hes6 induces neuronal differentiation of NSCs by suppressing Hes1 activity without controlling Hes1 expression [40]. Therefore, CLA may promote neuronal differentiation by suppressing Hes1 activity and supporting Mash1 activity by increasing the Hes6 mRNA expression (Fig. 7). In neuronal differentiation of NSCs, DHA characteristically represses the expression of Hes1 without the presence of Hes6. It is therefore assumed that the mechanism of CLA is different from that of DHA.

5. Conclusions

CLA increases the expression of Hes6 mRNA and promotes neuronal differentiation by activating $p21^{cip1}$ and $p27^{kip1}$ to arrest cell cycle. Therefore, CLA is speculated to control cell fate and to be useful in regenerative therapies for neurodegenerative diseases such as Alzheimer's and Parkinson's disease, particularly by controlling differentiation of NSCs pre- and post-transplantation.

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