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Suppression of amyloid-β secretion from neurons by *cis*-9, *trans*-11-octadecadienoic acid, an isomer of conjugated linoleic acid

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Abstract

Two common conjugated linoleic acids (LAs), cis-9, trans-11 CLA (c9,t11 CLA) and trans-10, cis-12 CLA (t10,c12 CLA), exert various biological activities. However, the effect of CLA on the generation of neurotoxic amyloid-β (Aβ) protein remains unclear. We found that c9,t11 CLA significantly suppressed the generation of Aβ in mouse neurons. CLA treatment did not affect the level of β-site APP-cleaving enzyme 1 (BACE1), a component of active γ-secretase complex presenilin 1 amino-terminal fragment, or Aβ protein precursor (APP) in cultured neurons. BACE1 and γ-secretase activities were not directly affected by c9,t11 CLA. Localization of BACE1 and APP in early endosomes increased in neurons treated with c9,t11 CLA; concomitantly, the localization of both proteins was reduced in late endosomes, the predominant site of APP cleavage by BACE1. The level of CLA-containing phosphatidylcholine (CLA-PC) increased dramatically in neurons incubated with CLA. Incorporation of phospholipids containing c9,t11 CLA, but not t10,c12 CLA, into the membrane may affect the localization of some membrane-associated proteins in intracellular membrane compartments. Thus, in neurons treated with c9,t11 CLA, reduced colocalization of APP with BACE1 in late endosomes may decrease APP cleavage by BACE1 and subsequent Aβ generation. Our findings suggest that accumulation of c9,t11 CLA-PC/LPC in neuronal membranes suppresses production of neurotoxic Aβ in neurons.

Introduction

Linoleic acid (LA), an essential fatty acid in humans, can be esterified to form phospholipids, a major component of the cell membrane (**Whelan and Fritsche 2013**). Conjugated linoleic acid (CLA) is present in meat products from ruminants. Symbiotic bacteria living in the rumens of these animals generate various CLA isomers by bio-hydrogenating polyunsaturated fatty acids such as LA. Cis-9, trans-11 CLA (c9,t11 CLA), and trans-10, cis-12 CLA (t10,c12 CLA) are the most common naturally occurring products (**Parodi 1976; Grinari et al., 2000**). CLA exerts a range of biological activities: the intake of CLA is beneficial for cardiovascular health and may also help to prevent cancers and inflammation (**Ha et al., 1987; Lee et al., 1994; Yu et al., 2002**). The biological activities of these compounds are also observed in the central nervous system: in animal studies, CLA exerts beneficial effects on brain disorders. CLA protects neurons from excitotoxicity caused by excess glutamate (**Hunt et al., 2010**), improves or preserves memory (**Gama et al., 2015; Binyamin et al., 2019**), and prevents age-dependent neurodegeneration (**Monaco et al., 2018**). CLA administered by feeding and gavage can be incorporated into the brain (**Fa et al., 2005; Vemuri et al., 2017**). However, in many studies, CLA has been used as a mixture of isomers; consequently, the detailed biological activities of individual CLA isomers remain unclear (**Noone et al., 2002; Hubbard et al., 2003; Bissinauth et al., 2008**).

Alzheimer's disease (AD) is the most common neurodegenerative disorder in aged individuals with dementia. The two major pathological hallmarks of AD are senile plaques and neurofibrillary tangles (NFTs) in the brain (**Scheltens et al., 2016**). Senile plaques are formed by extracellular accumulation of their principal component, amyloid-β (Aβ), whereas NFTs are intracellular aggregates of highly phosphorylated tau, a microtubule-associated protein (**Mucke & Selkoe, 2012**). Soluble Aβ oligomers are thought to trigger neuronal impairment by attacking synapses prior to the appearance of pathological marks in the brain (**Beniloval et al., 2012**), implying that Aβ production is a primary cause of AD onset.

Aβ is generated from amyloid-β protein precursor (APP) by serial proteolytic cleavages. APP is a type I membrane protein; in neurons, it contains 695 amino acids (APP695), whereas other tissues express the splicing variants APP770 and APP751 at lower levels (**Iijima et al., 2000**). All APP isoforms are subject to post-translational modifications, including *N*-glycosylation in the ER and *O*-glycosylation and phosphorylation in the Golgi complex. Mature APP (mAPP) with *N*- and *O*-glycosylation is cleaved by secretases in the late secretory pathways, whereas immature APP (imAPP) with *N*-glycosylation alone resides in the ER and *cis*-Golgi compartment (**Suzuki & Nakaya, 2008; Thinakaran & Koo, 2008**).

APP is cleaved by α-secretase (ADAM 10/ ADAM17), primarily on the plasma membrane, to generate the membrane-associated carboxy-terminal fragment CTFα; this is termed the non-amyloidogenic pathway because α-secretase cleaves APP within the Aβ sequence. In the amyloidogenic pathway, APP that escapes from cleavage on the plasma membrane is subject to endocytosis and alternative cleavage by β-secretase (BACE1) in

endosomes to generate CTFβ (**Cole and Vassar, 2008**). CTFβ, including the entire Aβ sequence, is further cleaved by the γ-secretase complex, yielding Aβ peptides that are secreted into the extracellular milieu. The γ-secretase complex, a membrane-embedded multimeric aspartic protease comprising presenilin 1 or 2 (PS1 or PS2), nicastrin (NCT), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2), cleaves APP CTF at the ε-site to release the APP intracellular domain fragment (AICD) into the cytoplasm (**Steiner et al. 2008**). The remaining membrane-associated N-terminal region is subject to further processing by the carboxypeptidase-like activity of the γ-secretase complex to generate various types of Aβ with different carboxyl-terminal γ-cleaved sites, including the strongly neurotoxic and aggregation-prone Aβ42 (**Qi-Takahara et al., 2005; Takami & Funamoto 2012**). Mutations of AD causative genes, including *PSENs* and *APP*, alter Aβ generation (**Mullan et al., 1992; Goate et al., 1991; Forman et al., 1997**), and a protective Icelandic mutation of *APP* decreases β-cleavage and facilitates β'-cleavage of APP, thereby decreasing production of neurotoxic Aβ1-40 and Aβ1-42, and increasing production of N-terminal truncated Aβ11-40 and Aβ11-42 (**Jonsson et al., 2012; Kimura et al., 2016**). Accordingly, Aβ generation is a primary cause of AD.

Although it remains controversial whether Aβ generation is altered in patients with sporadic AD, several lines of evidence support the idea that APP processing or APP secretase activities are altered in these cases (**Yanagida et al., 2009; Kakuda et al., 2012; Kakuda et al., 2020; Hata et al., 2011; Hata et al., 2020**). One cause of changes in APP cleavage and secretase activity is membrane lipid composition. Experimental alteration of the proportions of various membrane lipids can alter the C-terminal speciation of Aβ (**Qintero-Monzon et al., 2011; Holmes et al., 2012**); in addition, the cholesterol levels in membrane microdomains (detergent-resistant membranes, DRMs), where active γ-secretase complex cleaves substrate in the post-Golgi and endosomal membranes (**Vetrivel et al., 2004; Hur et al., 2008**), are reduced in AD brains (**Molander-Melin et al., 2005; Hata et al., 2020**). Because the substrates and enzymes related to Aβ generation are all membrane proteins that are subject to membrane trafficking in neurons (**Suzuki et al., 2006; Hata et al., 2016**), the lipid and fatty acid composition of neuronal membrane may play important roles in the metabolism and functions of AD-related membrane proteins, and thus in cognitive functions (**Snowden et al., 2017**).

In this study, we investigated whether the most common naturally occurring CLA isomers, c9,t11 CLA and t10,c12 CLA, can influence amyloid-β (Aβ) generation in primary cultured mouse neurons. To this end, we used highly pure samples of each isomer in all experiments.

Experimental procedures
Animals and primary cultured neurons and glia
All animal studies were conducted in compliance with the ARRIVE guidelines (approved #18-0168 in the Animal Studies Committee of Hokkaido University). C57BL/6J mice

(RRID:IMSR_JAX:000664; CLEA-Japan, Tokyo, Japan) and $App^{NL-F/NL-F}$ mice (C57BL/6-App<tm3(NL-F)Tcs, RRID:IMSR_RBRC06343) (**Saito et al., 2014**) were housed in a specific pathogen-free (SPF) environment with a microenvironment vent system (Allentown Inc., Allentown, NJ, USA) under a 12 h/12 h light/dark cycle with free access to food and water. Three to five male or female siblings were housed in each cage (floor space: 535 cm^2); cages were equipped with micro-barrier tops. Female mice (20–25 g body weight; 3–4 months of age) with genotypes discriminated by individual identification numbers were crossed with male mice (25–30 g body weight; 3–4 months of age). Minimal numbers of pregnant mice were sacrificed by cervical dislocation to harvest embryos (approximately 150 embryos from 30 dams were used in all studies, including preliminary experiments). In total, ten C57BL/6J and two $App^{NL-F/NL-F}$ pregnant mice were used in studies of Aβ generation and related biochemical analyses; immunocytochemical studies used five C57BL/6J pregnant mice, and LC-MS analyses used 15 C57BL/6J pregnant mice.

Embryonic mouse cortical and hippocampal neurons were cultured as previously described (**Chiba et al., 2014**). Briefly, the cortex and hippocampus of mice at embryonic day 15.5 were dissected, and neurons were dissociated in a buffer containing papain and cultured at 5×10^4 cells/cm^2 in Neurobasal Medium (Cat#21103049, Gibco/Thermo Fisher Scientific, Waltham, MA, USA) containing 2% (v/v) B-27 Supplement (Cat#17504044, Gibco/Thermo Fisher Scientific), Glutamax I (4 mM, Cat#35050061, Gibco/Thermo Fisher Scientific), heat-inactivated horse serum (5% v/v, Cat# 26050088, Gibco/Thermo Fisher Scientific), and penicillin-streptomycin (Cat#35050061, Gibco/Thermo Fisher Scientific) on Costar 6-well plates (Cat#3516, Corning, Corning, NY, USA) or Nunc Lab-Tek II Chambered Coverglass (Nalgene Nunc/Thermo Fisher Scientific, Rochester, NY, USA) coated with poly-L-lysine hydrobromide (Cat#P2636, Sigma-Aldrich, St. Louis, MO, USA). Half of the volume of the culture media was replaced with fresh media twice a week. 5-Fluoro-2'-deoxyuridine (FdU, Cat# F0503, Sigma-Aldrich) was added to the cultured media for the first 3–4 days *in vitro* (DIV) to prevent the growth of glial cells (**Hui et al., 2016**).

Neurons cultured for the indicated periods were treated with LA (Cat#L1376, Sigma-Aldrich), c9,t11 CLA (Cat# 16413, Sigma-Aldrich), or t10,c12 CLA (Cat#90145, Cayman Chemical, Ann Arbor, MI, USA). In a separate study, neurons were cultured for 24 h with LA, c9,t11 CLA, or t10,c12 CLA (10 μM) in the presence of human Aβ40 (20 nM, Cat#4307-v, Peptide Institute, Osaka, Japan). To quantify exogenous human Aβ40, the levels of Aβ in the medium were assayed by sandwich ELISA (sELISA). Cell viability was evaluated by the MTT assay (Cat #M009, Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and cell toxicity was evaluated using the LDH Cytotoxicity Detection Kit (Cat #MK401; Takara Bio, Shiga, Japan).

Antibodies, immunoblotting, immunostaining, and Aβ assay

Antibodies used in this study were raised against BACE1 (APP β-site–cleaving enzyme 1), which cleaves APP to generate Aβ in late endosomes; EEA1 (early endosome antigen 1), a marker of the early endosome; and Rab7, a late endosome-/lysosome-associated small GTPase. Information about purchased antibodies used in the present study is listed in Supplementary Table 1. Custom-made antibodies used in the present study will be shared upon reasonable request. Rabbit polyclonal anti-APP (1:10,000, 369; **Oishi et al., 1997**) and rabbit polyclonal PS1 NTF (1:10,000, Ab14; **Xu et al., 1998**) antibodies were kindly supplied by Dr. S. Gandy. The amounts of protein were measured using a BCA protein assay kit (Cat#23225, Thermo Fisher Scientific). Proteins were resolved by Tris-glycine buffered sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (8% and 15% polyacrylamide gels) and transferred onto nitrocellulose membrane (Cat# P/N66485, Paul Corp., Pensacola, FL, USA) for immunoblotting. Membranes were blocked in 5% non-fat dry milk (barcode 4 902220 354665, Morinaga Milk Industry, Tokyo, Japan) in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.05% Tween 20 [sc-29113, Nacalai Tesque, Kyoto, Japan]), probed with the indicated antibodies in TBS-T, and washed with TBS-T. Immunoreactive proteins were detected using Clarity Western ECL substrate (Cat#1705061, Bio-Rad, Hercules, CA, USA) and quantitated on a LAS-4000 (FUJIFILM, Tokyo, Japan). Results are derived from independent experiments, and numbers of experiments (n) are indicated in the figure legends.

Primary neurons from wild-type mice were cultured *in vitro* for 9–12 days (DIV 9-12) and treated with 10 μM LA or CLA for 24 h. Neurons were fixed with 4% paraformaldehyde in PBS (Cat#161-20141, FUJIFILM Wako Pure Chemical), treated with 0.2% Triton X-100 (Cat#12969-25, Nacalai Tesque) in PBS, and blocked with 1% horse serum (Cat# 26050088, Gibco/Thermo Fisher Scientific) and 1% goat serum (Cat#16210064, Gibco/Thermo Fisher Scientific) in PBS. The neurons were incubated with the indicated primary antibodies for 12 h, followed by incubation with secondary antibodies, Alexa Fluor 488-conjugated donkey anti-mouse IgG, Alexa Fluor 546-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-rabbit IgG, and Alexa Fluor 546-conjugated goat anti-rabbit IgG (**Supplementary Table 1**). Fluorescence images were acquired on a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan), and quantitative analysis and colocalization efficiency were performed with Fiji/ImageJ and the Colco2 Fiji plugin (ImageJ-Fiji-ImgLib; http://Fiji.sc or http://imageJ.net).

Mouse monoclonal anti-Aβ40 (4D1) and Aβ42 (4D8) carboxy-terminal specific antibodies were as described (**Tomita et al., 1998**). The Fab' fragment of rabbit polyclonal anti-mouse Aβ(1-16) IgG conjugated with horseradish peroxidase was used for the detection of mouse Aβ40, the major Aβ isoform of neurons. Biotinylated mouse monoclonal anti-human Aβ(1–16) IgG 82E1 was used for detection of human Aβ. Mouse Aβ40 was quantified by sELISA consisting of 4D1 and anti-mouse Aβ(1–16). Human Aβ42, the dominant isoform generated in neurons of *App^{NL-F/NL-F}* mouse with familial AD mutations in the humanized

Aβ sequence of the mouse *App* gene (**Saito et al., 2014**), was quantified by sELISA consisting of 4D8 and 82E1 (**Mizumaru et al., 2009**).

In vitro β-secretase assay

BACE1 activity was examined using the β-secretase activity assay kit (Cat. #ab65357, Abcam). Briefly, neurons were homogenized with BACE1 extraction buffer (contents are not opened), and the lysate (100 μg protein) including endogenous BACE1 or 4 μg of recombinant human BACE1 (rBACE1, amino acid sequence Glu46–Tyr460 in the mature form of BACE1) (Cat. # 931-AS; R&D Systems, Minneapolis, MN) in extraction buffer was incubated at 37°C for 1 h with a secretase-specific peptide conjugated to two reporter molecules. After the reaction was stopped, Ex/Em (335 nm/495 nm) was measured. Notably, rBACE1 lacks a part of the transmembrane region and the entire cytoplasmic region (C-terminal 40 amino acids) of the type I membrane protein, and is C-terminally tagged with 10-His (for a review of the BACE1 structure, see **Dislich & Lichtenthaler, 2012**).

In vitro γ-secretase assay

Neurons were suspended in buffer H (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% [v/v] glycerol) including protease inhibitors [5 mg/ml each of chymostatin (Cat#4063), leupeptin (Cat#4041), and pepstatin A (Cat# 4397) (Peptide Institute Osaka, Japan)] by passing the cells 10 times through a 30-gauge needle, and then centrifuged at $100,000 \times g$ (Beckman Optima L-100XP, 70.1 Ti rotor) for 1 h at 4°C. The resultant precipitates were resuspended in the same buffer and incubated for 2 h at 37°C with or without 10 μM N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT: Sigma-Aldrich), a γ-secretase inhibitor. The membrane suspensions were centrifuged at $10,000 \times g$ for 10 min, and Aβ40 levels were quantified by sELISA. The Aβ40 level in reaction mixtures containing DAPT was defined as background.

Neuronal toxicity/viability assays

In the presence or absence of LA and CLA, neurons were subjected to the MTT assay (Dojindo Molecular Technologies, Kumamoto, Japan), which measures cell viability, and the LDH assay (LDH Cytotoxicity Detection Kit; Takara Bio, Shiga, Japan), which measures the level of LDH leaked into the medium, a reflection of cellular damage.

Lipid extraction, fractionation, PLA reaction, and LC-MS/MS analysis

Neurons (DIV 10, ~5×10^6 cells) derived from wild-type mice were treated with 10 μM LA or CLA for 24 h, harvested, and quickly frozen in liquid nitrogen. Phospholipids, including phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), were extracted using acidic methanol (pH 4.0) with high extraction yield (**Satomi et al., 2017; Zhao & Xu, 2010,**

Okudaira et al., 2014). PC from the cell extract was then fractionated by thin layer chromatography.

To obtain 2-acyl LPC (sn-2-LPC) from PC, the PC fraction was incubated for 2 h at 37°C with a reaction mixture containing 78 mM phosphate (pH 7.4), 10% diethyl ether, 2 mM $CaCl_2$, 0.05% TritonX-100, and 1 U/µl *Rhizomucor miehei* PLA_1 (Cat#9001-62-1, Sigma-Aldrich). After the enzymatic reaction, nine volumes of methanol were added to stop the reaction, the mixture was centrifuged (21,500 ×g, 5 min), and the supernatant was stored at −80 °C. The resultant 2-acyl LPC (sn-2-LPC) was subjected to spontaneous acyl-migration as described previously (**Kawana et al., 2019; Okudaira et al., 2014**). Briefly, the 2-acyl LPC in methanol (pH 7.0) was incubated at 37°C until more than 90% of the 2-acyl LPC with a specific fatty acid was converted to the LPC with the fatty acid at the sn-1 position, as confirmed by LC-MS. To hydrolyze PC to produce 1-acyl LPC (sn-1-LPC), fractionated PC was incubated for 2 h at 25°C with a reaction mixture containing 50 mM Tris-HCl (pH 9.0), 2.5 mM $CaCl_2$, 0.05% TritonX-100, and 0.1 U/µl *Apis mellifera* PLA_2 (Cat#P9279, Sigma-Aldrich). The reaction was stopped by addition of nine volumes of methanol, and the samples were stored as described above. LC-MS/MS analysis of LPC was performed as described previously (**Kano et al., 2019; Fujita et al., 2021**).

Statistical analysis

Data are expressed as the means ± SD. Statistical differences were assessed using Student's t-test or one-way ANOVA, combined with the Tukey–Kramer post-hoc test and Dunnett's test for multiple comparisons (GraphPad Prism 8 software). A *p*-value of <0.05 was considered significant. No sample size calculation or tests for normal distribution or outliers were performed. Culture size and neuronal cell numbers were determined as described in previous publications on biochemical analyses including Aβ quantification (**Kwak et al., 2020**), immunocytochemical analysis (**Sobu et al., 2017**), and LC-MS (**Okudaira et al., 2014**). The study was not pre-registered. No randomization or blinding was performed.

Results

Suppression of Aβ generation in neurons treated with c9,t11 CLA

The structure of LA and common CLA isoforms used in this study are shown in **Fig. 1A**. To explore the effect of CLA on Aβ generation, we treated mixed cortical and hippocampal neurons (DIV 7–10) from wild-type (WT) mice with the indicated concentrations (0.1, 1, or 10 μM) of LA, c9,t11 CLA, and t10,c12 CLA along with vehicle (DMSO) for 24 h. The level of Aβ40, a major endogenous Aβ isoform, in the medium was quantified (**Supplementary Figure S1**). Only 10 μM c9,t11 CLA decreased the Aβ level in medium relative to vehicle (DMSO); lower concentrations of c9,t11 CLA (0.1 or 1 μM) did not exert a significant effect. Because various bioactive effects of CLA were observed in *in vitro* experiments with higher concentrations (>10 μM) of CLA (**Hunt et al., 2010; Lee et al., 2013**), the *in vitro* doses we used tended to be higher than the *in vivo* levels. We confirmed this effect in different batches of neurons treated with 10 μM of LA and CLA (**Fig. 2**).

We again treated mixed cortical and hippocampal neurons (DIV7−10) from WT mice for 24 h with 10 μM LA, c9,t11 CLA, or t10,c12 CLA or vehicle alone (DMSO), and then assessed secretion of endogenous mouse Aβ40 (**Fig. 2A**). Generation of mouse Aβ40 was significantly lower in neurons treated with c9,t11 CLA than in neurons treated with LA and DMSO, indicating that the suppression of Aβ generation by c9,t11 CLA was reproducible. By contrast, treatment with t10,c12 CLA had no effect on Aβ generation. Levels of endogenous mouse Aβ42 were too low to quantify in cultured neurons.

To further explore the effect of CLA on Aβ generation, we treated mixed cortical and hippocampal neurons (DIV 14–17) from human APP knock-in mice (*App^{NL-F/NL-F}*) with 10 μM LA, c9,t11 CLA, t10,c12 CLA, or DMSO for 48 h, and then quantified the secretion of humanized Aβ42 into the medium (**Fig. 2B**). Again, c9,t11 CLA decreased Aβ42 generation significantly relative to DMSO, whereas t10,c12 CLA did not.

We next assessed the viability of neurons after treatment with CLA and LA. To this end, we treated primary cultured neurons (DIV 12) from WT mice with the indicated concentration of LA, c9,t11 CLA, and t10,c12 CLA, along with vehicle alone (DMSO), and then examined cell viability by MTT assay and toxicity by LDH assay. In the MTT assay, we observed no significant differences among neurons treated with CLA and LA at any concentration (**Fig. 2C**). Furthermore, the levels of lactate dehydrogenase (LDH), an indicator of cytotoxicity and plasma membrane injury, were identical in cultures treated with LA and CLA (**Fig. 2D**). These data indicate that treatment of neurons with LA or CLA does not affect cell viability or cause toxicity; thus the decrease in Aβ generation in neurons treated with c9,t11 CLA was not due to changes in cell viability.

c9,t11CLA does not promote Aβ degradation in medium of primary cultured neurons and glial cells

Extracellular Aβ is subject to clearance by cellular uptake and proteolytic degradation (**Saido and Leissring, 2012**). The lower levels of Aβ in the medium of neurons treated with c9,t11 CLA could have been the result of faster clearance of Aβ from the medium. To explore this possibility, we investigated whether Aβ clearance or degradation was facilitated by CLA treatment. For this purpose, we treated primary cultured neurons (DIV10–12) of WT mice with LA, c9,t11 CLA, t10,c12 CLA, or DMSO (vehicle) for 24 h in the presence of human Aβ40. The collected media were subjected to human Aβ40-specific sELISA to quantify Aβ40 remaining. Media from neurons (**Fig. 3A**) contained 10–15% of the original levels of Aβ, less than in cell-free media (~20%); however, the magnitudes of the decrease were almost identical in neurons treated with LA and CLA. We also examined the levels of Aβ adsorbed on or incorporated into neurons. We quantified Aβ40 in lysates of neurons after culture in the presence of exogenous human Aβ40 and one wash with PBS (**Fig, 3B**). LA, c9,t11 CLA, and t10, c12 CLA did not affect the adsorption or incorporation of Aβ40 relative to vehicle alone (DMSO), and the levels detected in lysates were much lower than the amounts of Aβ [100%] administered in the medium. Because 10–15% of the Aβ remained in the media in the presence of neurons, a large fraction of exogenous Aβ in the medium must have been cleared during the incubation (24 h). However, the ability to clear Aβ from the medium was similar in neurons treated with LA, c9,t11 CLA, and t10,c12 CLA, indicating that c9, t11 CLA does not increase the neuronal capacity for degradation, incorporation, or clearance of medium Aβ.

Because glial cells secrete Aβ-degrading enzymes (**Kidana et al., 2018**), we investigated whether glial cells contribute to the decrease in medium Aβ (**Supplementary Fig. S2**). We treated primary cultured glia of WT mice with LA, c9,t11 CLA, t10,c12 CLA, or DMSO for 48 h in the presence of human Aβ40. The Aβ content of the collected media was examined by immunoblotting with human Aβ-specific antibody. Aβ levels were slightly lower than in cell-free media alone; however, the magnitudes of the decrease were almost identical in glial cells treated with LA and CLA at various concentrations (10 μ and 100 μM). Glial viability was not affected by treatment with LA or CLA. Together, these results demonstrate that the ability of glial cells to remove or degrade Aβ from the medium is not increased by c9,t11 CLA.

Taken together, these findings indicate that the decrease in the Aβ level in the culture medium of neurons treated with c9,t11 CLA is due to suppression of Aβ generation.

c9,t11 CLA decreases BACE1 activity in neuronal lysate, but not *in vitro*

To investigate how neurons treated with c9,t11 CLA decrease Aβ generation, we first assessed the level of β-site APP-cleaving enzyme (BACE1), which is the enzyme primarily responsible for amyloidogenic cleavage of APP (**Vassar et al., 1999; Kimura et al., 2016**). Because CLA alters expression of BACE1 but not ADAM10/α-secretase in SH-SY5Y cells (**Li et al., 2011**), we examined protein expression of APP, BACE1, and presenilin 1 amino-terminal fragment (PS1 NTF), a catabolic component of the active γ-secretase complex, in neurons treated with CLA and LA. The levels of these proteins, as well as α-tubulin (used as a loading control), were

measured by immunoblotting with the corresponding antibodies (**Fig. 4A**). Cellular levels of APP, BACE1, and PS1 NTF were almost identical among neurons treated with LA, c9,t11 CLA, and t10,c12 CLA, and no significant differences were observed (**Fig. 4B–D**). These results suggest that differences in the levels of substrate APP and APP-cleaving enzymes are not the primary cause of the reduction in Aβ generation in neurons treated with c9,t11 CLA.

Next, we examined endogenous BACE1 activity. Specifically, we assayed lysates of WT mouse neurons treated with CLA and LA for BACE1 activity *in vitro*. We treated primary cultured neurons (DIV 9–12) from WT mice with 10 μM LA, c9,t11 CLA, or t10,c12 CLA for 24 h, and assayed their lysates for BACE1 activity (**Fig. 5A**). BACE1 protein level was not affected by treatment with CLA or LA (**Fig. 4**), but lysates prepared with this assay system revealed that BACE1 enzyme activity was slightly but significantly reduced in neurons treated with c9,t11 CLA, but not t10,c12 CLA, relative to those treated with LA (**Fig. 5A**).

We next investigated whether c9,t11 CLA directly affects BACE1 activity *in vitro* (**Fig. 5B**). To this end, we incubated recombinant BACE1 (rBACE1) with LA, c9,t11 CLA, t10,c12 CLA, or DMSO alone, and assayed enzymatic activity following the addition of the BACE1 substrate. In the presence of LA or CLA, rBACE1 activity was slightly higher than in the vehicle (DMSO) control. However, we observed no remarkable differences in rBACE1 activity among enzyme solutions containing LA, c9,t11 CLA, and t10,c12 CLA (**Fig. 5B**). These results clearly show that c9,t11 CLA does not directly and specifically activate rBACE1. The slight increase of BACE1 activity in the presence of LA and CLA may be due to the stabilization of rBACE1 by fatty acids. Notably, rBACE1 lacks a large part of the single transmembrane and following cytoplasmic region, although the catalytic domain in the amino-terminal and luminal region is completely conserved. Therefore, the effect of c9,t11 CLA may be mediated through the hydrophobic transmembrane region of BACE1 (**Dislich & Lichtenthaler, 2012**).

We also investigated the effect of c9,t11 CLA on the activity of γ-secretase, which generates Aβ following APP cleavage by BACE1. For the *in vitro* γ-secretase assay, the membrane fraction including the γ-secretase complex of WT mouse neurons treated with LA, c9,t11 CLA, and t10,c12 CLA, or vehicle alone (DMSO) was incubated, and endogenous Aβ40 generated by γ–secretase cleavage was quantified (**Supplementary Figure S3**). No significant differences in Aβ40 generation were observed among membranes from neurons treated with LA, c9,t11 CLA, and t10,c12 CLA, and the levels were identical to those without treatment (DMSO). This observation suggests that the activity of the γ-secretase complex is not significantly influenced by treatment with CLA.

When neurons are treated with specific fatty acids, membrane lipid components such as glycerophospholipid are modified by incorporation of fatty acids into phospholipid (**Fig. 1B and C**). Such membrane lipid modification and/or exchange may influence the interactions of proteins with phospholipids in the membrane, thereby altering the intracellular localization of membrane proteins. Indeed, the increase in unique acyl chain composition in neuronal membranes regulates the local distribution of membrane protein components within a specific

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membrane compartment (**Kuge et al., 2014**). Therefore, we next explored the localization of BACE1 and APP in neurons treated with LA and CLA, and compared the phospholipid compositions among samples.

Reduced localization of APP with BACE1 in late endosomes of neurons treated with c9,t11 CLA

APP cleavage by BACE1 occurs in detergent-resistant membrane domains (DRMs) or lipid rafts (**Cordy et al., 2003; Puglielli et al., 2003; Kalvodova et al., 2005; Saito et al., 2008**) of acidic organelles following plasma membrane endocytosis of APP (**Cole and Vassar, 2008**). Hence, we first examined the localization of APP and BACE1 in neurons treated with CLA or LA, and compared the colocalization efficiency of APP with BACE1 among the samples (**Fig. 6**). Colocalization of APP with BACE1 in neurons is shown (**Fig. 6A (1)**). Moderate colocalization with a Pearson's R value of ~0.5 was observed in neurons treated with LA (a value of 1.0 indicates perfect colocalization, 0 indicates random localization, and -1 indicates perfect exclusion). However, Pearson's R value was significantly lower in neurons treated with c9,t11 CLA than in those treated with LA, whereas t10,c12 CLA had no effect (**Fig. 6B (1)**). The observation suggests that colocalization of BACE1 with APP decreased in neurons treated with c9,t11 CLA.

We next examined the colocalization of BACE1 with Rab7, a late endosomal marker (**Fig. 6A (2)**). Interestingly, colocalization between BACE1 and Rab7 was significantly reduced in neurons treated with c9,t11 CLA, but not t10,c12 CLA or LA (**Fig. 6B (2)**), suggesting less localization of BACE1 in late endosomes, where BACE1 cleaves APP in an acidic milieu. We also examined colocalization of BACE1 with EEA1, an early endosomal marker (**Fig. 6A (3)**). In contrast to Rab7, colocalization of BACE1 with EEA1 markedly increased in neurons treated with c9,t11 CLA relative to those treated with t10,c12 CLA or LA (**Fig. 6B (3)**). These observations suggest that membrane transport of BACE1 into late endosomes from early endosomes may be delayed, and that BACE1 tends to remain in early endosomes, resulting in weaker BACE1 activity under neutral pH.

We also analyzed the localization of APP with Rab7 and EEA1 (**Fig. 6A (4) and (5)**). Colocalization of APP with Rab7 decreased significantly in neurons treated with c9,t11 CLA, whereas colocalization of APP with EEA1 was greater in neurons treated with c9,t11 CLA than in those treated with t10,c12 CLA or LA (**Fig. 6B (4) and (5)**). These alterations were identical to those observed for BACE1. Overall, these findings imply that the decrease in colocalization of APP with BACE1 in late endosomes, where BACE1 actively cleaves APP, causes the reduction in Aβ generation in neurons treated with c9,t11 CLA.

Altered phospholipid ratio in neurons treated with CLA

The regulation of the transition from early to late endosomes and membrane protein trafficking into the late endosome from the early endosome is controversial, and the details of how APP

and BACE1 are regulated in such membrane trafficking remain unknown. In general, membrane lipid composition affects the localization and function of membrane proteins. Therefore, we used a reverse-phase LC/MS system to analyze phosphatidylcholine (PC), a major lipid constituent of the membrane, in neurons treated with LA or CLA. In neurons cultured in the presence of LA (18:2) or CLA (18:2), the peak of the corresponding PC species (*i.e.,* 18:2-containing PC species) increased (data not shown). However, we could not determine the level of PC-containing CLA because CLA- and LA-containing PC could not be satisfactorily separated by our reverse-phase LC. Therefore, we focused our analysis on lysophosphatidylcholine (LPC), which has only one acyl group at the *sn*-1 or *sn*-2 position of the glycerol backbone (**Fig. 1C**).

In neurons treated with DMSO (vehicle), we observed two peaks of LA-LPC corresponding to 1-acyl-LPC (sn-1-LA-LPC) and 2-acyl-LPC (sn-2-LA-LPC) (**Okudaira et al., 2014**) (**Supplementary Figure S4A**). The levels of both 1-acyl-LPC and 2-acyl-LPC were significantly higher in cells treated with LA than in those treated with control DMSO (**Supplementary Figure S4B**), suggesting that exogenous LA is incorporated into LPC. In cells treated with c9,t11 CLA or c10,t12 CLA, we detected an extra peak with a delayed retention time (**Supplementary Figure S4A**), which was identified as 1-acyl-CLA-LPC (sn-1-CLA-LPC) (**Fujita et al., 2021**). The levels of sn-1-c9,t11 CLA and sn-1-t10,c12 CLA were significantly higher in cells treated with CLA than in those treated with control DMSO (**Supplementary Figure S4B**). Unfortunately, the peak of sn-2-CLA-LPC overlapped with that of sn-1-LA-LPC in this procedure.

To confirm that CLA was incorporated into PC and changed its composition, we isolated PC from membrane lipid extract of neurons treated with LA, CLA, or DMSO, and then reacted it with phospholipase 1 (PLA_1) (**Fig. 7A**) or phospholipase 2 (PLA_2) (**Fig. 7B**), yielding 2-acyl-LPC (sn-2-LPC) or 1-acyl-LPC (sn-1-LPC), respectively. The 2-acyl-LPC (sn-2-LPC) was further converted into 1-acyl-LPC (sn-1-LPC) and analyzed as 1-acyl-LPC (**Fig. 7A**), as 2-acyl-CLA-LPC could not be separated from 1-acyl-LA-LPC (**Supplementary Figure S4A**). This comparative study showed once again that the level of LA-LPC was significantly higher in cells treated with LA than in those treated with control DMSO (see 18:2 (LA) on the left of **Fig. 7A and B**; the amount of 18:2 (LA) differed significantly between neurons treated with LA and those treated with DMSO ($p<0.01$ in panel A and $p<0.0001$ in panel B)). Similarly, the levels of 2-acyl-CLA-LPC (quantified as converted 1-acyl-LPC in the right panel in **Fig. 7A**) and 1-acyl-CLA-LPC (right in **Fig. 7B**) were remarkably elevated in cells treated with c9,t11 CLA or t10,c12 CLA (enlarged in the right of panels A and B), indicating that exogenous CLA, as well as LA, can be incorporated into membrane PC. In general, polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4) and docosahexaenoic acid (22:6), are preferentially incorporated at the *sn-2* position of phospholipids (**Fig. 1B**), as confirmed by the observation that LPC with PUFA was barely detected in the PLA2 reaction (**Fig. 7B**). It should be noted that CLA-LPC and LA-LPC were present at significant levels in both the PLA_1 and PLA_2

reactions, suggesting that CLA, as well as LA, can be incorporated in either the sn-1 or the sn-2 position of PC in neurons.

Discussion

In this study, we examined the effect of CLA on Aβ generation in primary cultured neurons. Many previous studies aimed at exploring the biological activities of CLA *in vivo* and *in vitro* used an isomeric mixture consisting largely of c9,t11 CLA and t10,c12 CLA (~50:50 ratio with several minor isomers) (**Bhattacharya et al., 2006; den Hartigh 2019**). By contrast, we used highly purified (>95%) c9,t11 CLA and t10,c12 CLA as well as pure mouse neurons.

We found that c9,t11 CLA, but neither t10,c12 CLA nor LA, decreased Aβ generation in WT mouse neurons and mouse neurons expressing humanized Aβ from the mouse *App* gene. This was not due to direct suppression of Aβ-generating enzymes (BACE1 and γ-secretase complex) or attenuated expression of these proteins in neurons treated with c9,t11 CLA. Alternatively, we observed reduced localization of APP and BACE1 in late endosomes/lysosomes with the Rab7 marker in which APP is subject to amyloidogenic primary processing by BACE1 in neurons treated with c9,t11 CLA, but not in neurons treated with t10,c12 CLA. Furthermore, we demonstrated that both isomers of CLA were incorporated into glycerophospholipids such as PC. Neuronal membranes containing c9,t11 CLA-glycerophospholipids may modify membrane protein trafficking in a manner distinct from membranes containing LA- or t10,c12 CLA-glycerophospholipids. Overall, colocalization of APP with BACE1 significantly decreased in neurons. We could not determine whether colocalization of N-terminal truncated active BACE1 with mature APP decreased, as non-active BACE1 with pro-peptide and immature APP (which is not subject to proteolytic processing) are located throughout the early secretory pathway of membrane compartments. At a minimum, however, the localization of APP and BACE1 in early and late endosomes involves mature APP and active BACE1. Therefore, we consider that the reduced localization of APP with BACE1 in late endosome may significantly contribute to the decrease in production of Aβ.

Another Aβ-generating enzyme, the γ-secretase complex, also consists of membrane proteins, and its activity is also influenced by membrane lipid composition (**Quintero-Monzon et al., 2011; Holmes et al., 2012; Hata et al., 2020; Kakuda et al., 2020**). However, we could not detect alteration of γ-secretase activities in neurons treated with LA or CLA. Therefore, we did not analyze whether the localization of γ-secretase complex was altered in neurons treated with these compounds. The complexity of this enzyme was another factor: the γ-secretase complex comprises four components, presenilin 1 or 2 (PS1 or PS2), nicastrin (NCT), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2), which are all membrane proteins. Furthermore, in the active γ-secretase complex, PS1 or PS2 are cleaved, and the presence of both the N- and C-terminal fragments in the complex is essential for activity (**Steiner et al., 2008**). Although γ-secretase activity in neurons was not influenced with LA and

CLA, we cannot rule out the possibility that the membrane localization of some γ-secretase components may have been altered.

We and others have shown that the activities of CLA are also beneficial to the central nervous system and neurons (**Fa et al., 2005**). Notably in this regard, the level of CLA-LPC is significantly elevated in the brain of mice fed a diet containing c9,t11 CLA (**Fujita et al., 2021**). Here, we showed that CLA was incorporated into PC and was present as CLA-PC/LPC in the neuronal membrane. In general, CLA-LPC constituted less than 1% of total LPC in the neuronal membrane, but the level significantly increased in neurons treated with CLA. Although almost identical levels of c9,t11 CLA and t10,c12 CLA were incorporated into PC of membranes, the structure of c9, t11 CLA-PC/LPC may differ from that of t10,c12 CLA-PC/LPC, and may influence interactions with membrane proteins. Overall, our findings indicate that membranes containing c9,t11 CLA-glycerophospholipid are likely to confine APP and BACE1 to early endosomes, decrease amyloidogenic cleavage of APP by BACE1 in late endosomes, and decrease Aβ generation in the neuron.

A diet containing CLA increases PLA_2 expression and activity in the rat hippocampus, which correlates with memory improvement (**Gama et al., 2015**). In conjunction with our findings, this observation suggests that elevated levels of PLA_2 contribute to the formation of CLA-glycerophospholipids. Furthermore, in a mouse model of neuropsychiatric lupus, CLA supplementation suppressed age-associated neuronal damage *in vivo* (**Monaco et al., 2018**), and c9,t11 CLA increased the proliferation of neural progenitor cells *in vitro* (**Wang et al., 2011**). Administration of punicic acid, from which CLA is generated as a metabolite, preserves memory and decreases Aβ accumulation in a mouse model of AD (**Binyamin et al., 2019**). Our findings and evidence obtained by others strongly support the idea that among the common CLA isomers, c9, t11CLA may prevent neurodegenerative disease in aged populations. The most common age-associated neurodegenerative disease with dementia is AD, and the neurotoxicity of Aβ oligomers is thought to trigger the impairment of neurons. Therefore, c9,t11 CLA may be effective against neurological diseases such as AD.

In a separate study in which an AD mouse model was fed c9,t11 CLA, c9,t11 CLA-LPC was detected in brain membranes, concomitant with a reduction in amyloid quantity (**Fujita et al., 2021**). It remains unclear whether the biological effects of c9,t11 CLA observed *in vivo* are due to the same effect observed in cultured neurons in this study. Nonetheless, taken together with Fujita's report, our results indicate that intake of high-purity c9,t11 CLA protects neurons in aged subjects from injury by toxic Aβ.

Author Contributions

T.S., Y.S., J.A., H.K., T.T., S.N., and T.S. participated in the study design. S.H., K.K., H.S., and S.K. performed biochemical studies with neuron and glia. H.S., Y.S., and H.T. performed immunocytochemical studies and statistical analysis. K.K. and J.A. performed LC-MS/MS analysis. T.S., Y.S., and T.S. prepared animal studies. S.H., H.T., J.A., T.T., and T.S. wrote the paper. All authors read and approved the final manuscript.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Figure S1. Aβ peptides in medium generated from primary cultured neurons treated with various concentrations of LA and CLA.

Supplementary Figure S2. Aβ clearance in medium of glia treated with LA and CLA.

Supplementary Figure S3. γ-Secretase activity in the membrane of neurons treated with LA and CLA.

Supplementary Figure S4. LC-MS/MS analysis of LPC in neurons treated with LA and CLA.

Supplementary Table 1. Purchased antibodies used in this study.

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Figure legends

Figure 1. Structures of linoleic acid, conjugated linoleic acid, and a typical glycerophospholipid with lysophospholipids.

(A) Structure of linoleic acid (LA, 18:2) and the major isomers of conjugated linoleic acid, *cis* 9, *trans*11 CLA (c9,t11 CLA) and *trans* 10, *cis*12 CLA (t10,c12 CLA), used in this study.

(B) Structure of a typical glycerophospholipid. The glycerol backbone structure of glycerophospholipid is shown in *yellow.* In general, the sn-1 position contains a saturated fatty acid (SFA) or monounsaturated fatty acid (MUFA), whereas the sn-2 position contains a MUFA or polyunsaturated fatty acid (PUFA). In some glycerophospholipids, MUFA and PUFA are linked at both the sn-1 and sn-2 positions. *X* indicates the headgroup.

(C) Structure of lysophospholipid. Lysophospholipid (in this case, phosphatidylcholine or PC) lacks fatty acid at either the sn-1 or sn-2 position due to diacylation of the phospholipid. In neurons treated with CLA, the unsaturated fatty acids c9,t11 CLA and t10,c12 CLA are detected at both the sn-1 and sn-2 positions (see **Figure 7**). Glycerophospholipid structures were modified from a review (**Yamashita et al., 2014**).

Figure 2. Aβ generation and viability of neurons treated with LA and CLA.

(**A, B**) Aβ peptides in medium generated from primary cultured neurons derived from hippocampus and cortex of wild-type (**A**) and *App*^NL-F/NL-F^ (**B**) mouse embryos. (**A**) Neurons of wild-type mice were cultured for 7−10 days (DIV 7-10, 5×10^5 neurons). Neurons were cultured for an additional 24 h in a fresh medium containing 10 μM LA, c9,t11 CLA, or t10,c12 CLA, and endogenous mouse Aβ40 in the medium was quantified. (**B**) Neurons of APP-KI (*App*^NL-F/NL-F^) mice were cultured for 14–17 days (DIV 14–17, 5×10^5 neurons). After the media was replaced, the cells were cultured for an additional 48 h as described above, and human Aβ42 in the medium was quantified. Aβ levels are indicated as ratios relative to the level of Aβ generated by neurons treated with vehicle alone (DMSO), which was assigned a reference value of 1.0. (**C, D**) Cell viability and neurotoxicity. Neurons of wild-type mice were cultured for 12 days (DIV 12, 2×10^4 neurons). Neurons were further cultured for 24 h in a fresh medium containing the indicated concentrations (μM) of LA, c9,t11 CLA, or t10,c12 CLA, and cell viability (**C**) and cell toxicity (**D**) were examined. Minus (-) indicates vehicle (DMSO). Conditioned media for Aβ quantification and cell viability tests with the indicated cell numbers were performed.   The results are those of two to four independent cell culture preparations with two to six cell cultures in another well of plates per preparation. All values of the biological replicates were combined and subjected to statistical analysis. Statistical analysis of results (numbers inside circles denote number of experiments) was performed by Dunnett's multiple comparison test. P-values are provided for comparison with neurons treated with vehicle alone (-) (means ± S.D., n=12–19 (**A**), n=5–8 (**B**), n=12 (**C**), n=9, (**D**); *, p<0.05 ; **, p<0.01).

Figure 3. Aβ clearance from the medium by neurons and Aβ levels in neurons treated with LA and CLA

Neurons of wild-type mice were cultured for 10 to 12 days (DIV10–12, 1.4×10^5 neurons), and further cultured in fresh medium including 10 μM LA, c9,t11 CLA, and t10,c12 CLA, or vesicle alone (DMSO) with human Aβ40 (10 nM [~17 ng]) for 24 h. 'Cell-free' indicates medium containing Aβ40 in the absence of neurons. **(A)** Levels of Aβ40 in the medium were quantified by human Aβ40-specific sELISA, and the remaining Aβ40 in the medium is indicated as a yield. The levels of Aβ40 remaining in the medium of neurons treated with LA and CLA were compared with the value of neurons treated with vehicle (DMSO). Independent cell culture preparations were performed three times with three to five cell cultures in another well of plates per preparation. All values were combined and subjected to statistical analysis with the indicated numbers of independent biological replicates (numbers inside circles denote number of experiments). No significant differences were observed between vehicle control and LA, c9,t11 CLA or t10,c12 CLA (Dunnett's multiple comparison test), except that the Aβ40 level was higher in medium without neurons. P-values (****, $p < 0.0001$) are shown for comparison between LA, c9,t11 CLA, or t10,c12 CLA and cell-free media (means ± S.D., n=10–14).

(B) Levels of Aβ40 in neuronal lysates were quantified as described above. Independent cell culture preparations were performed three times with three to four cell cultures in another well of plates per preparations. All values were combined and subject to statistical analysis with the indicated number of independent biological replicates (numbers inside circles denote number of experiments). No significance was detected in the comparisons between LA, c9,t11 CLA or t10,c12 CLA and DMSO (vehicle alone) (means ± S.D., n=11).

Figure 4. Protein expression of neurons treated with LA and CLA.

Immunoblotting **(A)** and quantification **(B–D)** of protein levels of APP, BACE1, PS1 NTF, and α-tubulin in neurons treated with LA, c9,t11 CLA, and t10,c12 CLA. Primary cultured neurons derived from hippocampus and cortex of wild-type mouse embryos (DIV 10–12, 5×10^5 neurons) were further cultured for 24 h with 10 μM LA, c9,t11 CLA, and t10,c12 CLA. These neurons were lysed, and the lysate (15 μg protein) was analyzed by immunoblotting with anti-APP, BACE1, PS1 NTF, and α-tubulin antibodies. The numbers in panel A indicate molecular size (kDa). The densities of each protein band were quantified and normalized against the density of α-tubulin. The levels of protein in cells treated with CLA were compared with the level in cells treated with LA, which was assigned a reference value of 1.0. Independent western blots were performed three times with two or three cell cultures in another well of plates per preparations. All values were combined and subject to statistical analysis with the indicated number of independent biological replicates (numbers inside circles denote number of experiments). Statistical analysis was performed using Dunnett's multiple comparison test; no significant difference was detected (means ± S.D., n=8).

Figure 5. BACE1 activity in the lysate of neurons treated with LA and CLA, and activity of recombinant human BACE1 in the presence of LA and CLA.

(**A**) Primary cultured neurons derived from hippocampus and cortex of wild-type mouse embryos (DIV 9–12, 4×10^6 neurons) were cultured for 24 h with 10 μM LA, c9,t11 CLA, or t10,c12 CLA. BACE1 activity in lysates (100 μg protein) of neurons was examined, and activities of neurons treated with CLA are indicated as ratios relative to the activity of neurons treated with LA, which was assigned a reference value of 1.0. Independent cell cultures were performed four times with two to four cell cultures in another well of plates per preparations. All values were combined and subjected to statistical analysis with the indicated number of independent biological replicates (numbers inside circles denote number of experiments). Statistical analysis was performed using Dunnett's multiple comparison test. P-values are provided for the comparison with neurons treated with LA (means ± S.D., n=11; *, $p < 0.05$).

(**B**) Human recombinant BACE1 (rBACE1) was assayed in the presence or absence of 10 μM LA, c9,t11 CLA, or t10,c12 CLA. The activity of rBACE1 in the absence of LA and CLA (DMSO) was assigned a reference value of 1.0, and the relative activities of rBACE1 in the presence of LA and CLA are indicated as relative ratios. Independent enzyme assays were performed three times with one or two mixtures. All values were combined and subjected to statistical analysis with the indicated number of independent biological replicates (numbers inside circles denote number of experiments). Statistical analysis was performed using Dunnett's multiple comparison test. P-values are provided for the comparison with rBACE1 without LA and CLA (means ± S.D., n=5; **, $p < 0.01$; ***, $p < 0.001$).

Figure 6. Colocalization of BACE1 and APP with Rab7 and EEA1 in neurons treated with LA and CLA.

(**A**) Immunostaining. Primary cultured neurons derived from hippocampus and cortex of wild-type mouse embryos (DIV 10–12, 2×10^4 neurons) were cultured for 24 h with 10 μM LA (first row), c9,t11 CLA (second row), or t10,c12 CLA (third row), and subjected to immunostaining with a combination of the indicated antibodies. (1) Mouse anti-APP (green) and rabbit anti-BACE1 (red) antibodies. (2) Rabbit anti-BACE1 (red) and mouse anti-Rab7 (green) antibodies. (3) Rabbit anti-BACE1 (red) and mouse anti-EEA1 (green) antibodies. (4) Rabbit anti-APP (red) and mouse anti-Rab7 (green) antibodies. (5) Rabbit anti-APP (red) and mouse anti-EEA1 (green) antibodies. Scale bar, 10 μm.

(**B**) Colocalization efficiency. The colocalization rate of proteins was calculated from each frame of images (15,750 μm^2) of neurons and is indicated as Pearson's coefficient. Independent cell stainings were performed two or three times per cell preparation, and four to seven frames were acquired from each well. All values were combined and subject to statistical analysis with the indicated number of independent biological repeats (numbers inside circles denote number of experiments). (1) Colocalization of BACE1 with APP. (2) Colocalization of BACE1 with Rab7. (3) Colocalization of BACE1 with EEA1. (4) Colocalization of APP with Rab7. (5)
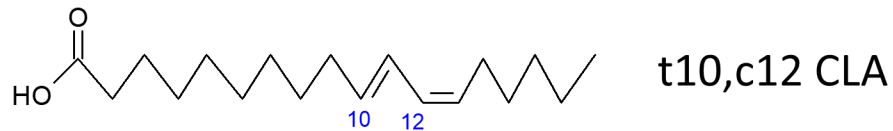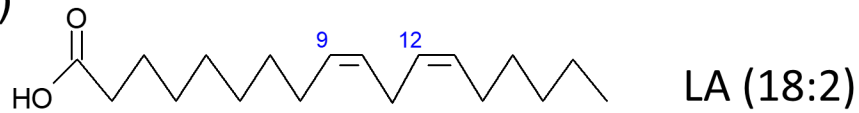
Colocalization of APP with EEA1. Statistical analysis was performed using Dunnett's multiple comparison test. P-values are provided for the comparison with LA treatment (means ± S.D., n=8–20; *, p<0.05; ****, p<0.0001).

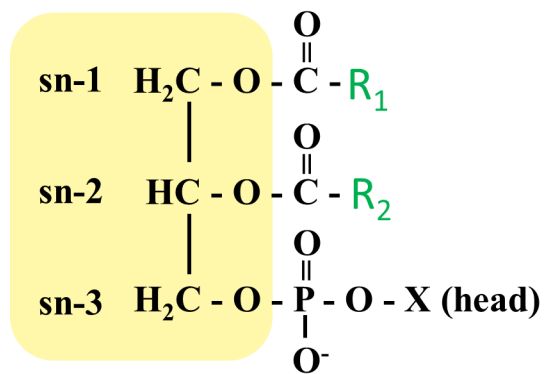**Figure 7. LC-MS/MS analysis of PC-derived LPC in neurons treated with LA and CLA.** Primary cultured neurons derived from the hippocampus and cortex of wild-type mouse embryos (DIV 10–12, 4×10^6 neurons) were cultured for 24 h with 10 µM LA (yellow column), c9,t11 CLA (blue column), or t10,c12 CLA (red column), and vehicle alone (DMSO, white column). Lipids were extracted from neurons, and PC fraction was prepared by TLC. The PC fraction was treated with PLA_1 to generate sn-2-LPC, followed by treatment of the spontaneous acyl-migration to generate sn-1-LPC (A). The PC fraction was also treated with PLA_2 to generate sn-1-LPC (B). Generated sn-1-LPC were subjected to analysis with LC-MS/MS.
(A) Comparison of LPC with fatty acid at sn-1 position (1-acyl LPC, left), which reflects the levels of sn-2-LPC before treatment for spontaneous acyl-migration. The ratios of CLA-LPC are enlarged (right). **(B)** Comparison of LPC with fatty acid at the sn-1 position (1-acyl LPC, left), which reflects the levels of sn-1-LPC. The ratios of CLA-LPC are enlarged (right). Amounts are indicated as percent of total LPC. Three independent MS analysis were performed. All values were combined and subject to statistical analysis with the indicated number of independent technical replicates (numbers inside circles denote number of experiments). Statistical analysis was performed using Dunnett's multiple comparison test. P-values are provided for the comparison with DMSO control (means ± S.D., n= 3; **, p<0.01; ****, p<0.0001).
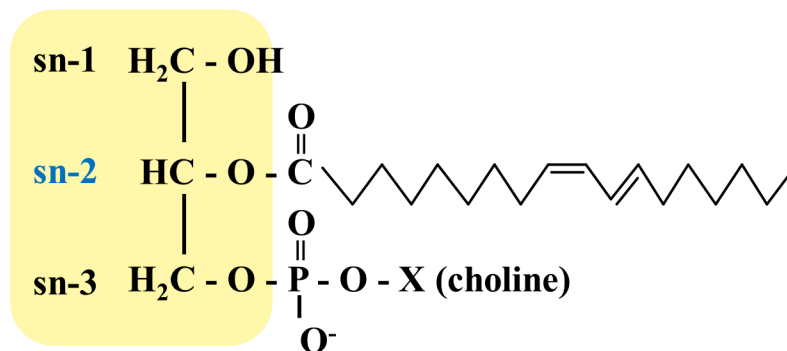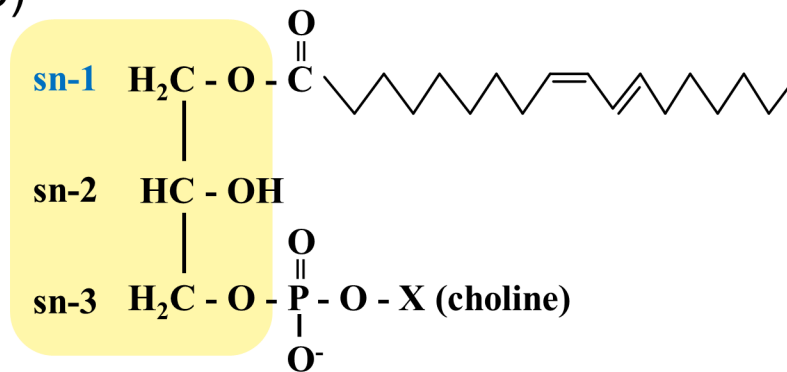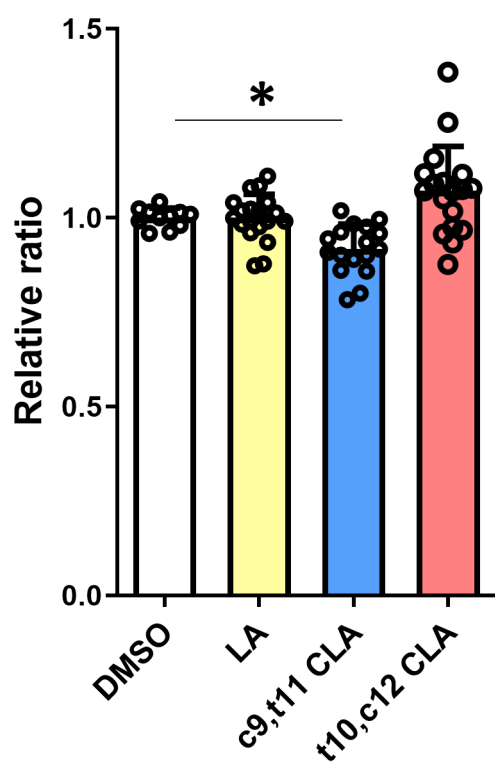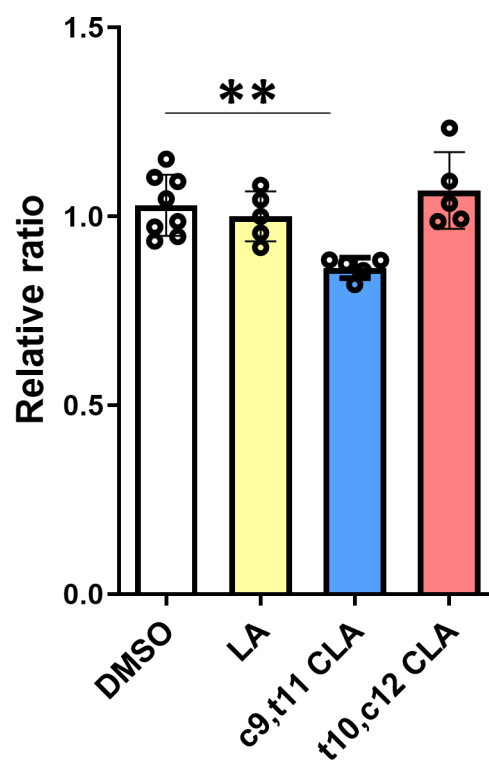
(A)

LA (18:2)

c9,t11 CLA

t10,c12 CLA

(B)

sn-1 $H_2C - O - \overset{\displaystyle O}{\overset{\displaystyle \|}{C}} - R_1$

sn-2 $HC - O - \overset{\displaystyle O}{\overset{\displaystyle \|}{C}} - R_2$

sn-3 $H_2C - O - \overset{\displaystyle O}{\underset{\displaystyle O^-}{\overset{\displaystyle \|}{P}}} - O - X \text{ (head)}$

(C)

sn-1 $H_2C - O - \overset{\displaystyle O}{\overset{\displaystyle \|}{C}} \sim\!\!\sim\!\!\sim$

sn-2 $HC - OH$

sn-3 $H_2C - O - \overset{\displaystyle O}{\underset{\displaystyle O^-}{\overset{\displaystyle \|}{P}}} - O - X \text{ (choline)}$

sn-1 $H_2C - OH$

sn-2 $HC - O - \overset{\displaystyle O}{\overset{\displaystyle \|}{C}} \sim\!\!\sim\!\!\sim$

sn-3 $H_2C - O - \overset{\displaystyle O}{\underset{\displaystyle O^-}{\overset{\displaystyle \|}{P}}} - O - X \text{ (choline)}$

(A) Mouse Aβ40

(B) Humanized Aβ42

(C) MTT

(D) LDH

(A)

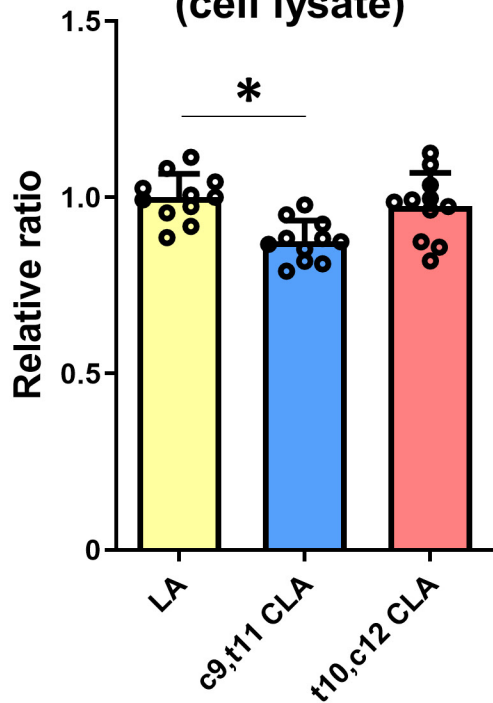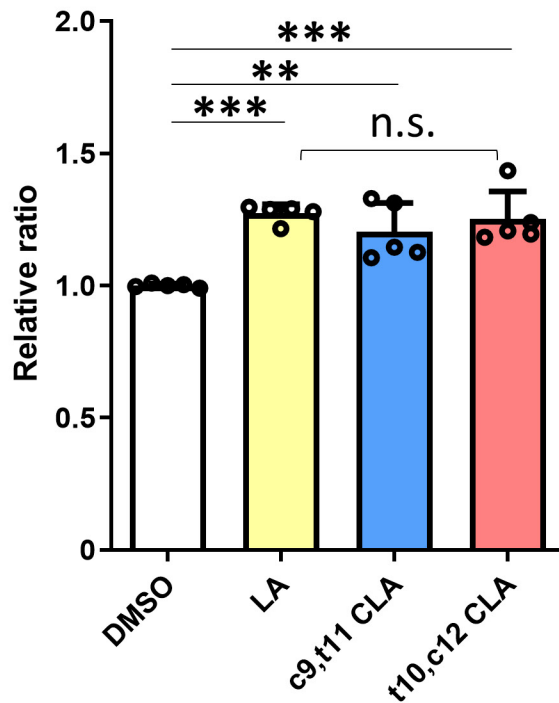| | LA | 9,11-CLA | 10,12-CLA | |
APP				116.25
BACE1				66.2
PS1 NTF				31
αTub				66.2
				45

(B) **APP**

(C) **BACE**

(D) **PS1**
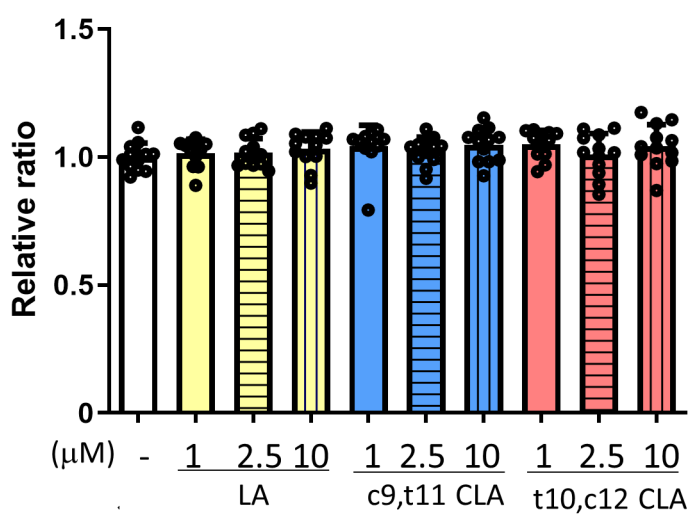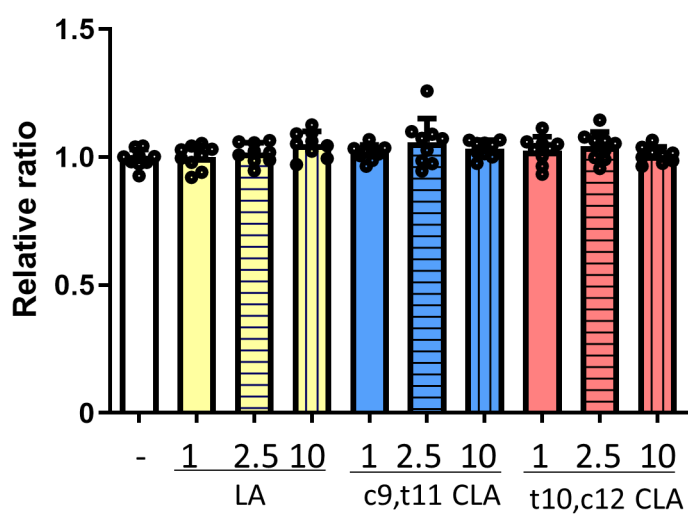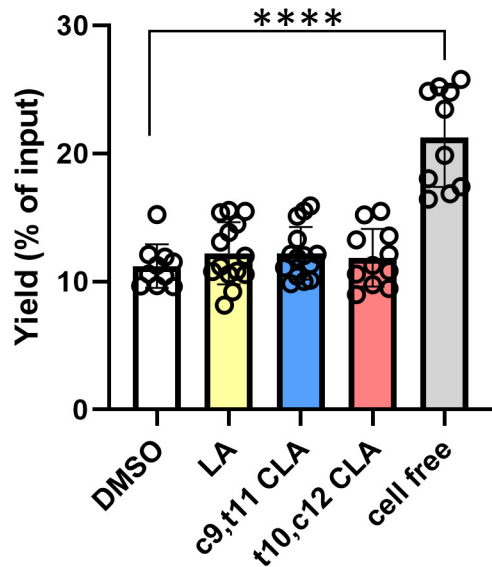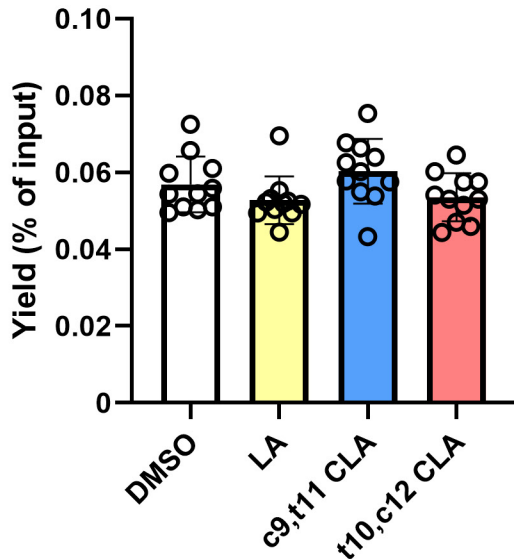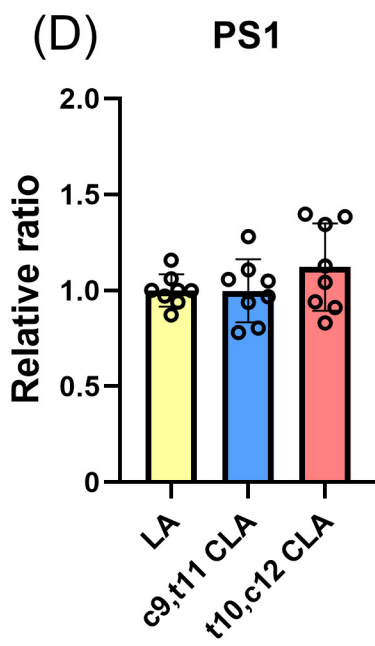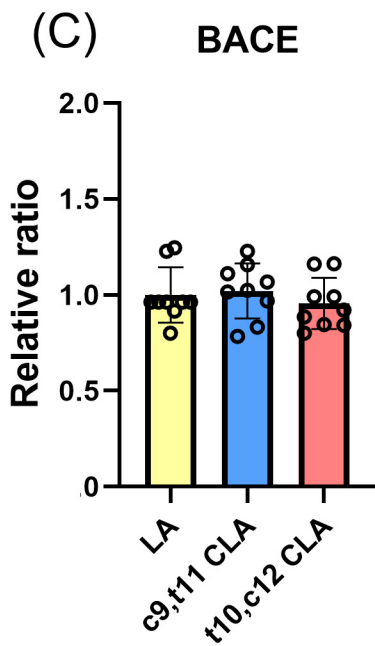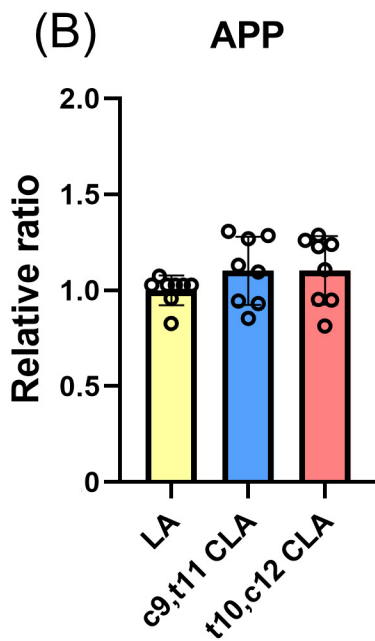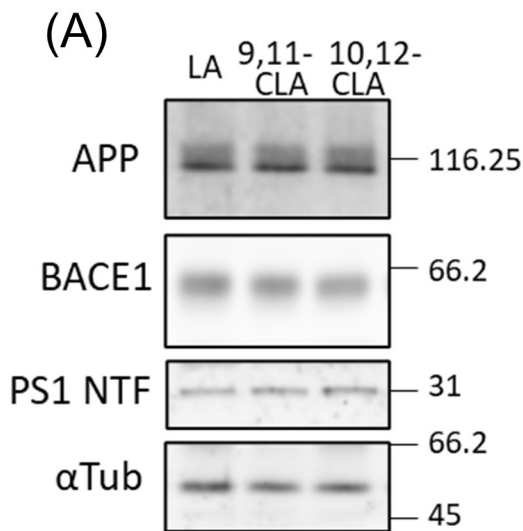
(A) BACE1 activity (cell lysate)

(B) rBACE1 activity

(A)

| | (1) APP / BACE1 | (2) BACE1 / Rab7 | (3) BACE1 / EEA1 | (4) APP / Rab7 | (5) APP / EEA1 |

Rows: LA, c9,t11 CLA, t10,c12 CLA

(B)

(1) APP/BACE1 — Pearson's R value
(2) BACE1/Rab7 — Pearson's R value
(3) BACE1/EEA1 — Pearson's R value
(4) APP/Rab7 — Pearson's R value
(5) APP/EEA1 — Pearson's R value

Legend:
LA
c9,t11 CLA
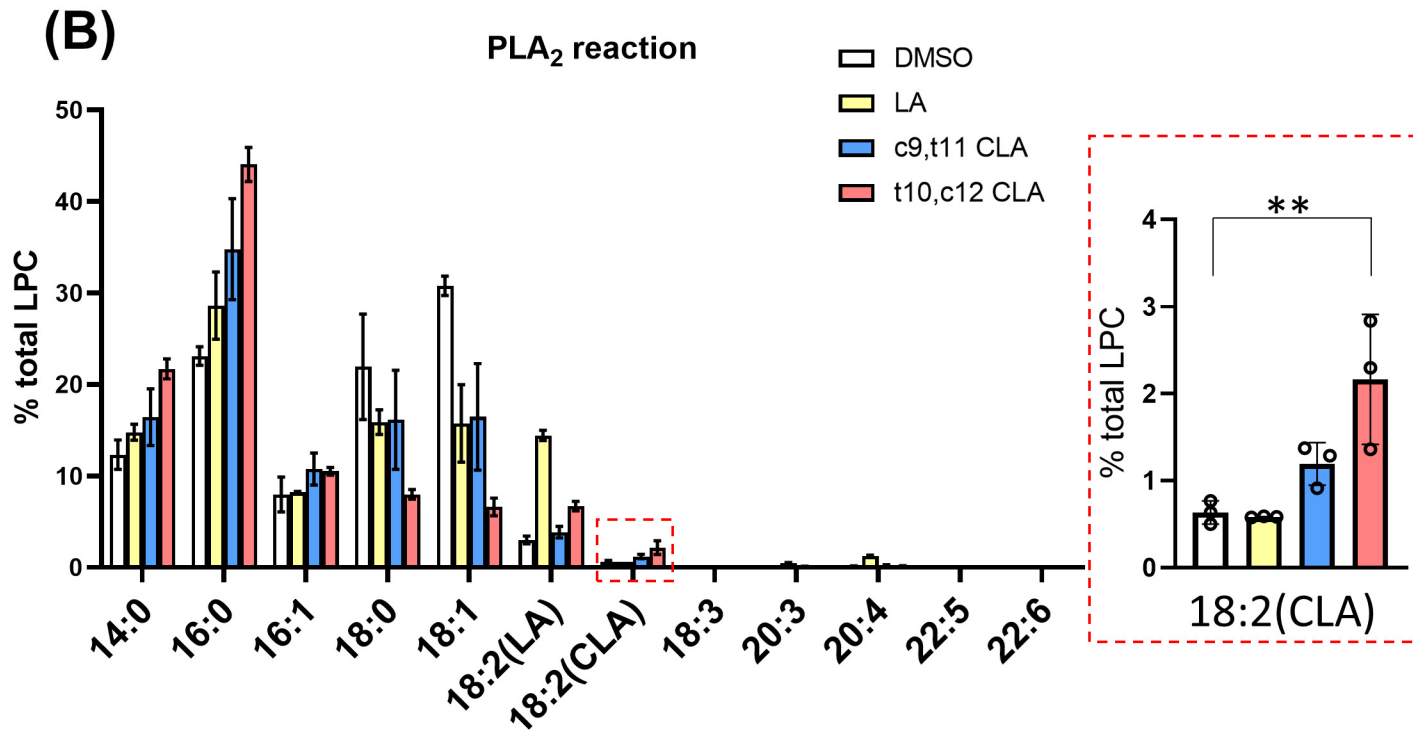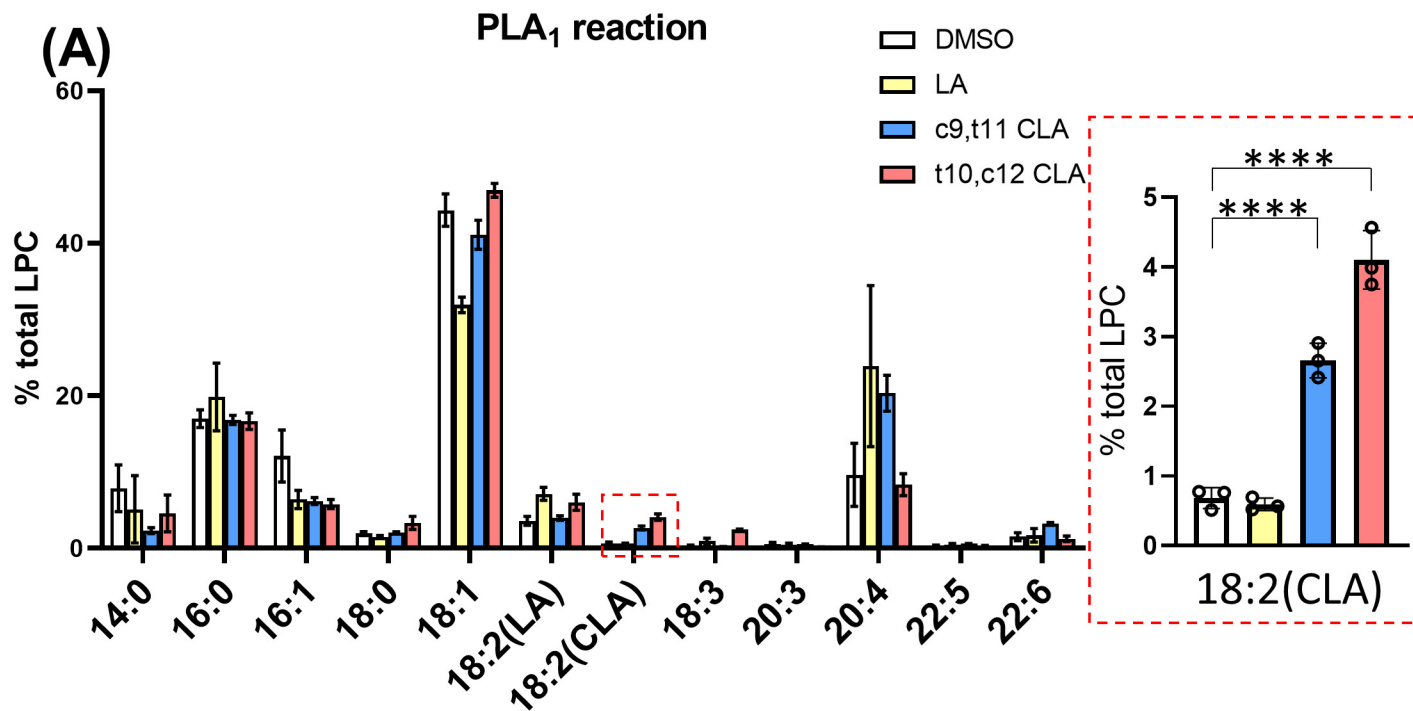t10,c12 CLA

(A) PLA$_1$ reaction

(B) PLA$_2$ reaction
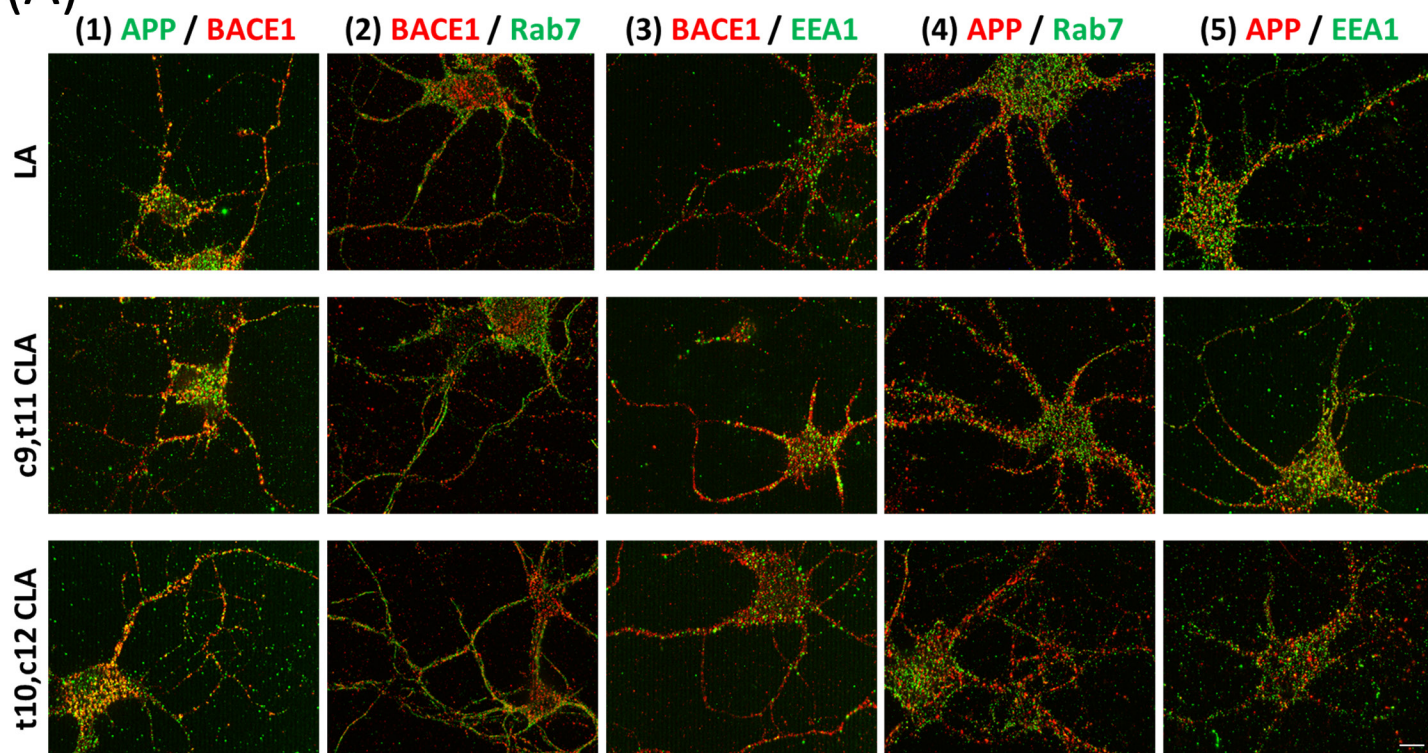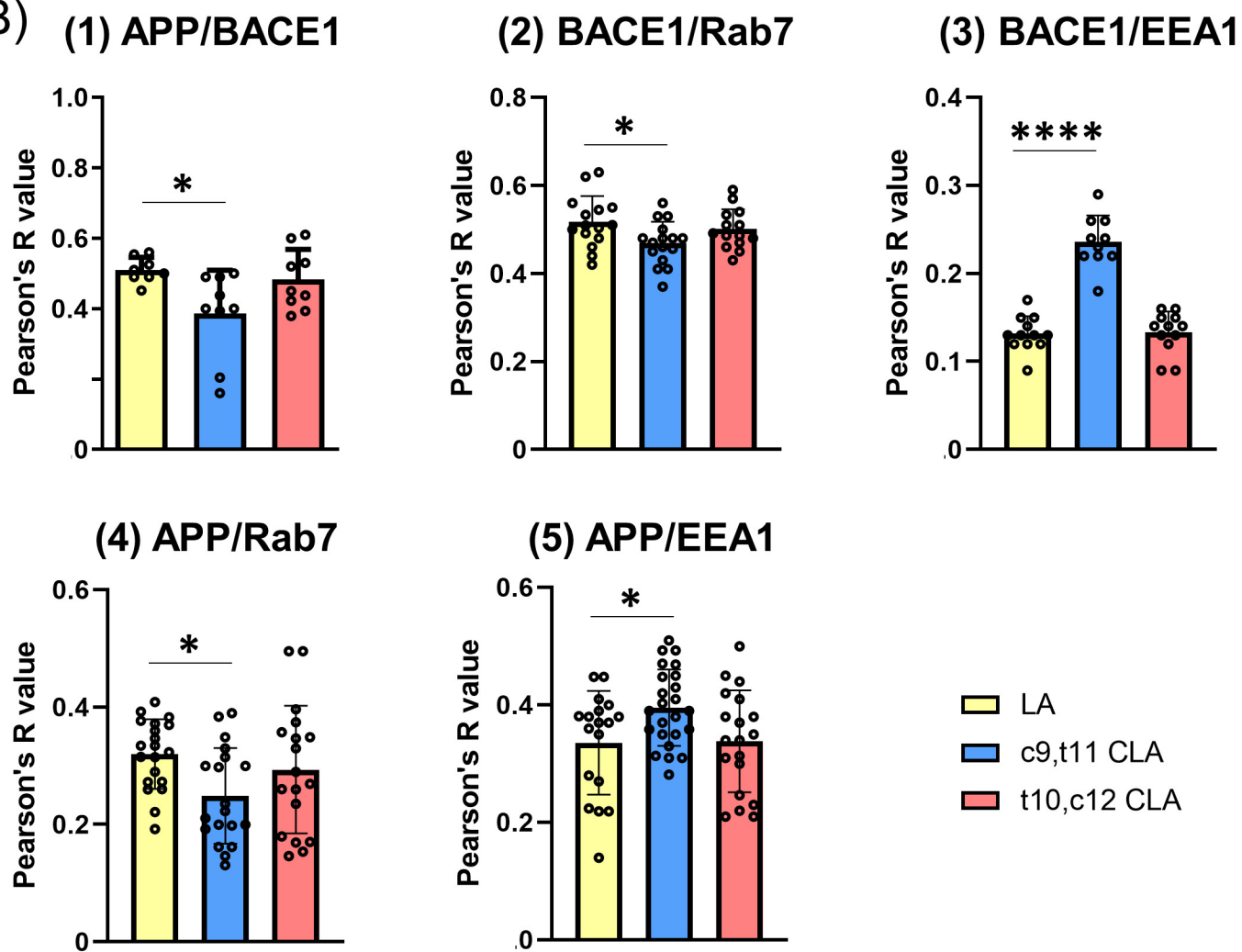
Supplementary Information

Suppression of amyloid-β secretion from neurons by *cis*-9, *trans*-11-octadecadienoic acid, an isomer of conjugated linoleic acid

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E-mail: tsuzuki@pharm.hokudai.ac.jp, Phone: +81-11-706-3250, Fax: +81-11-706-4991

Running title: Suppression of Aβ generation by CLA
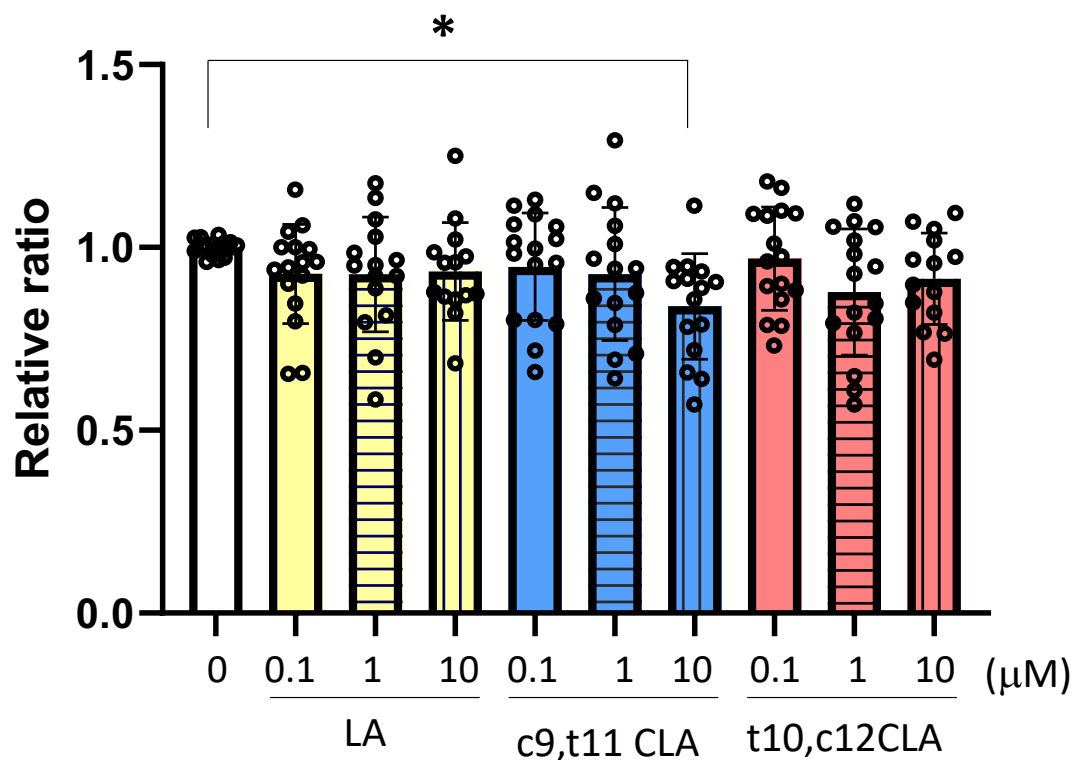
Supplementary Figure S1. Aβ peptides in medium generated from primary cultured neurons treated with various concentrations of LA and CLA.

Mouse hippocampus and cortex neurons were prepared from wild-type mouse embryos and cultured for 7−10 days (DIV 7−10, 1.4×10^5 neurons). Neurons were cultured for an additional 24 h in fresh medium containing the indicated concentrations (0.1, 1, or 10 μM) of LA, c9,t11 CLA, or t10,c12 CLA, or with vehicle alone (DMSO, 0 μM). Endogenous mouse Aβ40 in the medium was quantified. Aβ levels are indicated as ratios relative to the level of Aβ generated by neurons treated with vehicle alone (DMSO, 0 μM), which was assigned a reference value of 1.0. Statistical analysis was performed using Dunnett's multiple comparison test. P values are relative to neurons treated with vehicle alone. (means ± S.D., n=14−16; *, p<0.05).
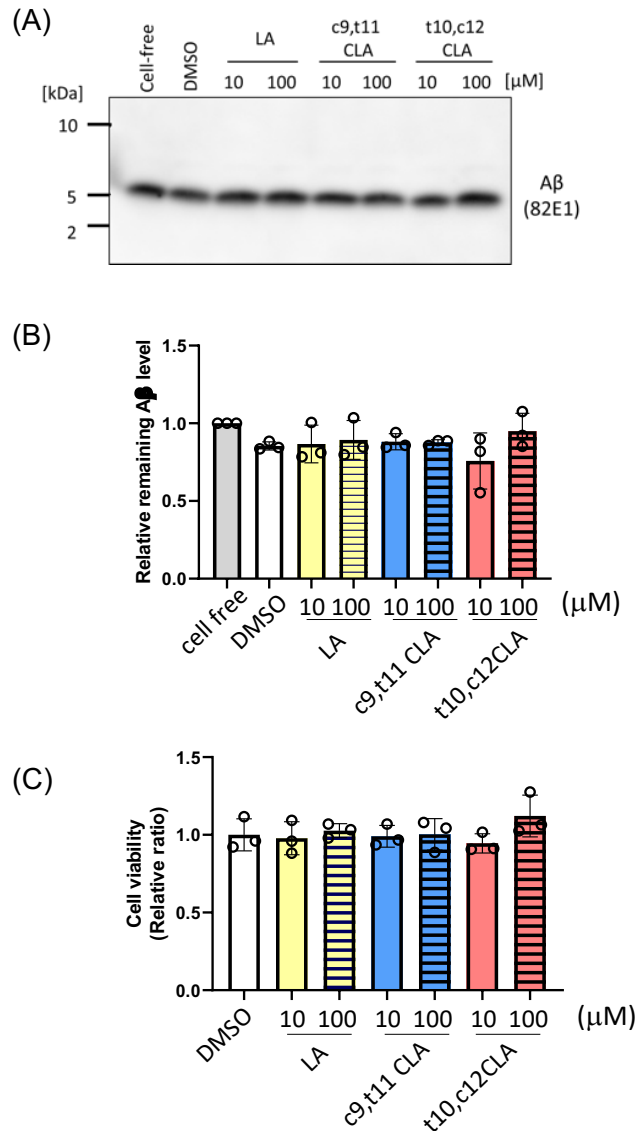
Supplementary Figure S2. Aβ clearance in medium of glia treated with LA and CLA.

Primary cultured glial cells were prepared from cortex of 1–2 day post-natal mouse brains and cultured for 15 days. Glial cells (largely astrocytes, 5×10^5 cells/well) of wild-type mice were cultured for 48 h in fresh medium containing 10 or 100 μM LA, c9,t11 CLA, or t10,c12 CLA with 20 nM Aβ40.

(A, B) Aβ in medium was quantified by immunoblotting with anti-human Aβ antibody (82E1). Band densities are indicated as ratios relative to the level of Aβ in medium in the absence of cells, which was assigned a reference value of 1.0. Numbers (panel A, left side) indicate molecular size (kDa).

(C) Cell viability was assayed by staining with Alamar blue (Cat. #DAL1025, Thermo Fisher Scientific, Waltham, MA. USA) and is indicated as the ratio relative to the level in cells without treatment (DMSO), which was assigned a reference value of 1.0. No significant difference was observed between LA, c9,t11 CLA, and t10,c12 CLA (Dunnett's multiple comparison test; n=3).
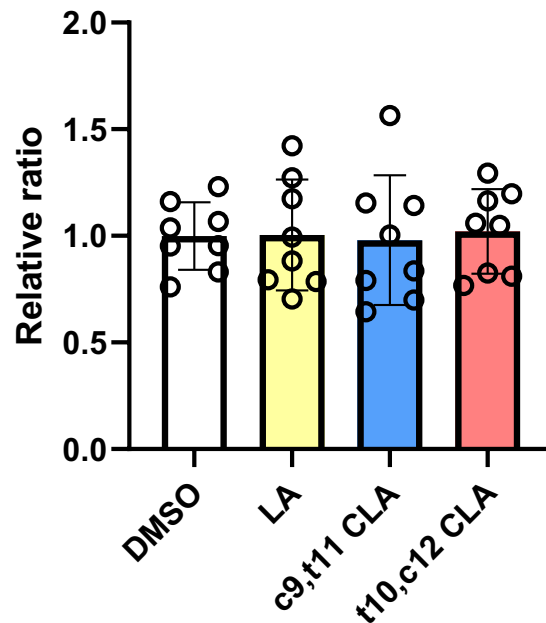
Supplementary Figure S3. γ-Secretase activity in membranes of neurons treated with LA and CLA.

Mouse hippocampus and cortex neurons were prepared from wild-type mouse embryos and cultured for 10–12 days (DIV 10–12, 4×10^6 neurons). Neurons were cultured for an additional 24 h in fresh medium containing 10 μM LA, c9,t11 CLA, or t10, c12 CLA or with vehicle alone (DMSO). Neurons were harvested, membrane fractions were prepared, and *in vitro* γ-secretase assays were performed. Mouse Aβ40 levels are indicated as ratios relative to the level in cells without treatment (DMSO), which was assigned a reference value of 1.0. No significant difference was observed between LA, c9, t11 CLA, and t10,c12 CLA (Dunnett's multiple comparison test; n=8).
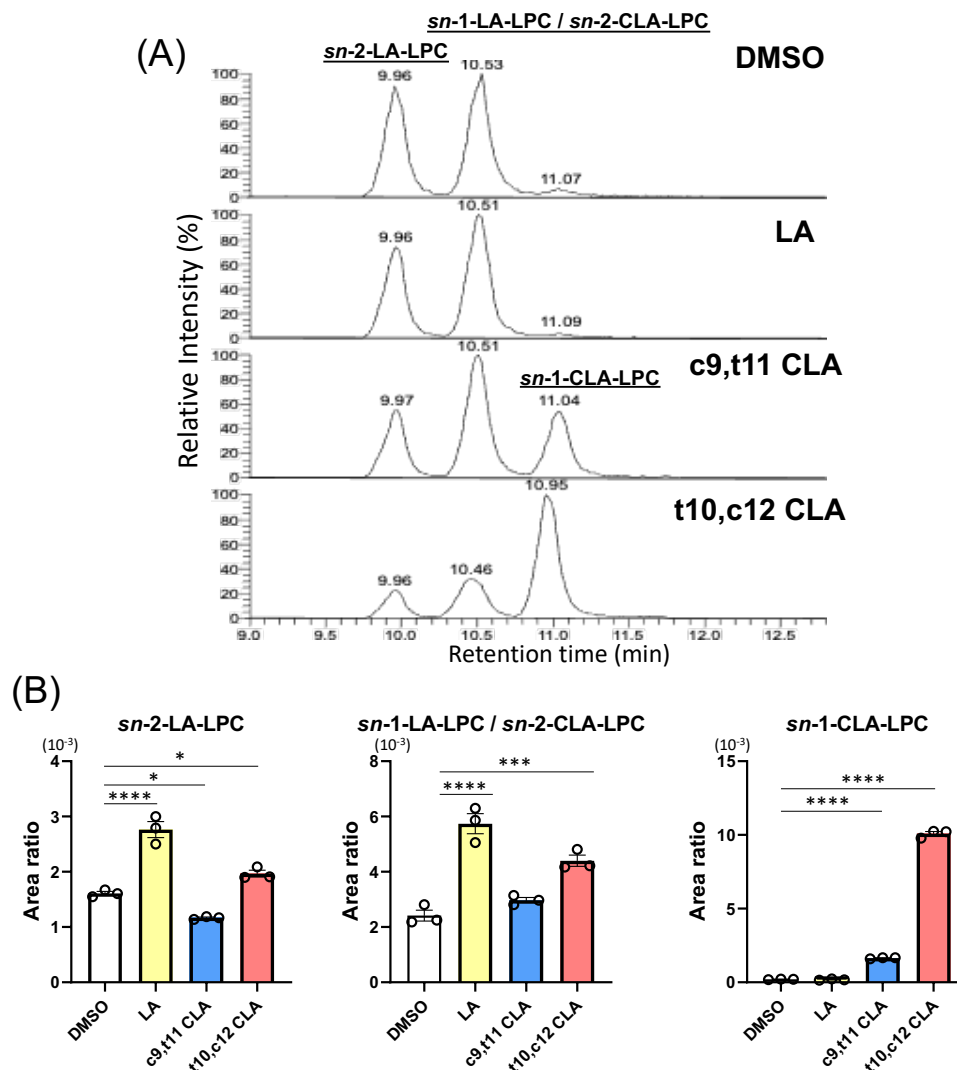
Supplementary Figure S4. LC-MS/MS analysis of LPC in neurons treated with LA and CLA.

(A) Representative elution profiles of sn-2-LA-LPC, sn-1-LA-LPC/sn-2-CLA-LPC, and sn-1-CLA-LPC. LPC from neurons treated with 10 μM linoleic acid (LA), c9,t11 CLA, or t10,c12 CLA, or with vehicle alone (DMSO) was analyzed by LC-MS/MS. sn-1-LA-LPC/sn-2-CLA-LPC indicates the mixture of 1-LA-LPC and 2-CLA-LPC.

(B) Comparison of sn-2-LA-LPC, sn-1-LA-LPC/sn-2-CLA-LPC, and sn-1-CLA-LPC levels in neurons treated with LA and CLA. Peak areas are calculated relative to the area of the internal standard, 17:0-LPC (1 μM), and are indicated as area ratios (1.0 for 17:0-LPC) relative to that of control neurons (DMSO). Statistical analysis was performed using Dunnett's multiple comparison test. P values are relative to the DMSO control (means ± S.E., n= 3; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, ****, $p<0.0001$).

Supplementary Table 1. Purchased antibodies used in this study

Antibody name	Supplier	Catalogue #	RRID	Host	Dilution
monoclonal anti-α-tubulin (10G10)	Fujifilm-Wako (Osaka, Japan)	017-25031	n/a	Mouse	1:2000
monoclonal anti-BACE1(D10E5)	Cell Signaling Technologies (Danvers, MA, USA)	5606	AB_1903900	Rabbit	1:500
monoclonal anti-EEA1 (clone 14)	BD Biosciences (San Jose, CA, USA)	610457	AB_397830	Mouse	1:500
polyclonal anti-EEA1 (H-300)	Santa Cruz Biotechnology, (Dallas, TX, USA)	c-33585	AB_2277714	Rabbit	1:500
monoclonal anti-Rab7	Abcam, (Cambridge, UK)	ab50533	AB_882241	Mouse	1:500
monoclonal anti-Rab7 (D95F2)	Cell Signaling Technologies (Danvers, MA, USA)	9367	AB_1904103	Rabbit	1:500
monoclonal anti-APP (22C11)	Merck Millipore/Sigma, (Burlington, MA, USA)	Mab348	AB_94882	Mouse	1:500
Alexa Fluor 488-conjugated donkey anti-mouse IgG	Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA)	A-21202	AB_141607	Donkey	1:500
Alexa Fluor 546-conjugated goat anti-mouse IgG	Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA)	A-11003	AB_2534071	Goat	1:500
Alexa Fluor 488-conjugated donkey anti-rabbit IgG	Abcam (Cambridge, UK)	ab150065	n/a	Donkey	1:500
1. Alexa Fluor 54-conjugated goat anti-rabbit IgG	Invitrogen/Thermo Fisher Scientific (Waltham, MA, USA)	A-11010	AB_2534077	Goat	1:500
Fab' fragment of rabbit polyclonal anti-mouse Aβ(1-16) IgG conjugated with horseradish peroxidase	IBL (Fujioka, Japan)	27720	n/a	Rabbit	1:30
Biotinylated mouse monoclonal anti-human Aβ(1–16) IgG 82E1	IBL (Fujioka, Japan)	10326	AB_1630806	Mouse	0.05 μg/mL