Cholesterol-mediated Neurite Outgrowth Is Differently Regulated between Cortical and Hippocampal Neurons*\textsuperscript{S}

Mihee Ko, Kun Zou, Hirohisa Minagawa, Wenxin Yu, Jian-Sheng Gong, Katsuhiko Yanagisawa, and Makoto Michikawa\textsuperscript{1}

From the Department of Alzheimer’s Disease Research, National Institute for Longevity Sciences, 36-3 Gengo, Morigoka, Obu, Aichi 474-8522, Japan

The acquisition of neuronal type-specific morphogenesis is a central feature of neuronal differentiation and has important consequences for region-specific nervous system functions. Here, we report that the cell type-specific cholesterol profile determines the differential modulation of axon and dendrite outgrowths in hippocampal and cerebral cortical neurons in culture. The extent of axon and dendrite outgrowths is greater and the polarity formation occurs earlier in cortical neurons than in hippocampal neurons. The cholesterol concentrations in total homogenate and the lipid rafts from hippocampal neurons are significantly higher than those from cortical neurons. Cholesterol depletion by \( \beta \)-cyclodextrin markedly enhanced the neurite outgrowth and accelerated the establishment of neuronal polarity in hippocampal neurons, which were similarly observed in nontreated cortical neurons, whereas cholesterol loading had no effects. In contrast, both depletion and loading of cholesterol decreased the neurite outgrowths in cortical neurons. The stimulation of neurite outgrowth and polarity formation induced by cholesterol depletion was accompanied by an enhanced localization of Fyn, a Src kinase, in the lipid rafts of hippocampal neurons. A concomitant treatment with \( \beta \)-cyclodextrin and a Src family kinase inhibitor, PP2, specifically blocked axon outgrowth but not dendrite outgrowth (both of which were enhanced by \( \beta \)-cyclodextrin) in hippocampal neurons, suggesting that axon outgrowth modulated by cholesterol is induced in a Fyn-dependent manner. These results suggest that cellular cholesterol modulates axon and dendrite outgrowths and neuronal polarization under culture conditions and also that the difference in cholesterol profile between hippocampal and cortical neurons underlies the difference in neurite outgrowth between these two types of neurons.

Neurons contain two types of processes, axons and dendrites, which are structurally and functionally distinct and play different roles in the maintenance of brain functions. There are studies showing the significant role of lipids in the formation of neuronal polarity; it has been shown that phospholipids regulate neurite outgrowth in cultured neurons\textsuperscript{1} and that the correct distribution of axonal membrane proteins requires the formation of sphingomyelin/cholesterol-rich microdomains, lipid rafts, and the maturation of the axonal plasma membrane requires the up-regulation of sphingomyelin synthesis\textsuperscript{2}. Cholesterol also plays a prominent role in raft-mediated trafficking and sorting, because cholesterol depletion by methyl-\( \beta \)-cyclodextrin impedes trafficking from the trans-Golgi network to the apical membrane\textsuperscript{4}. It has been shown that cholesterol modulates dendrite outgrowth\textsuperscript{5}, that its deficiency enhances phosphorylation of tau and axonal depolymerization\textsuperscript{6}, and that axonal regeneration is dependent on local cholesterol reutilization \textit{in vivo}\textsuperscript{7}. In addition, cholesterol supplied as glial lipoproteins stimulates the axon outgrowth of central nervous system neurons\textsuperscript{8,9}. Moreover, previous studies have shown that glia-derived cholesterol is essential for synaptogenesis and synaptic plasticity\textsuperscript{10,11}. These lines of evidence suggest that membrane lipids play essential roles in neurite outgrowth and the formation of synapse neuronal polarity.

The region-specific difference in the development of Alzheimer disease pathologies is known. For example, the initial amyloid-\( \beta \) protein deposition occurs in poorly myelinated areas of the basal neocortex and spreads into adjoining areas and the hippocampus, whereas the formation of neurofibrillary tangles, which contain hyperphosphorylated tau, preferentially occurs in the transentorhinal region and hippocampus in the absence of amyloid deposits\textsuperscript{12–14}. Previous studies have suggested that the altered cholesterol metabolism is associated with the development of Alzheimer disease (for review, see Ref. 15) via the modulation of amyloid-\( \beta \) synthesis\textsuperscript{16,17}. Other lines of evidence suggest that cholesterol plays essential roles in the modulation of tau phosphorylation\textsuperscript{6,18,19}, neurofibrillary tangle formation\textsuperscript{20}, and neuronal survival\textsuperscript{21,22}. These lines of evidence suggest that Alzheimer disease pathologies preferentially developing in specific brain regions may be explained by a region-specific difference in the lipid profile. However, the region-specific profiles of lipids in neurons and their effects on neuronal functions remain to be clarified. The present study was designed to determine whether there is any difference in the profiles of lipids in primary cultured neurons isolated from different regions, namely, the mouse cerebral cortices and hippocampus, and whether neuronal function, including neurite outgrowth and polarity formation, is modulated by cellular lipids.

**MATERIALS AND METHODS**

**Cell Culture**—Neuron-rich cultures were prepared from the cerebral cortices and hippocampi of rat brains on embryonic day 18. The isolated cerebral cortices and hippocampi were incubated in 2 ml of Heps-buffered saline solution containing 0.25% trypsin-EDTA for 20 min at 37 °C. The tissues were then washed three times in Dulbecco’s modified Eagle’s medium (DMEM)\textsuperscript{2} containing 10% fetal bovine serum. The tissues in 1 ml of DMEM containing 10% fetal bovine serum were sub-

---

* This work was supported by Grants H14-10 (Comprehensive Research on Aging and Health) and H17-004 (Research on Human Genome and Tissue Engineering) from the Ministry of Health, Labor, and Welfare of Japan and by the Program for Promotion of Fundamental Studies in Health of the National Institute of Biomedical Innovation, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Experimental Procedures.

\textsuperscript{1} To whom correspondence should be addressed. Tel.: 81-562-46-2311; Fax: 81-562-46-8569; E-mail: michi@nils.go.jp.

\textsuperscript{2} The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; \( \beta \)-CD, methyl-\( \beta \)-cyclodextrin; Mes, 4-morpholineethanesulfonic acid.
**Cholesterol Modulates Neuronal Development**

jected to gentle pipetting using a micropipette (Gilson) 10 times and to further pipetting using a fire-polished glass Pasteur pipette 10 times. The volume of solution containing dissociated tissues was adjusted to 10 ml by adding DMEM containing 1% fetal bovine serum, and the tissues were obtained as pellets by centrifugation at 800 rpm for 5 min. The samples were washed two times in DMEM by centrifugation at 800 rpm for 5 min. The dissociated cells were suspended in feeding medium (consisting of DMEM/F12 (50:50%) nutrient mixture and N2 supplements) and plated onto poly-D-lysine-coated 12-well plates at a cell density of $5 \times 10^3$/cm$^2$. More than 99% of the cultured cells were identified as neurons by immunocytochemical analysis using a monoclonal antibody against microtubule-associated protein 2, a neuron-specific marker, on day 3 of culture (21).

**Morphological Analysis**—The cultured neurons were washed three times in PBS and incubated in PBS containing 4% paraformaldehyde for 15 min at room temperature. The cells were then washed three times in PBS and incubated in PBS containing 0.2% Triton X-100 and 1% bovine serum albumin for 15 min at room temperature. The cells were washed three times in PBS and incubated with a monoclonal anti-β-tubulin antibody (Covance, Berkeley, CA) at 2 μg/ml overnight at 4°C. The cultured cells were then washed in PBS three times, and the anti-β-tubulin antibody bound to neuronal β-tubulin was visualized using an ABC kit (Vector Laboratories, Burlingame, CA). The photographs of stained neurons were captured using a charge-coupled device camera (DC500) (Leica Microsystems GMBH, Wetzar, Germany) attached to a phase-contrast microscope (Olympus IX70, Olympus Co., Ltd., Tokyo, Japan). The length of axons and dendrites/cell and the ratio of neurite number/cell were determined using an image analyzer (KS400, Karl Zeiss Co., Ltd., Jena, Germany). Longest-axon length, axonal plexus length, and total dendrite length are defined in supplemental Fig. S1.

**Lipid Analysis**—For the extraction of cellular lipids, dried cells were incubated in hexane/isopropanol (3:2 v/v) for 1 h at room temperature. The solvent in each plate was collected and dried under N$_2$ gas. The organic phases were redissolved in 400 μl of chloroform, and a 150-μl sample was transferred onto 96-well polypropylene plates (Corning Coster, Corning, NY) and dried under air flow. The dried lipids were then dissolved in 20 μl of isopropanol. The concentration of cholesterol was determined using a cholesterol determination kit, LTCII (Kyowa Medex, Tokyo), and the concentration of phospholipids was determined using a phospholipid determination kit, PLB (Wako, Osaka, Japan) as described previously (23).

**Immunoblot Analysis**—Immunoblot analysis was performed as described previously (18). The primary antibodies used were mouse monoclonal antibodies, Fyn sc-434 (1: 1,000 dilution, Santa Cruz Bio-technology, Inc.), Ibotillin (1:1000 dilution, BD Biosciences), and NACM (1:1000 dilution, Chemicon International, Inc.). GM1 was detected using cholera toxin B-conjugated horseradish peroxidase (1:10,000 dilution, Chemicon International, Inc.). After rinsing and incubation in the presence of an appropriate peroxidase-conjugated secondary antibody, the bands were detected with an ECL kit (Amersham Biosciences). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce).

**Cholesterol Depletion and Drug Treatment**—A stock solution of methyl-β-cyclodextrin (β-CD) (Sigma-Aldrich) was prepared by dissolving β-CD in DMEM/F12 medium at a concentration of 100 mM. For cholesterol depletion, neurons were treated with β-CD at a final concentration of 5 mM for 10 min at 37°C, washed in the culture medium three times, and then cultured further for 5, 24, or 48 h. Cholesterol (Sigma-Aldrich) and PP2 (Calbiochem) were dissolved in 100% ethanol to prepare stock solutions at concentrations of 7 μg/ml and 5 μm, respectively. Cholesterol and PP2 solutions were diluted and used at final concentrations of 7 μg/ml and 5 μm, respectively.

**Lipid Raft Fractions**—Sucrose gradients were prepared by established methods with modifications (24, 25). Neurons were rinsed with PBS and scraped and homogenized with 1 ml of Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 mM NaCl) containing 1% Triton X-100, a mixture of protease inhibitors, Complete$^\text{TM}$, and phosphatase inhibitors. Extracts containing 350 μg of proteins were subjected to sucrose gradient analysis. Gradients were centrifuged for 20 h at 44,800 rpm at 4°C in a SW 50.1 rotor (Beckman Instruments). Fractions (400 μl) were collected sequentially starting from the top of the gradient. The extraction of lipids and the subsequent determination of the concentrations of cholesterol and phospholipid in each sample were carried out according to previously described methods (23).

**Triton X-100-soluble and -insoluble Fractions**—Cultured neurons (1 × 10$^6$ cells) were washed three times with ice-cold PBS and scraped in 200 μl of 1% Triton X-100 in Mes-buffered saline. Neurons were lysed by pipetting 10 times followed by ultrasonication for 5 min at a high level at 4°C in a Bioruptor (Cosmo Bio, Tokyo). Triton X-100-soluble and -insoluble fractions were separated by centrifugation at 100,000 × g for 60 min using a TL-100 rotor (Beckman Instruments). The resulting pellet (Triton X-100-insoluble fraction) was washed with PBS three times and resuspended in 100 μl of radioimmune precipitation assay buffer; the resultant supernatant was used in lipid analysis. The supernatant, i.e. the Triton X-100-soluble fraction, was used in lipid analysis.

**Statistical Analyses**—All statistical analyses were conducted using the StatView 5.0 software package (Abacus Concepts Inc., Berkeley, CA). The data are expressed as means ± S.E., and statistical significance was assessed by an analysis of variance followed by post-hoc Fisher’s PLSD test. A value of $p < 0.05$ was considered statistically significant.

**RESULTS**

**Differential Neurite Outgrowth in Cultured Neurons Prepared from the Hippocampi and Cortices of Rat Embryos**—Primary neurons were prepared from the hippocampi and cortices of rat embryos on embryonic day 18 and maintained for 1, 3, and 5 days in serum-free N2-supplemented culture medium. The cultures were then stained with the anti-β-tubulin antibody. Cortical neurons immunostained with the anti-tubulin antibody on culture days 1, 3, and 5 are shown in Fig. 1, a, c, and e, respectively. Hippocampal neurons immunostained with the same antibody on culture days 1, 3, and 5 are shown in Fig. 1, b, d, and f, respectively. Cortical neurons had longer axons and dendrites than hippocampal neurons at each time point examined. The longest-axon length, axonal plexus length, dendrite length, and number of neurites were determined as described under "Materials and Methods" (see also supplemental Fig. S1). The longest-axon length and total dendrite length were greater in cortical neurons than in hippocampal neurons; these lengths increased with culture time in both types of neurons (Fig. 1, g and i). The axonal plexus length of cortical neurons on culture day 5 was greater than that of hippocampal neurons (Fig. 1h). In contrast, the number of neurites per cortical neuron on culture day 5 was similar to the number of neurites per hippocampal neuron (Fig. 1j).

**Differential Lipid Profiles in Cultured Neurons Prepared from the Hippocampi and Cortices of Rat Embryos**—We next determined the concentrations of cholesterol and phospholipids in these neurons. The cholesterol concentration in hippocampal neurons was significantly higher than in cortical neurons (Fig. 2a); however, the concentration of phospholipids was similar between these two types of neurons (Fig. 2b). The concentrations of cholesterol and phospholipids in Triton X-100-soluble and -insoluble fractions in each neuronal culture were also
determined. The cholesterol concentration in the Triton X-100-insoluble fraction from hippocampal neurons was higher than that from cortical neurons, whereas the concentration in the Triton X-100-soluble fraction from hippocampal neurons was similar to that from cortical neurons (Fig. 2c). There were no significant differences in the concentrations of phospholipids in the Triton X-100-soluble and -insoluble fractions between hippocampal and cortical neurons (Fig. 2d). Because cholesterol concentration in the Triton X-100-insoluble fraction was different between these two types of neurons, we next examined lipid distribution in the lipid raft fractions. The cholesterol concentration in the lipid raft fractions isolated from hippocampal neurons was higher than that from cortical neurons (Fig. 2e), whereas there was no significant difference in phospholipid concentration between these two types of neurons in culture (Fig. 2f).

Cholesterol Concentration-dependent Regulation of Neurite Outgrowth in Hippocampal and Cortical Neurons—To determine whether a higher cholesterol concentration is responsible for the shorter neurite outgrowth from hippocampal neurons than from cortical neurons, cholesterol concentration in both types of neurons was modulated by treatment with H9252-cyclodextrin and cholesterol. Interestingly, cholesterol depletion by H9252-cyclodextrin stimulated neurite outgrowth in hippocampal neurons (Fig. 3b and d), which made these neurons similar to cortical neurons without treatment (Fig. 3a and d). In contrast, β-cyclodextrin treatment decreased the extent of neurite outgrowth in cortical neurons (Fig. 3a and c). Cholesterol loading also decreased the extent of neurite outgrowth in cortical neurons (Fig. 3a and e), whereas it had no effects on neurite outgrowth in hippocampal neurons (Fig. 3b and f). Neuronal development in cultures has been defined from stages 1 to 5 (26). On the basis of these criteria, we determined the ratio of

FIGURE 1. Difference in neurite outgrowth between cortical and hippocampal neurons in culture. Primary neurons were prepared from cortices and hippocampi of rat brains on embryonic day 18 and plated on poly-D-lysine-coated 12-well plastic plates at a cell density of 5 × 10^3/well. a–f, the neurons were cultured in serum-free N2-supplemented medium (N2-medium) and fixed and immunostained with anti-β-tubulin antibody on the days indicated. Scale bar represents 100 μm. COR and HIP represent cortical neurons and hippocampal neurons, respectively. Neurons from stages 3–5 (26) were analyzed. Longest-axon length (g) and total dendrite length (i) per neuron on days 1, 3, and 5 in culture were determined. Axonal plexus length (h) and number of neurites (j) per neuron were determined on culture day 5. The number of neurons analyzed was 25 in each culture. *, p < 0.0001, and **, p < 0.005, between cortical and hippocampal neurons. Four independent experiments showed similar results.

FIGURE 2. Characterization of lipid profiles of cortical and hippocampal neurons. Cholesterol and phospholipid concentrations in cortical and hippocampal neurons cultured in N2-medium for 5 days were analyzed as described under “Materials and Methods.” The concentrations of cholesterol (a) and phospholipids (b) in hippocampal (HIP) and cortical (COR) neurons per mg of protein were determined. In addition, the concentrations of cholesterol (c) and phospholipids (d) in the Triton X-100-soluble and -insoluble fraction from hippocampal and cortical neurons were determined. The data represent mean ± S.E. The number of samples was six for each treatment. *, p < 0.05. Three independent experiments showed similar results. Isolation of lipid raft fractions from cultured hippocampal and cortical neurons was performed by sucrose density gradient ultracentrifugation, and cholesterol (e) and phospholipid (f) levels in each fraction are shown.
neurons at stages 4 and 5 to the total number of neurons. As shown in Fig. 3g, cholesterol depletion by β-cyclodextrin treatment or cholesterol loading had no significant effects on the development of cortical neurons (left panel). In contrast, β-cyclodextrin treatment stimulated the development of hippocampal neurons; however, cholesterol loading had no significant effects (Fig. 3g, right panel).

The effects of cholesterol depletion and loading on longest-axon length, axonal plexus length, and total dendrite length of cortical and hippocampal neurons were determined. Cholesterol depletion by treatment with β-cyclodextrin and cholesterol loading decreased the longest-axon length, axonal plexus length, and total dendrite length of cortical neurons (Fig. 4, a–c, respectively). In contrast, cholesterol depletion by treatment with β-cyclodextrin increased the longest-axon length, axonal plexus length, and total dendrite length of hippocampal neurons (Fig. 4, a–c, respectively), whereas cholesterol loading had no significant effects on these three parameters in hippocampal neurons (Fig. 4, a–c). In both types of neurons, these chemicals had no effects on neurite numbers (Fig. 4d).

Cholesterol concentrations in cultured neurons treated with β-cyclodextrin or cholesterol loading were determined. Cholesterol concentration in neurons was reduced at 5 h and was recovered at 24 h following treatment with β-cyclodextrin in both cortical and hippocampal neurons (Fig. 5a). Cholesterol concentration in neurons increased at 24 h following treatment with cholesterol in both cortical and hippocampal neurons (Fig. 5a), although phospholipid concentrations remained unchanged by these treatments (Fig. 5a). We further determined the longest-axon length, total dendrite length, and neurite length per cell in cortical and hippocampal neurons treated with β-CD or cholesterol. As shown in Fig. 5b, the neurite length was dependent on the treatments.

**Fyn, a Member of the Src Family, in Lipid Raft Fraction Is a Key Molecule Modulating Cholesterol-dependent Axon Outgrowth**—The observations mentioned above suggest that the difference in cholesterol concentration between cortical and hippocampal neurons could explain differences in neurite outgrowth and neuronal development between these two types of neurons. Because cholesterol concentrations differ in the Triton X-100-insoluble fraction and the lipid raft fraction but not in the Triton X-100-soluble fraction between these two types of neurons (Fig. 2), proteins that are localized in lipid rafts and also play a role in axon and dendrite outgrowths may underlie these differences between cortical and hippocampal neurons in terms of neurite outgrowth. Among the molecules examined, only the level of Fyn in the raft fraction was significantly high in hippocampal neurons treated with β-cyclodextrin, whereas that in total homogenates did not alter (Fig. 6a). The levels of flotillin and GM1 did not differ between cortical and hippocampal neurons following these treatments (Fig. 6a).

We next determined whether an inhibitor of Fyn signaling, PP2, inhibits the cholesterol deficiency-induced stimulation of axon and dendrite outgrowths in hippocampal neurons. The treatment of hippocampal neurons with 5 μM PP2 inhibited enhanced neurite outgrowth induced by β-cyclodextrin (Fig. 6b). However, PP2 did not have any effects on neurite growth in control and cholesterol-loaded neurons (Fig. 6b). The neurite outgrowth in hippocampal neurons treated with β-cyclodextrin or cholesterol in the presence or absence of PP2 was quantified. The treatment of hippocampal neurons with PP2 significantly inhibited the increase in longest-axon length and axonal plexus length induced by β-cyclodextrin (Fig. 6c, black bars); however, PP2 had no effect on neurite outgrowth in control neurons and cholesterol-loaded neurons (Fig. 6c, white bars). An inhibitory effect of PP2 on the
FIGURE 5. Cholesterol concentrations in hippocampal (HIP) and cortical neurons (COR) treated with cholesterol and β-cyclodextrin.

Cultured neurons were maintained in N2-medium for 3 days after plating, and the cultures were treated with β-CD or cholesterol as described in the legend for Fig. 3. a, the neurons were then harvested at 5 and 24 h following the treatment, and the concentrations of cholesterol and phospholipids in these cultures were determined as described under “Materials and Methods.” The data represent mean ± S.E. Six cultures for each treatment were counted. *, p < 0.01. Three independent experiments showed similar results. b, cultured neurons maintained in N2-medium for 3 days after plating were treated with 1 or 2 mM β-CD or 7 μg/ml cholesterol and maintained for another 2 days. Some of the cultures treated with β-CD were treated again with β-CD at the same concentration for 10 min at 37 °C and maintained for 1 additional day. All of the neurons were harvested on culture day 5 and then immunostained with the anti-tubulin antibody. Longest-axon length, total dendrite length, and neurite length per cell were determined. The data represent mean ± S.E. Twenty-five neurons were counted for each treatment. *, p < 0.005 versus 2 mM β-CD and cholesterol for cortical neurons; *, p < 0.005 versus cholesterol, no treatment (None), and 2 mM β-CD (twice) for hippocampal neurons. Two independent experiments showed similar results.

FIGURE 6. Involvement of Fyn signaling in cholesterol-dependent modulation of axonal outgrowth in hippocampal neurons. Cortical (COR) and hippocampal neurons (HIP) were maintained in N2-medium for 3 days after plating, and the cultures were treated with β-CD or cholesterol (CHOL) as described under “Materials and Methods.” a, the cultures were harvested after 5 days in vitro, and each sample was subjected to immunoblot analysis using antibodies specific for flotillin, GM1, and Fyn. b, hippocampal neurons were maintained in N2-medium for 3 days after plating, and the cultures were treated with β-CD or cholesterol as described under “Materials and Methods.” The hippocampal neurons were then maintained for another 2 days in the presence or absence of PP2, a Fyn signaling inhibitor, at a concentration of 5 μM. The cultures were then harvested and immunostained. c, longest-axon length, axonal plexus length, and dendrite length of the hippocampal neurons not treated (control (CONT)) or treated with β-CD or cholesterol in the presence or absence of PP2 were quantified. The data represent mean ± S.E. Twenty-five cells were analyzed for each treatment. *, p < 0.005; **, p < 0.0001. Three independent experiments showed similar results.
enhanced dendrite outgrowth induced by β-cyclodextrin was not observed (Fig. 6c, bottom panel).

**DISCUSSION**

This study showed that cholesterol concentration in hippocampal neurons was markedly higher than in cortical neurons and that the neurite outgrowth was significantly greater and the polarity formation occurred earlier in cortical neurons than in hippocampal neurons. In contrast, the phospholipid concentrations in these neurons were similar. Our finding that the cholesterol biosynthetic pathway is highly enhanced in hippocampal neurons compared with cortical neurons (supplemental Fig. S2) may explain the difference in cholesterol concentration between these two types of neurons.

Cholesterol depletion from hippocampal neurons markedly enhanced axon and dendrite outgrowth and accelerated the establishment of cell polarity. The morphological features appeared similar to those in cortical neurons, suggesting that a high concentration of cholesterol attenuates neurite outgrowth and polarity formation in hippocampal neurons in culture. The finding that both the loading of cholesterol and the depletion of cholesterol from the cortical neurons attenuated neurite outgrowth suggests that there may be an optimal concentration of cholesterol for cortical neurons to exhibit neurite outgrowth (Figs. 3 and 4). This is also the case for hippocampal neurons, because the neurite outgrowth was inhibited when cholesterol was further depleted from cultured neurons by treatment with β-CD twice (Fig. 5b).

The cholesterol concentration in the Triton X-100-insoluble fraction from hippocampal neurons was greater than that from cortical neurons, whereas cholesterol concentration in the Triton X-100-soluble fraction did not differ between these two types of neurons, indicating that the difference in the concentration of cholesterol in the Triton X-100-insoluble fraction may explain the neuron-specific difference in total cholesterol concentration between these two types of neurons. This notion is supported by the finding that cholesterol concentration in lipid rafts isolated from hippocampal neurons is higher than in those from cortical neurons (Fig. 2). In contrast to cholesterol concentration, phospholipid concentration did not show any difference between these two types of neurons. This also suggests that neuronal differentiation and neurite outgrowth in hippocampal neurons may be modulated by cholesterol in lipid rafts and raft-localized molecules and that the morphological difference between cortical and hippocampal neurons can be explained by the difference in cholesterol concentration between these neurons.

The mechanism by which a decreased level of cholesterol in lipid rafts modulates neurite outgrowth has not been completely understood. However, the finding that the enhancement of neurite outgrowth induced by β-cyclodextrin treatment is accompanied by the recruitment of Fyn to lipid rafts and that the Src family inhibitor PP2 inhibits axonal elongation induced by β-cyclodextrin treatment suggests that an increased level of raft-localized Fyn is in part involved in β-cyclodextrin-induced axonal elongation in hippocampal neurons. This notion is supported by previous studies showing that Src family kinases, including Fyn, play a critical role in axon outgrowth (27, 28) and that lipid raft-localized Fyn is more catabolically active than non-raft-localized Fyn (29, 30).

Another interesting point is that cellular cholesterol concentration modulates the development of hippocampal neurons but not that of cortical neurons (Fig. 3), although it modulates neurite outgrowth in both types of neurons (Figs. 4 and 5). As demonstrated previously (26), neurons initially establish several apparently identical, short processes. With culture time, one of the processes begins to grow very rapidly and becomes an axon, and the other processes then become dendrites. Interestingly, the establishment of neuronal polarity in hippocampal neurons depends on cellular cholesterol concentration; a decreased cholesterol concentration stimulates the establishment of neuronal polarity, whereas an increased concentration of cellular cholesterol inhibits it. This is not the case for cortical neurons. The present study does not provide any explanation for the discrepancy in the cell type-specific regulation of polarity formation mediated by cholesterol. This may suggest that cholesterol is not the only lipid responsible for the observed alterations and that membrane composition is relevant for cortical neurons. Further study is required to clarify the mechanism underlying the cell type-specific regulation of polarity formation mediated by cholesterol.

Recent studies have shown that cholesterol supplied as an apoE-lipoprotein complex to neurons via apoE receptors plays a critical role in synaptogenesis, neurite outgrowth, and neuronal repair (9–11). Most of the published literature is concerned with only a single neuronal subtype, which is not sufficient for the appreciation of the complex role of cholesterol in neurons. The present study shows that cholesterol demand and the optimal cholesterol concentration for neurite outgrowth depend completely on the neuronal type and that the mechanism underlying the effect of cholesterol on neuronal maturation involves the attainment of the optimal cholesterol concentration. There are issues that need to be elucidated to delineate the mechanisms underlying the neuronal type-specific regulation of neurite outgrowth by cellular cholesterol concentration, and it is required to confirm that the present findings are also the case in vivo. However, the present study suggests that the role of cholesterol in relation to neuronal phenotypes and functions should be elucidated in a neuron type- and brain region-specific manner.

**REFERENCES**

1. Schwarz, A., Rapaport, E., Hirschberg, K., and Puterman, A. H. (1995) *J. Biol. Chem.* 270, 10990–10998.
2. Ledesma, M. D., Simons, K., and Dotti, C. G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 3966–3971.
3. Ledesma, M. D., Brugger, B., Bunning, C., Wieland, F. T., and Dotti, C. G. (1999) *EMBO J.* 18, 1761–1771.
4. Simons, K., and Toonder, D. (2000) *Nat. Rev. Mol. Cell Biol.* 1, 31–39.
5. Fan, Q.-W., Yu, W., Gong, J.-S., Zou, K., Sawamura, N., Senda, T., Yanagisawa, K., and Michikawa, M. (2002) *J. Neurochem.* 80, 178–190.
6. Fan, Q.-W., Yu, W., Senda, T., Yanagisawa, K., and Michikawa, M. (2001) *J. Neurochem.* 76, 391–400.
7. Goodrum, J. F., Brown, J. C., Fowler, K. A., and Boulidn, T. W. (2000) *J. Neuropathol. Exp. Neurol.* 59, 1002–1010.
8. Karten, B., Vance, D. E., Campenot, R. B., and Vance, J. E. (2003) *J. Biol. Chem.* 278, 4168–4175.
9. Hayashi, H., Campenot, R. B., Vance, D. E., and Vance, J. E. (2004) *J. Biol. Chem.* 279, 14009–14015.
10. Mauch, D. H., Nagler, K., Schumacher, S., Goritz, C., Muller, E. C., Otto, A., and Pfiriger, F. W. (2001) *Science* 294, 1354–1357.
11. Koudinov, A. R., and Koudinova, N. V. (2001) *FASEB J.* 15, 1858–1860.
12. Braak, H., and Braak, E. (1997) *Neurobiol. Aging* 18, 351–357.
13. Price, J. L., and Morris, J. C. (1999) *Ann. Neurol.* 45, 358–368.
14. Katsumo, T., Morishima-Kawamura, M., Saito, Y., Yamanoouchi, H., Ishiura, S., Murayama, S., and Ishara, Y. (2005) *Neurology* 64, 687–692.
15. Simons, M., Keller, P., Dichgans, J., and Schulz, J. B. (2001) *Neurology* 57, 1089–1093.
16. Simons, M., Keller, P., De Strooper, B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 6460–6464.
17. Fassbender, K., Simons, M., Bergmann, C., Struyck, M., Lutjohann, D., Keller, P., Runz, H., Kohl, S., Bertsch, T., von Bergmann, K., Hennerici, M., Beyreuther, K., and Hartmann, T. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 5856–5861.
18. Sawamura, N., Gong, J.-S., Garver, W. S., Heidenreich, R. A., Ninomiya, H., Ohno, K., Yanagisawa, K., and Michikawa, M. (2001) *J. Biol. Chem.* 276, 10314–10319.
19. Bu, B., Li, J., Davies, P., and Vincent, I. (2002) *J. Neurosci.* 22, 6515–6525.
20. Suzuki, K., Parker, C. C., Pentchev, P. G., Katz, D., Ghetti, B., D’Agostino, A. N., and
Carstea, E. D. (1995) Acta Neuropathol. (Berl.) 89, 227–238
21. Michikawa, M., and Yanagisawa, K. (1999) J. Neurochem. 72, 2278–2285
22. Yu, W., Gong, J. S., Ko, M., Garver, W. S., Yanagisawa, K., and Michikawa, M. (2005) J. Biol. Chem. 280, 11731–11739
23. Gong, J. S., Kobayashi, M., Hayashi, H., Zou, K., Sawamura, N., Fujita, S. C., Yanagisawa, K., and Michikawa, M. (2002) J. Biol. Chem. 277, 29919–29926
24. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y. H., Cook, R. F., and Sargiacomo, M. (1994) J. Cell Biol. 126, 111–126
25. Sawamura, N., Ko, M., Yu, W., Zou, K., Hanada, K., Suzuki, T., Gong, J. S., Yanagisawa, K., and Michikawa, M. (2004) J. Biol. Chem. 279, 11984–11991
26. Dotti, C. G., Sullivan, C. A., and Banker, G. A. (1988) J. Neurosci. 8, 1454–1468
27. Meriane, M., Tcherkezian, J., Webber, C. A., Danek, E. I., Triki, I., McFarlane, S., Bloch-Gallego, E., and Lamarche-Vane, N. (2004) J. Cell Biol. 167, 687–698
28. Liu, G., Beggs, H., Jurgensen, C., Park, H. T., Tang, H., Gorski, J., Jones, K. R., Reichardt, L. F., Wu, J., and Rao, Y. (2004) Nat. Neurosci. 7, 1222–1232
29. Mukherjee, A., Arnaud, L., and Cooper, J. A. (2003) J. Biol. Chem. 278, 40806–40814
30. Shim, T., Nada, S., and Okada, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14897–14902

Cholesterol Modulates Neuronal Development