CHOLESTEROL-MEDIATED NEURITE OUTGROWTH IS DIFFRENTLY REGULATED BETWEEN CORTICAL AND HIPPOCAMPAL NEURONS

Mihee Ko, Kun Zou, Hirohisa Minagawa, Wenxin Yu, Jian-Sheng Gong, Katsuhiro Yanagisawa, and Makoto Michikawa*

From the Department of Alzheimer’s Disease Research, National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan
Abbreviated title: Cholesterol Modulates Neuronal Development
Address Correspondence: * Makoto Michikawa, M.D., Department of Alzheimer’s Disease Research, National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan,
TEL: +81 562 46 2311, FAX: +81 562 46 8569, E-mail: michi@nils.go.jp

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 β-cyclodextrin markedly enhanced the neurite outgrowth and accelerated the establishment of neuronal polarity in hippocampal neurons, which are similarly observed in nontreated cortical neurons, whereas the 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 β-cyclodextrin and a Src family kinase inhibitor, PP2, specifically blocked axon outgrowth, but not dendrite outgrowth, both of which were enhanced by β-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 cultured conditions, and that the difference in cholesterol profile between hippocampal and cortical neurons underlies the difference in neurite outgrowth between these two types of neuron.

INTRODUCTION

Neurons contain two types of process, 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 was shown that phospholipids regulate neurite outgrowth in cultured neurons (1), and that the correct distribution of axonal membrane

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proteins requires the formation of sphingomyelin/cholesterol-rich microdomains, lipid rafts, and the maturation of the axonal plasma membrane requires the upregulation of sphingomyelin synthesis (2,3). Cholesterol also plays a prominent role in raft-mediated trafficking and sorting, because cholesterol depletion by methyl-β-cyclodextrin impedes trafficking from the trans-Golgi-network to the apical membrane (4). It was shown that cholesterol modulates dendrite outgrowth (5) and its deficiency enhances phosphorylation of tau and axonal depolymerization (6), and that axonal regeneration is dependent on local cholesterol reutilization in vivo (7). In addition, cholesterol supplied as glial lipoproteins stimulates the axon outgrowth of central nervous system neurons (8,9). Moreover, previous studies showed that glia-derived cholesterol is essential for synaptogenesis and synaptic plasticity (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’s disease (AD) pathologies is known. For example, the initial amyloid β-protein (Aβ) deposition occurs in poorly myelinated areas of the basal neocortex and spreads into adjoining areas and the hippocampus, whereas the formation of neurofibrillary tangle (NFT), which consist of hyperphosphorylated tau, preferentially occurs in the transentorhinal region and hippocampus in the absence of amyloid deposits (12-14). Previous studies suggested that the altered cholesterol metabolism is associated with AD development (see review, (15)) via the modulation of Aβ synthesis (16,17). Other lines of evidence suggest that cholesterol plays essential roles in the modulation of tau phosphorylation (6,18,19), NFT formation (20), and neuronal survival (21,22). These lines of evidence suggest that AD 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 to determine whether neuronal function including neurite outgrowth and polarity formation are 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 hippocampus were incubated in 2 ml of Hepes-buffered saline solution (HBSS) containing 0.25% trypsin and EDTA for 20 min at 37°C. The tissues were then washed three times in DMEM containing 10% FBS. The tissues in 1 ml of DMEM containing 10% FBS were subjected to gentle pipetting using a micropipette (Gilson) ten times and further pipetting using a fire-polished glass Pasteur pipette ten times. The volume of solution containing dissociated tissues was adjusted to 10 ml by adding DMEM containing 10% FBS 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 the feeding medium and plated onto poly-o-lysine-coated 12-well plates at a cell density of 5 x 10^3/cm². The feeding medium consisted of Dulbecco’s
modified Eagle’s medium nutrient mixture (DMEM/F12; 50%: 50%) and N₂ supplements. 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% BSA for 15 min at room temperature. The cells were washed three times in PBS and incubated with a monoclonal anti-β-tubulin antibody (Covance, Barkley, 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, USA). The photographs of stained neurons were captured using a CCD 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 the Supplementry 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 N₂ 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 Ltd., Corning, NY) and dried under airflow. 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 that of phospholipids was determined using a phospholipid determination kit, PLB (Wako, Osaka, Japan) as previously described (23).

Immunoblot analysis- Immunoblot analysis was performed as previously described (18). The primary antibodies used were mouse monoclonal antibodies, Fyn sc-434 (1: 1,000 dilution, Santa Cruz Biotechnology, Inc., CA, USA), flotillin (1:1000 dilution, BD Biosciences, Inc., CA, USA), and NCAM (1:1000 dilution, Chemicon International, Inc., CA, USA). GM1 was detected using cholera toxin B-conjugated horseradish peroxidase (1:10,000, Sigma-Aldrich, St. Louis, MO, USA). After rinsing and incubation in the presence of an appropriate peroxidase-conjugated secondary antibody, the bands were detected with an ECL kit (Amersham Pharmacia Biotech, UK). 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, St. Louis, MO) was prepared by dissolving β-CD in the 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, St. Louis, MO) and PP2 (Calbiochem, CA) were dissolved in 100% ethanol to prepare stock solutions at concentrations of 7 mg/ml and 5 mM, 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 (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100, a mixture of protease inhibitors, Complete™, 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 Inc., CA). Fractions (400 μl) were sequentially collected 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 (1x10⁶ cells) were washed three times with ice-cold PBS and scraped in 200 μl of 1% Triton X-100 in MBS. Neurons were lysed by pipetting 10 times followed by ultrasonication for 5 min at a high level at 4°C in Bioruption (COSMO BIO, Tokyo, Japan). Triton X-100-soluble and -insoluble fractions were separated by centrifugation at 100,000 × g for 60 min, using a TLA-100 rotor (Beckman Instruments Inc., CA). The resulting pellet (Triton X-100-insoluble fraction) was washed with PBS three times and resuspended in 100 μl of RIPA buffer; the resultant supernatant was used in lipid analysis. The supernatant, that is, 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, USA). The data are expressed as means ± SEM, 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 hippocampus and cortices of rat embryos** - Primary neurons were prepared from hippocampi and cortices of rat embryos on embryonic day 18 and maintained for 1, 3, and 5 days in the 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 Figs. 1a, c, and e, respectively. Hippocampal neurons immunostained with the same antibody on culture days 1, 3, and 5 are shown in Figs. 1b, d, and f, respectively. Cortical neurons have longer axons and dendrites than hippocampal neurons at each time point examined. The longest-axon length, axonal plexus length, dendrite length, and the number of neurite were determined as described in the Materials and Methods (see also Supplementary Fig. S1). The longest-axon length and total dendrite length were greater in cortical neurons than in hippocampal neurons, which increased with culture time in both types of neuron (Figs. 1g, 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 that per hippocampal neuron (Fig. 1j).

**Differential lipid profiles in cultured neurons prepared from 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 that in cortical neurons (Fig. 2a); however, the concentration of phospholipids was similar between these two types of neuron (Fig. 2b). The concentrations of cholesterol and phospholipids in Triton-X-100-soluble and -insoluble fractions of 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 that 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 neuron, we next examined lipid distribution in the lipid raft fractions. The lipid raft fractions were isolated as described in the Materials and Methods. The cholesterol concentration in the lipid raft fractions isolated from hippocampal neurons was higher than that from cortical neurons (Fig. 2e), whereas there is no significant difference in phospholipid concentration between these two types of neuron in culture (Fig. 2f).

**Cholesterol-concentration-dependent regulation of neurite outgrowth in hippocampal and cortical neurons** - In order 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 neuron was modulated by the treatment with β-cyclodextrin and cholesterol. Interestingly, cholesterol depletion by β-cyclodextrin stimulated neurite outgrowth in hippocampal neurons (Figs. 3b, d), which made these neurons similar to cortical neurons without treatment (Figs. 3a, d). In contrast, β-cyclodextrin treatment decreased the extent of neurite outgrowth in cortical neurons (Figs. 3a, c). Cholesterol loading also decreased the extent of neurite outgrowth in cortical neurons (Figs. 3a, e), whereas it had no effects on neurite outgrowth in hippocampal neurons (Figs. 3b, f). Neuronal development in cultures has been defined from stages 1 to 5 (26). On the basis of these criteria, we determined the number ratio of 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 (Figs. 4a, b, and 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 (Figs. 4a, b, and c, respectively), whereas cholesterol loading had no significant effects on these three parameters in hippocampal neurons (Figs. 4a, b, and c). In both types of neuron, 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 the treatment with β-cyclodextrin in both cortical and hippocampal neurons (Fig. 5a). Cholesterol concentration in neurons increased at 24 h following the treatment with cholesterol in both cortical and hippocampal neurons (Figs. 5a). While phospholipid concentrations remained unchanged by these treatments (Figs. 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 CHOL. As shown in Fig. 5b, the neurite length was dependent on the treatments.

Fyn, a member of Src family, in lipid raft fraction is a key molecule modulating cholesterol-dependent axon-outgrowth. The above-mentioned observations 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 neuron. Because cholesterol concentrations are different 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 neuron (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 following these treatments between cortical and hippocampal neurons (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. 7b, black bars); however, PP2 had no effects on neurite outgrowth in control neurons and cholesterol-loaded neurons (Fig. 6c, white bars). The inhibitory effect of PP2 on the enhanced dendrite outgrowth induced by β-cyclodextrin was not observed (Fig. 6c, the lowest panel).

DISCUSSION

This study showed that cholesterol concentration in hippocampal neurons was markedly higher than 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 (Supplementary Fig. S2) may be responsible for the difference in cholesterol concentration between these two types of neuron.

Cholesterol depletion from hippocampal neurons markedly enhanced axon and dendrite
outgrowth and accelerated the establishment of cell polarity, and the morphological features appeared similar to those of 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 to 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 is greater than that from cortical neurons, whereas cholesterol concentration in the Triton X-100-soluble fraction did not differ between these two types of neuron, indicating that the difference in the concentration of cholesterol in the Triton-X-100-insoluble fraction can explain the neuron-specific difference in total cholesterol concentration between these two types of neuron. This notion is supported by the finding that cholesterol concentration in lipid rafts isolated from hippocampal neurons is higher than that from cortical neurons (Fig. 2). In contrast to cholesterol, phospholipid concentration did not show any difference between these two types of neuron. 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, suggest 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 catabolitically active than nonraft-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 it 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, while 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 this.

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 only on 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 completely depend 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 mechanisms underlying the neuronal-type-specific regulation of neurite outgrowth by cellular cholesterol concentration, and it is required to confirm our findings in vivo. However, the present study suggests that one should elucidate the role of cholesterol in relation to neuronal phenotypes and functions in a neuron type- and brain region-specific manner.

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FIGURE LEGENDS

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 x 10⁵/well. (a-f) The neurons were cultured in the serum-free N2-supplemented medium (N2-medium) and fixed and immunostained with the 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 to 5 (26) were analyzed. Longest-axon length (g) and total dendrite length (i) per neuron on 1, 3, and 5 days in culture were determined. Axonal plexus length (h) and the 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 the N2-medium for 5 days were analyzed as described in the “Materials and Methods”. COR and HIP represent cortical neurons and hippocampal neurons, respectively. The concentrations of cholesterol (a) and phospholipids (b) in hippocampal (HIP) and cortical (COR) neurons per mg protein were determined. In addition, the concentrations of cholesterol (c) and (d) phospholipids 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.

Figure 3. Cholesterol-dependent neurite outgrowth and polarity formation in cortical and hippocampal neurons. Cortical and hippocampal neurons were maintained in the N2-medium for 3 days and each culture was treated with β-CD at 5 mM for 10 min and washed in N2-medium 3 times and maintained for another 2 days. COR and HIP represent cortical neurons and hippocampal neurons, respectively. For cholesterol (CHOL) treatment, cultures maintained for 3 days were treated with CHOL at 7μg/ml and maintained for for another 2 days. The neurons were then fixed and processed for immunocytochemistry using the anti-tubulin antibody (a-f). Ratio of stages 4 and 5 neurons to total neurons in COR and HIP neurons was determined (g). The data represent mean ± S.E. Two hundred fifty neurons for each treatment were counted. *P<0.0001. Three independent experiments showed similar results.

Figure 4. Cholesterol-dependent modulation of axon and dendrite outgrowths in cortical and hippocampal neurons in culture. Cultured neurons were maintained in the N2-medium for three days after plating, and the cultures were treated with β-CD or CHOL as described in Figure 3. The neurons were then immunostained with the anti-tubulin antibody and the longest-axon length (a), axonal plexus length (b), total dendrite length (c), and number of neurite (d) per cell were determined.
COR and HIP represent cortical neurons and hippocampal neurons, respectively. The data represent mean ± S.E. Twenty-five neurons for each treatment were counted. *P<0.005 and **P<0.0001. Three independent experiments showed similar results.

Figure 5. Cholesterol concentrations in hippocampal and cortical neurons treated with cholesterol and β-cyclodextrin. Cultured neurons were maintained in the N2-medium for three days after plating, and the cultures were treated with β-CD or CHOL as described in Figure 3. (a) The neurons were then harvested at 5 h and 24 h following the treatment and the concentrations of cholesterol and phospholipids in these cultures were determined as described in the “Materials and Methods”. The data represent mean ± S.E. Six cultures for each treatment were counted. *P<0.01, **P<0.05. Three independent experiments showed similar results. (b) Cultured neurons maintained in the N2-medium for three days after plating were treated with 1 mM or 2 mM β-CD, or 7 μg/ml CHOL 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 another 1 days. All the neurons were harvested on culture day 5 and then immunostained with the anti-tubulin antibody. The longest-axon length, total dendrite length, and neurite length per cell were determined. The data represent mean ± S.E. Twenty-five neurons for each treatment were counted. *P<0.005 vs. 2 mM β-CD and CHOL for cortical neurons and *P<0.005 vs. CHOL, 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 and hippocampal neurons were maintained in the N2-medium for 3 days after plating, and the cultures were treated with β-CD or CHOL as described in the “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 flotilin, GM1, and Fyn. COR and HIP represent cortical neurons and hippocampal neurons, respectively. (b) Hippocampal neurons (HIP) were maintained in the N2-medium for 3 days after plating, and the cultures were treated with β-CD or CHOL as described in the “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. The longest-axon length, axonal plexus length, and dendrite length of the hippocampal neurons not treated (CONT) or treated with β-CD or CHOL in the presence or absence of PP2 were quantified (c). The data represent mean ± S.E. Twenty-five cells for each treatment were analyzed. *P<0.005 and **P<0.0001. Three independent experiments showed similar results.
Figure 1
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Axonal plexus length
Number of neurites/cell

Longest-axon length (mm/axon)
Total dendrite length (mm/cell)
Number of neurites/cell

Days in culture
0    1     2    3    4     5

0    1     2    3    4     5

COR HIP

*p, **p < 0.05, ***p < 0.01, ****p < 0.001
Figure 3
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- Ratio of stage 4/5 neurons (% of total neurons)

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* *
Figure 4
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a) COR HIP

Longest-axon length (mm/cell)

b) COR HIP

Axonal plexus length (mm/cell)

c) COR HIP

Total dendrite length (mm/cell)

d) COR HIP

Number of neurite/cell
Figure 6

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The longest axonal length (µm/cell)

Axonal plexus length (µm/cell)

Dendrite length (µm/cell)

Flotilin

GM1

Fyn

β-CD

β-CD + PP2

CHOL

CHOL + PP2

PP2(-)

PP2 (+)

**

*

HIP

CONT

PP2
CHOLESTEROL-MEDIATED NEURITE OUTGROWTH IS DIFFRENTLY REGULATED BETWEEN CORTICAL AND HIPPOCAMPAL NEURONS

Supplemental Materials

Mihee Ko, Kun Zou, Hirohisa Minagawa, Wenxin Yu, Jian-Sheng Gong, Katsuhiko Yanagisawa, and Makoto Michikawa

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

Correspondence should be addressed to:
Makoto Michikawa, M.D.
Department of Alzheimer’s Disease Research, National Institute for Longevity Sciences
36-3 Gengo, Morioka, Obu
Aichi 474-8522, Japan
TEL: +81 562 46 2311, FAX: +81 562 46 8569
E-mail: michi@nils.go.jp
Supplementary Figure S1.
Quantification of neurite length. The terms of "longest-axon length", "axonal plexus length", and "total dendrite length" are defined as 1, 1+2+3+4+5+6+7, and 8, respectively.
Supplemental Figure S2
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[**Diagram**

- **3 days**
  - HIP: 5
  - COR: 4

- **5 days**
  - HIP: *60 ± 10*
  - COR: 4

[^14C]-acetate incorporation into cholesterol
(Arbitrary unit/min/mg protein)
Supplemental Figure S2.
[\(^{14}\)C]acetate incorporation into cholesterol was determined in cultured neurons in 24-well dishes maintained for 3 and 5 days following the plating. The data represent the mean ± S.E.M. of four samples. *P<0.02.

Experimental Procedures
Quantification of de novo cholesterol synthesis. Neurons prepared from the rat cerebral cortices and hippocampus were cultured at a density of 1 x 10^5 cells/cm² on poly-D-lysine-coated 24-well plates. Cultured neurons maintained in a N₂-supplemented medium for 3 or 5 days were pulsed with 2 μCi/ml [\(^{14}\)C]acetate (1.64 x 10^5 photostimulated luminescence/nmol) at 37°C for 1 h. To determine the level of cholesterol biosynthesis from radioactive acetate, the cultures were washed in cold PBS and dried, and lipids were extracted by incubation with 500 ul of hexane/isopropanol (3:2 vol/vol) for 30 min, evaporated under a nitrogen stream, and analyzed by thin-layer chromatography using the mobile phase of hexane/diethyl ether/acetic acid (70:30:1 by volume). The radioactive lipids that comigrated with a purified standard of \(^{14}\)C-labeled cholesterol were quantified using a Bio-Imaging Analyzer System-2500 Mac (Fuji Photo Film Co., Ltd., Japan). Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. The amount of [\(^{14}\)C]acetate incorporated into cholesterol per minute per milligram of cellular protein was calculated.