Cholesterol-induced robust Ca oscillation in astrocytes required for survival and lipid droplet formation in high-cholesterol condition
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SUMMARY

Cholesterol, one of the major cell membrane components, stabilizes membrane fluidity and regulates signal transduction. Beside its canonical roles, cholesterol has been reported to directly activate signaling pathways such as hedgehog (Hh). We recently found that astrocytes, one of the glial cells, respond to Hh pathway stimulation by Ca signaling. These notions led us to test if extracellularly applied cholesterol triggers Ca signaling in astrocytes. Here, we found that cholesterol application induces robust Ca oscillation only in astrocytes with different properties from the Hh-induced Ca response. The Ca oscillation has a long delay which corresponds to the onset of cholesterol accumulation in the plasma membrane. Blockade of the Ca oscillation resulted in enhancement of astrocytic cell death and disturbance of lipid droplet formation, implying a possibility that the cholesterol-induced Ca oscillation plays important roles in astrocytic survival and cholesterol handling under pathological conditions of cholesterol load such as demyelination.

INTRODUCTION

Cholesterol is a major constituent of the plasma membrane, where it forms lipid raft, cholesterol-rich domain. The concept of lipid raft has attracted attention owing to the accumulation of various receptors thereby serving as hot spots for signal transduction (Lingwood and Simons, 2010). Several G-protein coupled receptors (GPCRs) and tyrosine kinase receptor families possess cholesterol-binding domains termed cholesterol consensus motif (CCM) or cholesterol recognition amino acid consensus (CRAC) motifs, which are considered to stabilize the conformation toward active or inactive receptor states (Cannarozzo et al., 2021; Gimpl, 2016; Faila and Chattopadhyay, 2010). On the other hand, direct activation of signaling pathways by cholesterol binding to effectors has been reported for some GPCRs. Smoothened, a receptor residing in the hedgehog (Hh) signaling pathway, has two cholesterol binding sites, and in addition to the authentic modulation of the receptor function, cholesterol directly activates smoothened and the downstream transcription factor Gli (Deshpande et al., 2019; Huang et al., 2016a; Luchetti et al., 2016).

Hh is a well-known morphogen in organogenesis and neurogenesis during development. In addition to the canonical Hh signaling pathway that activates Gli, non-canonical pathways not linked to Gli have been proposed: namely Type I non-canonical Hh signaling that originates from Patched1, a Hh receptor, but is unrelated to Gli activation through smoothened activation and Type II non-canonical Hh signaling that activates trimeric Gi proteins through smoothened activation (Adachi et al., 2019; Brennan et al., 2012; Jenkins, 2009). We recently identified another non-canonical Hh pathway where Sonic hedgehog, a Hh ligand, and smoothened agonist (SAG) evoke calcium (Ca) response in astrocytes (Adachi et al., 2019), in which smoothened activation, inositol trisphosphate (IP3) production and intracellular Ca release were required. In the course of investigation of this phenomenon, we found that extracellularly applied cholesterol evoked Ca oscillation in astrocytes through the phospholipase C (PLC) and IP3 receptor pathway with different characteristics from the Hh-induced Ca response. Histochemical characterization revealed that neurons and microglia did not respond to applied cholesterol by Ca response. The uprise timing of cholesterol accumulation in the plasma membrane was close to the Ca response lag. Blockade of the cholesterol-induced Ca oscillation resulted in decrease in lipid droplet formation and increased cell death of astrocytes. These results suggest that cholesterol may serve as a signal transducer which directly activates an
Figure 1. Ca oscillation evoked by extracellularly applied cholesterol in astrocytes in mouse hippocampal primary culture

(A) Mouse hippocampal primary culture. Top, a differential interference contrast image; bottom, fluorescence image with Fura-2. Scale bar, 100 μm.

(B) F\textsubscript{340}/F\textsubscript{380} ratio images of the same field of view in A along time. Scale bar, 100 μm.

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unknown signaling pathway that induces Ca oscillation in astrocytes. And the Ca signaling may protect astrocytes from cell death under high cholesterol conditions, such as demyelinating diseases.

RESULTS

Cholesterol/MCD complex induces Ca oscillation only in astrocytes in primary hippocampal culture

To test effects of extracellular cholesterol load to cells in the brain, we applied cholesterol extracellularly to hippocampal primary culture cells and responses were monitored by Ca imaging (Figure 1A). Because cholesterol is insoluble in water, it was conjugated with methyl-β-cyclodextrin (MCD). MCD binds with cholesterol and acts as a water-soluble cholesterol donor (Zidovetzki and Levitan, 2007). After a 10 min baseline period, cells were exposed to a cholesterol/MCD solution (250 μM cholesterol and 2.5 mM MCD, 1:10 molar ratio), and most of the cells exhibited strong Ca oscillation 10–20 min after the exposure (Figures 1B and 1C). During the 50-min period following the exposure, more than 90% of the cells manifested Ca transients (Figure 1Cb). MCD alone did not evoke such Ca oscillation; only spontaneous Ca transients were observed in some cells, which were also observed before the MCD application and in vehicle-treated control cells (Figures 1D and 1E). Ca response was evaluated by the frequency of Ca transients. Ca event frequency was compared among treatments after subtracting Ca event frequency during the baseline period from that after drug application in each cell (ΔFrequency; Adachi et al., 2019). Control vehicle-treated cells and MCD-treated cells showed similar ΔFrequency distributions, while cholesterol-treated cells showed an apparent increase in ΔFrequency (right shift in the cumulative curve, Figure 1F), indicating that extracellular cholesterol application induces robust Ca oscillation in hippocampal cells.

Because the primary culture contained neurons and glial cells, we examined the relationship between the cholesterol-induced Ca oscillations and neuronal excitability. Under 1 μM tetrodotoxin (TTX), a Na channel blocker, cholesterol induced Ca oscillation with even higher frequencies and smaller amplitudes than those without TTX (Figure S1), suggesting that neuronal excitability has some influence on the cholesterol-induced Ca oscillation. Next, we performed Ca imaging combined with post hoc immunohistochemistry to identify the responsible cell types. All the cells were stained either by anti-NeuN or by anti-glial fibrillary acidic protein (GFAP) antibodies (Figure 1G), in agreement with our previous study that the cell population of our hippocampal primary culture preparation is composed mostly of neurons and astrocytes, and that other cell types, such as oligodendrocytes and microglia, are ignorable (Adachi et al., 2019). GFAP-positive cells, namely astrocytes, exhibited cholesterol-induced Ca oscillation (Figure 1H), whereas NeuN-positive cells, neurons, responded to cholesterol with occasional small Ca transients (Figure 1I). The brown trace in Figure 1a shows a periodic signal fluctuation of low amplitude, which we consider to be a crosstalk of the strong fluorescence changes emitted from the overlapping astrocyte because the events in the neuron and the astrocyte are synchronized. The distribution patterns of ΔFrequency and Ca event amplitude of astrocytes both overlapped well with those of all cell populations, whereas those of neurons completely differed (Figures 1J and 1K). This apparent difference in Ca response between astrocytes and neurons promoted us to ask how general is this cholesterol-induced Ca response among cell types. We tested popular cell lines, C6, COS-7, HEK293T, HeLa, and NIH3T3, and none showed any Ca response to bath applied cholesterol (Figure S2). It was unexpected that the C6 cell line did not respond to cholesterol, because it is derived from rat glioma and shares many features with primary cultured astrocytes (Galland et al., 2019). These results suggest that the cholesterol-induced Ca oscillation is not a general response but rather limited in astrocytes and a small number of cell types, if any.
Features of cholesterol-induced Ca oscillation in astrocytes

Because astrocytes turned out to be the player of the cholesterol-induced Ca oscillation, we hereafter used primary astrocyte-rich glial cultures to characterize the phenomenon. Ca oscillation was induced in almost all cells in hippocampal glial culture with 250 μM cholesterol (Figure 2A), similarly to the primary neuron-astrocyte mixed culture (Figure 1). With 100 μM cholesterol, more than 60% of the cells showed Ca oscillation (Figure 2B), and no Ca oscillatory response was observed with 25 μM cholesterol (Figure 2C) and with 2.5 mM MCD without cholesterol (not shown). Cholesterol (250 μM) of cholesterol/MCD complex at 1:20 ratio, the ratio at which MCD is known to become saturated with cholesterol, did not induce strong Ca oscillation in astrocytes (Figure 2D) as seen with 1:10 cholesterol/MCD complex (Figure 2A). Frequency of Ca oscillation showed marked and moderate increases in the 250 and 100 μM cholesterol treated cells, respectively, compared to those of the 25 μM cholesterol, the sole MCD application and the 250 μM 1:20 cholesterol treatment (Figure 2E). The amplitude and half-width of Ca transients in 250 μM cholesterol were the largest, followed by 100 and 25 μM cholesterol, and MCD alone (Figures 2F and 2G). The Ca response had a 5–10 min lag to cholesterol application and reached a plateau frequency level within 15 and 25 min with 250 and 100 μM cholesterol, respectively (Figures 2H and 2I). During the plateau phases, the coefficient of variance of the Ca transient intervals was smaller in astrocytes treated with 100 and 250 μM cholesterol than that in MCD-treated control cells during a corresponding time range (Figure 2J), indicating that the interval of cholesterol-induced Ca oscillation was more regular under cholesterol. When cells with Ca oscillation frequencies greater than 0.005 Hz during these periods were selected, the distribution pattern of Ca event frequency of the 100 and 250 μM cholesterol groups became closer (Figure 2Ka), and mostly overlapped when the threshold was raised to 0.01 Hz (Figure 2La). The distribution of Ca event amplitudes of the 100 and 250 μM cholesterol groups overlapped with either frequency threshold (Figures 2Kb and 2Lb). Half-width of Ca transients was slightly longer in the 250 μM cholesterol group than in the 100 μM group (Figures 2Kc and 2Lc). These results suggest that the frequency of cholesterol-induced Ca oscillation has an upper limit, which was reached in most cells in 250 μM cholesterol, and in fewer cells in 100 μM cholesterol with longer delay than in 250 μM. In accordance with these results, free cholesterol quantity in cultured astrocytes was raised by 2- to 3-fold after 30 min incubation with 250 μM cholesterol compared with the vehicle control (Figure 2M). These properties of cholesterol-induced Ca oscillation seemed to be limited to hippocampal astrocytes: whereas primary astrocytes prepared from cortex and spinal cord, in which astrocytes predominated (Figure S3), also displayed cholesterol-induced Ca oscillation, the frequency and responding cell population were much smaller than those of hippocampal astrocytes (Figure S4). Post hoc immunostaining revealed that the Ca responses observed in the spinal cord culture was most represented by astrocytes, and the contribution of microglia, Iba1-positive cells, to the cholesterol-induced Ca oscillation was very small (Figures S4E–S4I). These results suggest that cholesterol-induced Ca oscillation may occur in astrocytes with different properties depending on the brain area. Hereafter, we used hippocampal astrocytes and 250 μM cholesterol, which showed stable Ca oscillations.

Differences between Hh- and cholesterol-induced Ca responses

We compared the cholesterol-induced Ca oscillation to the SAG-induced Ca response that we reported previously (Adachi et al., 2019). The Ca imaging data obtained from mouse hippocampal astrocyte culture in the previous study were reanalyzed with the same parameters used for the cholesterol-induced Ca oscillation.
Figure 3. Ca oscillation lasts at least 4 h in the presence of cholesterol

(A) Ca imaging traces from Cameleon(YC3.60)-expressing astrocytes. Ca imaging was performed for 10 min before (baseline, a) and 20 min after cholesterol application (b). Thereafter, 10 min Ca imaging was done every 1 h (c–h) from different field of views each time.

(B and C) Cumulative fractions of frequency (a) and amplitude (b) of Ca events with 250 μM cholesterol (B) and vehicle (C). *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney U test.

(D and E) Time course of frequency (D) and amplitude (E) of Ca events with cholesterol (red) and vehicle (gray). ‘+’ represents the average of cells in each culture batch, and lines show average of 5 batches. *p<0.05, **p<0.01, two-way repeated measures ANOVA and post hoc Steel-Dwass tests. Number of cells (number of cultures): cholesterol (base), 292(5); cholesterol (20–30 min), 295(5); cholesterol (60–70 min), 309(5); cholesterol (120–130 min), 309(5); cholesterol (180–190 min), 310(5); cholesterol (240–250 min), 310(5); cholesterol (300–310 min), 310(5).
oscillation in this study. The oscillation frequency in the cholesterol-induced Ca oscillation was much higher than that in the SAG-induced one with slightly larger amplitude (Figure S5A). The SAG-induced Ca response was completely blocked by a connexin gap junction and hemichannel inhibitor, carbenoxolone (CBX), indicating that it relies on the gap junction and/or hemi-channel activity (Adachi et al., 2019), whereas CBX only partially blocked the cholesterol-induced Ca oscillation (Figure S5B). These differences suggest that the features and underlying mechanisms of the cholesterol-induced Ca oscillation do not coincide with those of the SAG-induced Ca response, yet some are shared.

Long lasting cholesterol-induced Ca oscillation
Because the Ca oscillation under extracellular cholesterol appeared constant and did not decay for at least 30 min (Figure 2A), we examined the duration the Ca oscillation holds in the presence of cholesterol. For long-term Ca imaging, a protein-based Ca indicator, YC3.60, was expressed in astrocytes instead of loading Fura2, and culture medium containing 25 mM HEPES (pH 7.40) was used instead of HEPES-buffered saline (HBS). During the baseline period before cholesterol application, much more spontaneous Ca transients were observed than with HBS, presumably because of the presence of serum in the medium (Figure 3A). In this active spontaneous Ca activity background, cholesterol (250 μM) induced Ca oscillation of larger amplitudes than the baseline Ca transients with approximately 15 min lags, which lasted for the recording period up to 6 h (Figures 3AB–3H). Both the frequency and amplitude of Ca oscillation were highest around 20–30 min after cholesterol application and gradually decreased (Figures 3B, 3D, and 3E). Because long exposure of astrocytes to MCD without cholesterol, which was used as a control in previous experiments, reduced the viability of cells, vehicle (water) was used as a control. The time course of frequency decay was similar between the cell groups with or without cholesterol, except for the initial increase in the cholesterol-stimulated group. The increase in amplitude by cholesterol lasted for at least 4 h, whereas the Ca transient amplitude was constant in the control group (Figures 3B, 3C and 3E), thus we conclude that the cholesterol-induced Ca oscillation lasts for at least 4 h in the presence of cholesterol.

Washout of extracellular cholesterol enhances Ca oscillation
We found that washout of extracellularly applied cholesterol activates Ca responses in astrocytes. After a 10-min baseline in HBS, cholesterol was added to the bath, then, after 30 min incubation, it was washed out by superfusing HBS. This washout induced immediate enhancement rather than cessation of Ca oscillation evoked by the cholesterol (Figure 4A). The frequency of Ca events peaked during 0–30 min after washing and decreased gradually (Figure 4C). MCD without cholesterol conjugation did not show such Ca frequency increase after washout (Figures 4B and 4D).

Cholesterol accumulation in the plasma membrane may drive Ca oscillation
The observation that the Ca oscillation did not cease by washout of extracellular cholesterol hinted that the oscillation is driven not by direct action of extracellular cholesterol molecules to the cell surface but by cholesterol molecules newly embedded in the plasma membrane. Cholesterol level in the plasma membrane was examined using the filipin dye, which stains cholesterol mainly in the plasma membrane (Norman et al., 1972). Filipin signal gradually increased 10 min after cholesterol application, while it stayed at a lower level in vehicle-, MCD-, and 1:20 cholesterol/MCD-treated astrocytes (Figures 5A and 5B). The similarity of the timing that this filipin signal uprises and the 10-min lag of the cholesterol-induced Ca oscillation (Figure 2A) implies that the Ca oscillation may be ignited by cholesterol accumulated in the plasma membrane. Cholesterol content in the plasma membrane was kept high with 1:10 cholesterol after 24 h incubation (Figure 5B). When extracellular cholesterol was washed after 30 min incubation, the accumulated membrane cholesterol gradually decreased during the first 6 h and returned to the original level 24 h after the wash (Figure 5C).

Cholesterol-induced Ca oscillation requires the PLC-IP3 pathway
To investigate the molecular mechanisms underlying the cholesterol-induced Ca oscillations in astrocytes, pharmacological experiments were performed. Astrocytes are well known for Ca responses by various
stimuli, where Ca is mobilized from the extracellular space and from intracellular Ca stores. In the latter, sarco/ER Ca2+-ATPase (SERCA) pumps Ca2+ into ER and IP3 receptor and ryanodine receptor release the stored Ca2+ into cytosol. When astrocytes were preincubated with 2-aminoethoxydiphenyldorane (2-APB, 50 μM), an IP3 receptor inhibitor, the cholesterol-induced Ca oscillation was completely blocked (Figure 6A). Thapsigargin (Tg, 100 nM), a SERCA inhibitor, also inhibited Ca oscillation (Figure 6B). These results indicate that intracellular Ca release from ER through IP3 receptor is the key Ca source for the cholesterol-induced Ca oscillation. When astrocytes were preincubated with U-73122 (10 μM), an inhibitor of phospholipase C (PLC), which produces IP3, U-73122 itself evoked temporal Ca transients for several minutes, and following cholesterol application did not evoke Ca oscillation (Figure 6C). U-73343, an inactive analog of U-73122 did not inhibit the cholesterol-induced Ca oscillation (10 μM, Figures 6Cb and 6Cc). Without extracellular Ca, where 1 mM EGTA was added to Ca-free HBS, astrocytes showed cholesterol-induced Ca oscillation with prolonged intervals and smaller amplitudes compared to Ca oscillations evoked in Ca-containing HBS, but still with larger D Frequency than the MCD-applied control group in Ca-free medium (Figure 6D). These results indicate that the PLC-IP3 receptor pathway is the main source of Ca in the cholesterol-induced Ca oscillation.

**Cholesterol increases Ca event frequency in astrocytes in acute hippocampal slice**

Cultured astrocytes have different morphologies and features, including gene expression patterns, from those in the brain (Lange et al., 2012; Zhang and Barres, 2010). We asked if extracellularly applied cholesterol induces Ca responses in astrocytes in acute brain slice preparation, where the properties of astrocytes are closer to those in the brain. After 1–2 h incubation in 250 μM cholesterol, Ca imaging was conducted in dentate gyrus in hippocampal slices prepared from Mlc1-tTA:tetO-YC-Nano50 double transgenic (Mlc1-YC-Nano50) mice, in which a protein-based Ca indicator, YC-Nano50, is expressed under an astrocyte-specific Mlc1-tTA expression system (Figure 7Aa; Kanemaru et al., 2014). On top of the basal Ca activity, which was observed in control slices (Figures 7Ab), preincubation in cholesterol added more Ca transients (Figures 7Ac and 7B). Although the amplitude of Ca transients and Ca-increase area within the astrocytic arbor were similar between the cholesterol-incubated and control slices (Figures 7C and 7D), Ca increased loci within the cholesterol incubated astrocytes were closer to the cell body than the control; in other words, cholesterol induced Ca transients only in the central parts but not in peripheral regions within astrocytes.
Figure 5. Accumulation of cholesterol in plasma membrane by extracellularly applied cholesterol

(A) Time-lapse images of plasma membrane cholesterol levels reported by filipin dye (a). Time course changes of the filipin signal normalized to time 0 when reagents were applied (b). Scale bar, 100 μm. Number of samples were 3 for each trial. Table indicates statistical results between cholesterol 1:10 and other reagents (**p<0.05, ***p<0.01, two-way repeated measures ANOVA and post hoc Steel-Dwass test).

(B) Filipin images after 24 h incubation in vehicle, cholesterol-MCD 1:10 and 1:20 (a) and quantification normalized by vehicle-treated control (b, n = 3). **p<0.01, Mann-Whitney U test. Scale bar, 100 μm.

(C) Time course of filipin signal after washout of MCD alone or cholesterol after 30 min incubation (a). Filipin intensity was quantified and normalized by that of vehicle-treated control (b, n = 3, **p<0.01, ***p<0.001, two-way repeated measures ANOVA and post-hoc Steel-Dwass test). Scale bar, 100 μm.

(Figure 7E). These results suggest that astrocytes in the brain may respond to extracellular cholesterol increase by Ca transients.

Blockage of cholesterol-induced Ca oscillation reduces viability of astrocytes

We examined whether extracellular cholesterol affects viability of astrocyte by staining with a dead cell marker, Ethidium Homodimer III (EthD-III) (Overmeyer et al., 2008), and an apoptosis marker, annexin-V. Since MCD without cholesterol reduced the viability of astrocytes, as mentioned above, vehicle (water) was used as a control. After 24 h incubation, EthD-III positive dead cells accounted for less than 10% of total astrocytes in the control vehicle medium and 25 μM cholesterol, and the rate increased to 30% under 250 μM cholesterol (Figures 8Aa and 8Ab), whereas 1:20 cholesterol/MCD did not alter EthD-III positive cell rate or cell density (Figure S6). When 2-APB (10 μM) was added to the cholesterol incubation, the EthD-III-positive cell rate remarkably increased with the high cholesterol concentrations, 100 and 250 μM. 2-APB itself did not have an apparent effect on the cell death rate. On the other hand, the cholesterol and/or the additional 2-APB did not obviously affect the ratio of annexin-V positive cells (Figure 8Ac), suggesting that the astrocyte death caused by the high concentrations of extracellular cholesterol and additional 2-APB was not because of apoptosis. Cell density, another index of viability, was decreased under 100 and 250 μM cholesterol incubation, which was accelerated by the addition of 2-APB (Figure 8Ad). The finding that 2-APB enhanced the deteriorating effect of cholesterol on astrocytes implies that Ca oscillation, which should have been blocked under 2-APB, may have an ameliorating effect on the survival of astrocytes under high extracellular cholesterol concentration.

The expression of GFAP in astrocytes is known to be promoted under stress conditions, including trauma and ischemia in the brain (Eng and Ghirnikar, 1994), and activation of GFAP expression was reported in cultured astrocytes after incubation in 50 μM cholesterol with ethanol as solvent for 48 h (Avila-Muñoz and Arias, 2015). Immunohistochemistry showed stronger GFAP signals in astrocytes treated with 250 μM cholesterol conjugated with MCD for 48 h in accordance with the previous report, without noticeable morphological changes compared to control astrocytes that were incubated with vehicle (Figure 8Bb). RT-PCR revealed an apparent increase in gfas transcripts in astrocytes treated with 50 μM ethanol dissolved- and 250 μM MCD-conjugated-cholesterol for 48 h, but not in those incubated for 24 h (Figure 8Bb). Compared to the rise time of GFAP protein expression (12 h) reported for cocaine or lipopolysaccharide (LPS) stimulation (Brahmachari et al., 2006; Yang et al., 2016), the increase in GFAP transcription under extracellular cholesterol took much longer time (48 h).

Ca oscillation plays a role in lipid droplet accumulation in astrocytes under high extracellular cholesterol

Cells store excess cholesterol in lipid droplets (Ikonen, 2008). Astrocytes remarkably increased the number of lipid droplets under excess extracellular cholesterol within 24 h (Figure 9). The intensity of Nile red, a lipid droplet marker, increased according to cholesterol concentration, and discernible bright dots, namely lipid droplets, appeared with 100 and 250 μM cholesterol (Figures 9Aa and 9Ab). When extracellular cholesterol was washed after 30 min incubation with 250 μM cholesterol, apparent lipid droplet formation appeared 6 h after the wash, which decreased to the baseline level at 24 h (Figure S7). When 2-APB, which blocks Ca oscillation, was added to the cholesterol incubation, lipid droplets became inconspicuous, but the overall Nile red signal tended to become even stronger, with the lipid droplet size affected especially in 25 μM cholesterol (Figure 9). Although the comparisons of the lipid droplet size between the 2-APB-incubated and DMSO control groups indicate statistical significances in the 25, 100 and 250 μM cholesterol conditions, the overall size distribution patterns of the two groups in 100 and 250 μM cholesterol look similar (Figure 9Bc). We consider that the lipid droplet size was not essentially affected by the 2-APB treatment in 100 and 250 μM cholesterol, but the statistics flagged them because of the large number of lipid droplets
to be compared (see the legend to Figure 9). These results suggest that Ca oscillation may play an important role in lipid droplet formation in astrocytes in excess cholesterol conditions.

**DISCUSSION**

In this study, we revealed that extracellular cholesterol application induces robust Ca oscillation only in astrocytes and that the cholesterol-induced Ca oscillation may be required for the survival of astrocytes under extracellular high cholesterol stress. Because the cholesterol-induced Ca oscillation lasted even after extracellular cholesterol washout and the rise time of cholesterol content in plasma membrane was close to the lag of Ca oscillation start, we consider that the cholesterol-induced Ca oscillation is triggered by the increase in cholesterol embedded in the plasma membrane. Our results further imply that the cholesterol-induced Ca oscillation may be required for the survival of astrocytes in a high-cholesterol environment by properly handling internalized cholesterol by storing them as lipid droplets.

The fact that cholesterol-induced Ca oscillation requires activation of the PLC-IP3 receptor pathway suggests that accumulated cholesterol in the plasma membrane may activate plasma membrane-resident
receptors that activate PLC, such as GPCRs and receptor tyrosine kinases. The notion that several GPCRs and receptor tyrosine kinases have cholesterol-binding domains (Gimpl, 2016; Paila and Chattopadhyay, 2010) supports this idea. Furthermore, it was reported that cholesterol application increases PLC expression in the plasma membrane within 30 min (Chun et al., 2013). It is tempting to elucidate the molecular mechanism that transduces cholesterol load in the plasma membrane to the PLC activity and leads to Ca oscillation in astrocytes. Our data imply that smoothened, which possesses cholesterol-binding domains and is activated by cholesterol (Deshpande et al., 2019; Huang et al., 2016a; Luchetti et al., 2016), is not the sole candidate for the membrane receptors responsible to the cholesterol-induced Ca oscillation in astrocytes because there are differences in Ca response properties, such as Ca response kinetics and participation of gap junction. The fact that the cholesterol-induced Ca oscillation was not observed in neurons (Figure 1) and numbers of cell lines including the glioma-derived C6 cell line (Figure S2) but only in astrocytes implies that the putative plasma membrane receptor(s) that detects cholesterol increase in the plasma membrane may be expressed selectively in astrocytes. And the difference in the properties of cholesterol-induced Ca oscillation among astrocytes from hippocampus, cortex and spinal cord (Figures 2 and S4) may also reflect differences in the expression level of the putative cholesterol-sensitive receptor and/or its downstream signaling elements, as the heterogeneity of astrocytes among brain regions and even in the same region is obvious (Batiuk et al., 2020).

In this study, we revealed that blockade of cholesterol-induced Ca oscillation exacerbated astrocytic death under high cholesterol load. Although cholesterol-induced apoptotic cell death has been reported in vascular smooth muscle cell, pancreatic β cell, and the SH-SYSY human neuroblastoma cell line (Huang et al., 2016b; Lu et al., 2011; Yin et al., 2000), the astrocytic death observed in this study was not apoptotic, as the apoptotic marker, annexin-V, was negative. However, it has been reported that cholesterol accumulation induces necroptosis, a regulated form of necrosis, in SH-SYSY cells (Funakoshi et al., 2016). Detailed mechanisms that led to cell death observed in this study are intriguing. It is noteworthy that the two phenomena, lipid droplet formation and prevention of astrocytic death from cholesterol load, were both related to the cholesterol-induced Ca oscillation. It would be tempting to determine whether lipid droplet formation is correlated with the avoidance of cell death.

Figure 7. Cholesterol raises frequency of Ca events in astrocytes in hippocampal slice
(A) YC-Nano50 expressing astrocytes in dentate gyrus in a hippocampal slice (a) and typical traces from vehicle-incubated control (b) and cholesterol-incubated (c) astrocytes. Scale bar, 100 μm.
(B) Ca transient frequency of vehicle- and cholesterol-treated astrocytes. ***p<0.001, Mann-Whitney U test.
(C–E) Distribution of Ca increase area within astrocytic arbor (C), amplitude of Ca event (D), and distance between Ca-increase locus and cell body (E). ***p<0.001, Mann-Whitney U test. Number of cells (number of slices/number of mice): cholesterol, 85(8/3), vehicle, 67(6/3).

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Lipid droplets are organelles that store excess neutral lipids, such as triacylglycerol and sterol ester. Excess cholesterol in the cell is esterified by acyl-CoA: cholesterol O-acyltransferase (ACAT), and lipid droplet biogenesis occurs at the ER membrane (Olzmann and Carvalho, 2019). In this study, Nile red staining

![Cell death and GFAP expression induction in cholesterol treated astrocytes](image)

**Figure 8. Cell death and GFAP expression induction in cholesterol treated astrocytes**

(A) Cell viability assay after 24 h cholesterol incubation with 2-APB or DMSO as a vehicle control by staining with EthD-III (dead cell marker, green) and Hoechst (nuclear marker, magenta; a). Ab and c: proportion of EthD-III (b) and Annexin-V (p = 0.68, Kruskal-Wallis test; c) positive cells (black bars, 2-APB treatment; gray bars, DMSO treatment). Ad: cell density normalized by the vehicle control without 2-APB. Five independent experiment sets were performed. Scale bar, 100 μm. *p<0.05, **p<0.001, Kruskal-Wallis test and post hoc Steel-Dwass test.

(B) Astrocytes stained with anti-GFAP antibody (white) and DAPI (magenta) after incubation with 250 μM cholesterol conjugated with MCD, 50 μM cholesterol dissolved in ethanol, and vehicle (addition of equivalent volume of water to the medium) for 24 and 48 h (a). Scale bar, 100 μm. Bb: relative gfap mRNA expression levels by real-time PCR (n = 4, respectively). ***p<0.001, Kruskal-Wallis test and post hoc Steel-Dwass test.

See also Figure S6.
revealed that droplet formation by cholesterol was depressed in 2-APB treated astrocytes; however, the signal level was rather increased with an even distribution pattern throughout the cytoplasm. Because Nile red stains neutral lipids, cholesterol per se is not stained; it must be esterified to be stained (Fowler and Greenspan, 1985). Thus, the cholesterol-induced Ca oscillation may play a role in lipid droplet biogenesis and possibly in cholesterol esterification. Phospholipase D1 (PLD1) is reported to be necessary for lipid droplet biogenesis and is located downstream of the extracellular signal-regulated kinase (ERK) signaling pathway (Andersson et al., 2006; Marchesan et al., 2003). It has been reported that the activities of ERK and an ERK activator, Ras, are positively regulated by the Ca oscillation frequency in HeLa cells (Kupzig et al., 2005). It is possible that the cholesterol-induced Ca oscillation promotes lipid droplet biogenesis through activation of the Ras-ERK signal, together with different scenarios that involve a variety of Ca-activated enzymes, including calcineurin, calpain, and Ca\(^{2+}\)/calmodulin-dependent protein kinases (Bagur and Hajnoczky, 2017), and downstream factors, such as nuclear factor of activated T-cells (NFAT), NF-\(\kappa\)B, and c-jun N-terminal kinase (JNK) (Crabtree, 1999; Dolmetsch et al., 1997, 1998).

Under physiological conditions, extracellular cholesterol is conveyed by apolipoprotein, and the lipoprotein-cholesterol complex is captured by low-density lipoprotein (LDL) receptor families on the plasma
membrane and taken up by endocytosis. In this study, we used cholesterol/MCD complex instead of lipoprotein-cholesterol complex, and we confirmed that the cholesterol content in astrocytes was increased by cholesterol quantification (Figure 2M) and filipin staining (Figure 5). Therefore, cholesterol may have been taken up by astrocytes through a different mechanism from that for lipoprotein-cholesterol complex uptake. Free cholesterol transfer from low- and high-density lipoproteins to the plasma membrane has been reported in several cell types (Fong and Angel, 1989; Wustner et al., 2004; Axmann et al., 2019). Under an LDL overload condition where cholesterol content in aortic smooth muscle cells approximately doubled, the route of 40% of the increased cholesterol was estimated to be via free cholesterol transfer to the cell surface (Slotte et al., 1988). It is likely that free cholesterol transfer from lipoproteins to plasma membrane may occur under high cholesterol conditions, which would induce Ca oscillation and subsequent events in astrocytes in the brain as observed in this study.

The 2- to 3-fold increase in free cholesterol content by incubation with 250 μM cholesterol (Figure 2M) overlaps with the free cholesterol content ranges reported in pathological conditions: about 1.5-fold higher cholesterol content in brain tissues of a hypercholesterolemia model mouse (Paul and Borah, 2017) and 1.5- to 3-fold higher accumulation of unesterified free cholesterol in a Niemann-Pick disease type C model fibroblasts (Newton et al., 2020; Xu et al., 2012). Thus, the cholesterol increase by incubation with cholesterol observed in this study could be relevant to pathological conditions.

Intracellular Ca increase has potential to produce reactive oxygen species (ROS) (Reynolds and Hastings, 1995; Tauffenberger and Magistretti, 2021), and cholesterol is susceptible to oxidation reactions to be transformed to oxysterols (Murphy and Johnson, 2008). Both ROS and oxysterols are reactive and influence variety of signaling pathways. It would be possible that the cholesterol-induced Ca oscillation activated ROS production, and the increased ROS, together with oxysterols converted from cholesterol by ROS, played some roles in the phenomena observed in this study, namely long-lasting Ca oscillation and lipid droplet formation. Further studies would be awaited.

The involvement of cholesterol in neurodegenerative diseases has been demonstrated. In Alzheimer’s disease (AD), amyloid β (Aβ) production is reported to be promoted by increased membrane cholesterol in both neurons and astrocytes (Avila-Muñoz and Arias, 2015; Wang et al., 2021), and AD-related proteins such as amyloid precursor protein (APP), Aβ, and β-site APP cleaving enzyme 1 (BACE-1) are highly expressed in hypercholesterolemic model brains (Chen et al., 2016; Refolo et al., 2000; Thirumangalakudi et al., 2008), which was ameliorated by statin, a cholesterol biosynthesis blocker (DeKosky, 2005; Langness et al., 2020). These results suggest that cholesterolemia can cause cholesterol accumulation in the brain, leading to the AD pathology. Also the blood concentration of 24-OHC, a cholesterol metabolite mainly produced in the brain, is increased in patients of AD and multiple sclerosis (Lütjohann et al., 2000; Zhoritsky et al., 2016), suggesting that excess extracellular free cholesterol conditions may exist in pathological brains because of demyelination or neuronal death. Taking these lines of evidence into consideration, it would be intriguing to imagine that astrocytes under neurodegenerative diseases especially accompanying demyelination may be exposed to high extracellular cholesterol, and thus, the astrocytic reactions to cholesterol load revealed in this study, that is Ca oscillation leading to survival of astrocytes possibly through uptake and storage of cholesterol as lipid droplets, are taking place as an early step in the etiology. Further studies that link the cholesterol-induced events in astrocytes revealed in this study and neurodegenerative diseases are awaited.

**Limitation of the study**

In this study, we revealed that extracellularly applied cholesterol induces strong Ca oscillation in astrocytes, however two major questions remain. The first one is the identity of the receptor to cholesterol. It can be narrowed down to receptor(s) that activate PLC. Receptor screening is needed as a next step. The second question is whether the cholesterol-induced Ca oscillation observed in cultured astrocyte actually occurs in astrocytes in physiological and pathological brains. In this study, cholesterol was fed to cells by cholesterol/MCD complex which is not a natural composition but possesses high capacity for providing cholesterol in aqueous environment. Because the cholesterol content in the myelin in the brain is very high, extracellular cholesterol content in brain parenchyma could be raised very high on demyelination. Future works should examine whether astrocytes in the brain exhibit robust Ca oscillation because of extracellular cholesterol load on demyelination.
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105138.

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AUTHOR CONTRIBUTIONS
Conceptualization, C.A. and T.I.; Methodology, C.A. and T.I.; Software, T.I.; Validation, C.A. and T.I.; Formal Analysis, C.A. and T.I.; Investigation, C.A. and S.O.; Data Curation, C.A.; Writing – Original Draft, C.A. and T.I.; Writing – Review and Editing, C.A. and T.I.; Visualization, C.A.; Supervision, T.I.; Project Administration, T.I.; Funding Acquisition, C.A. and T.I.

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The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-NeuN | Millipore | Cat# MAB377; RRID: AB_2298772 |
| Goat polyclonal anti-GFAP | Abcam | Cat# ab3553; RRID: AB_880202 |
| Rabbit polyclonal anti-Iba1 | FUJIFILM Wako | Cat# 019-19741; RRID: AB_839504 |
| Donkey anti-mouse IgG H&L (Alexa Fluor 594) | Abcam | Cat# ab150108; RRID: AB_2732073 |
| Chicken anti-goat IgG (H + L) antibody, Alexa Fluor 488 conjugated | Molecular probes | Cat# A-21467, RRID: AB_141893 |
| Donkey anti-Rabbit IgG (H + L) Highly cross-absorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-21207; RRID: AB_141637 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Cholesterol | Nacalai tesque | 08721-62, CAS: 57-88-5 |
| Methyl-ß-cyclodextrin | Sigma-Aldrich | 332615, CAS: 128446-36-6 |
| Tetrodotoxin | LATOXAN | L8503, CAS: 4368-28-9 |
| 2-APB | Tokyo Chemical Industry | D0281; CAS: 524-95-8 |
| Thapsigargin | Nacalai tesque | 33637-31; CAS: 67526-95-8 |
| U-73122 | Cayman chemical | 70740; CAS: 112648-68-7 |
| U-73343 | Cayman chemical | 17339; CAS: 142878-12-4 |
| Carbenoxolone | Sigma-Aldrich | C4790, CAS: 7421-40-1 |
| Fura 2-AM solution | Dojindo | 343-05401; CAS: 108964-32-5 |
| Nile red | Thermo Fisher Scientific | N-1142 |
| Filipin | Sigma-Aldrich | F9765 |
| **Critical commercial assays** |        |            |
| Apoptotic/Necrotic/Healthy Cells Detection Kit | PromoCell | PK-CA707-30018 |
| Amaxa Basic Nucleofector Kit for primary mammalian glial cells | Lonza | Cat# VPI-1006 |
| ISOGEN II RNA extraction kit | NiPPON GENE | 311-07361 |
| THUNDERBIRD SYBR qPCR/RT kit | Toyobo | QPS201/FSQ101 |
| AmpexTM Red Cholesterol Assay Kit | Invitrogen | A12216 |
| **Experimental models: Cell lines** |        |            |
| Rat: C6 cells | JCRB | Cat# IFO50110 |
| African green monkey: COS-7 cells | N/A | N/A |
| Human: HEK293T cells | N/A | N/A |
| Human: HeLa cells | N/A | N/A |
| Mouse: NIH/3T3 cells | N/A | N/A |
| **Experimental models: Organisms/strains** |        |            |
| Mouse: Scl:ICR | Japan SLC | N/A |
| Mouse: Mc1-tTA::tetO-YC-Nano50 | Kanemaru et al., 2014 | N/A |
| **Oligonucleotides** |        |            |
| Primer: mouse gfap forward: 5’-TTGCTGGAGGGCGAGAAAAA-3’ | This paper | N/A |
| Primer: mouse gfap reverse: 5’-TGCTTTTGCCCTCCAAG-3’ | This paper | N/A |
| Primer: β-actin forward: 5’-GCTCTAGCACCATTGAGAAC-3’ | This paper | N/A |
| Primer: β-actin reverse: 5’-ACTCTCCTGCTTGATCCAC-3’ | This paper | N/A |

(Continued on next page)
# RESOURCE AVAILABILITY

## Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takafumi Inoue (inoue.t@waseda.jp).

## Materials availability

This study did not generate new unique reagents.

## Data and code availability

- All data reported in this article will be shared by the lead contact on request.
- This article does not report original code.
- Any additional information required to reanalyze the data reported in this article is available from the lead contact on request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Animals

Female 4-8-week-old Mlc1-tTA:tetO-YC-Nano50 mice (Kanemaru et al., 2014) gifted from Dr. K. F. Tanaka (Keio University) were used in this study. Mice were housed under a constant ambient temperature and relative humidity. Less than five mice were housed per cage under a 12-h light/dark cycle and provided food and water ad libitum. Genotyping was conducted using the primer sets reported previously (see the key resources table). Animal care was in accordance with guidelines outlined by the Institutional Animal Care and Use Committee of Waseda University. The animal experiment protocol was approved by the Committee on the Ethics of Animal Experiments of Waseda University. All efforts were made to minimize the number of animals used and their suffering during experiments.

### Primary cell cultures and cell lines

All primary cell cultures were prepared from embryonic day (E) 17 ICR mice purchased from Sankyo Labo Service Corporation (Tokyo, Japan). Primary hippocampal neuron-astrocyte co-culture was harvested in Neurobasal-A medium supplemented with NeuroBrew-21, L-glutamine and penicillin/streptomycin, and primary glial cultures were maintained in DMEM/Ham’s F12 medium supplemented with 5% horse serum, 5% fetal bovine serum, L-glutamine and penicillin/streptomycin. All cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cell cultures were incubated at 37°C and 5% CO₂. No cell authentication was performed.

## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primer: genotyping tetOup: 5’-AGCAGAGCTCGTATAGTGAAACGGT-3’ | Kanemaru et al., 2014 | N/A |
| Primer: genotyping intronlow: 5’-AAGGGCAGGATGAGCAGACGGATTG-3’ | Kanemaru et al., 2014 | N/A |
| Primer: genotyping MlcU-657: 5’-AAATTCAGGAAGCCTGCTGCTGCTG-3’ | Kanemaru et al., 2014 | N/A |
| Primer: genotyping mTA24L: 5’-CAGAGTTGACACCTTGAGTTG-3’ | Kanemaru et al., 2014 | N/A |
| Recombinant DNA | | |
| Plasmid: YC3.60 | Nagai et al., 2004 | N/A |
| Software and algorithms | | |
| TI Workbench | Inoue, 2018 | http://inouelab.biomed.sc.waseda.ac.jp/inouelab-web/tiwb.html |
| R (version 4.0.3) | Ihaka and Gentleman, 1996 | https://www.r-project.org/ |
| Igor Pro | WaveMetrics | http://www.wavemetrics.com/products/igorpro/igorpro.htm |
| | | RRID:SCR_000325 |
METHOD DETAILS

Reagents

Cholesterol (08721-62, Nacalai tesque, Kyoto, Japan)/methyl-β-cyclodextrin (MCD, 332615, Sigma-Aldrich, Tokyo, Japan) complex was prepared as described previously (Huang et al., 2016a) with the molar ratio of MCD to cholesterol 10:1 or 20:1. Briefly, 40 mM cholesterol in EtOH and 40 mM MCD in water were mixed 1:10 or 1:20, and evaporated under vacuum for 1-2 weeks. The dried complex was dissolved in water to set the cholesterol concentration at 2.5 mM and stored at 4°C. TTX (Latoxan Laboratory, Portes lès Valence, France) was dissolved in 12.7 mM citrate solution at 1 mM and stored at 4°C. 2-APB (D0281, Tokyo Chemical Industry, Tokyo, Japan) was dissolved in Dimethyl Sulfoxide (DMSO, D2650, Sigma-Aldrich) at 50 mM and stored at −20°C. Thapsigargin (Tg, 33637-31, Nacalai tesque) was dissolved in DMSO at 100 μM and stored at −20°C. U-73122 and U-73343 (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in DMSO at 2 mM and stored at −20°C. Carbenoxolone (CBX; C4790, Sigma-Aldrich) was dissolved in DMSO at 100 mM and stored at −20°C.

Cell culture

Primary hippocampal neuron-astrocyte co-culture

Dissected hippocampi from E17 ICR mice were digested with 0.25% papain (Worthington Biochemical Corp., Lakewood, NJ, USA) containing 1% DNase (Roche, Basel, Switzerland) in Glucose mix [0.4% BSA (Sigma-Aldrich) and 0.04% L-cysteine (Nacalai tesque) in phosphate-buffered saline (PBS)] at 37°C for 5 min. Dissociated cells were seeded on poly-ethyleneimine (PEI, Sigma-Aldrich)-coated round glass coverslips (12 mm in diameter, 1 × 10⁵ cells/cover slip, Matsunami Glass, Tokyo, Japan) in 24 well-plate in Neurobasal-A medium (GIBCO, Tokyo, Japan) containing 2% NeuroBrew-21 (Miltenyi Biotec, Tokyo, Japan), 1% L-glutamine (GIBCO), 5 U/mL penicillin and 5 μg/mL streptomycin (Nacalai tesque). Day in vitro (DIV) 10–15 cultures were used for experiments.

Primary hippocampal glial culture

Four hippocampi taken from two E17 ICR mice were digested with 0.25% papain containing 1% DNase in Glucose mix at 37°C for 5 min. Dissociated cells were processed and used for experiments as described for hippocampal neuron-astrocyte co-culture.

Primary cortical glial culture

A cortical hemisphere taken from E17 ICR mouse was digested with 0.25% papain containing 1% DNase in Glucose mix at 37°C for 30 min. Dissociated cells were processed and used for experiments as described for hippocampal glial culture.

Primary spinal cord glial culture

A spinal cord was isolated from an E17 ICR mouse by hydraulic extrusion (Richner et al., 2017) and digested with 0.25% papain containing 1% DNase in Glucose mix at 37°C for 20 min. Dissociated cells were processed and used for experiments as described for hippocampal glial culture.

Cell lines

C6 (JCRB Cat#IFO50110, RRID:CVCL_0194), COS-7, HEK293T, HeLa and NIH/3T3 cell lines were seeded on 10-cm dishes in DMEM containing 10% fetal bovine serum, 5 U/mL penicillin and 5 μg/mL streptomycin. After confluence, cells were collected using 0.05% trypsin in Ca and Mg free HBSS and plated on Matrigel (BD Biosciences, NJ, USA)-coated round glass coverslips with the same culture medium at 5 × 10⁵ cells/cover slip. Cultures at 70–90% confluency were used for Ca imaging.

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde/PBS (Nacalai tesque) for 15 min, permeabilized with 0.25% Triton X-100 (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) in PBS for 10 min and blocked with 1% bovine
serum albumin (BSA, A9647, Sigma-Aldrich) in PBS for 1hr at room temperature (RT). Cells were incubated with primary antibodies (1:500 dilution in 1% BSA) overnight at 4°C and with secondary antibodies (1:1000 dilution in 1% BSA) for 1 h at RT. Primary antibodies used were: anti-NeuN (Cat#: MAB377, Millipore, Tokyo, Japan, RRID:AB_2298772), anti-GFAP (Cat#: ab53554, Abcam, Tokyo, Japan, RRID:AB_880202), and anti-Iba1 (Cat# 019-19741, FUJIFILM Wako Shibayagi Corp, Shibukawa, Japan; RRID:AB_839504). Secondary antibodies used were: Alexa Fluor 594 donkey anti-mouse IgG H&L (Cat# ab150108, Abcam, RRID:AB_2732073), Alexa Fluor 488 chicken anti-goat IgG H&L (Cat# A-21467, Molecular Probes, RRID:AB_141893), and Alexa Fluor 594 donkey anti-rabbit IgG (H+L) (Cat# A-21207, Thermo Fisher Scientific, RRID:AB_141637). Nuclei were stained with DAPI (sc-3598, Santa Cruz Biotechnology, Dallas, TX, USA). Images were obtained with an inverted microscope (IX71, Olympus, Tokyo, Japan) with a 20×3 objective (UApo/340, N.A. 0.75, Olympus) and a cooled-CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan).

Filipin staining
Cells were fixed with 4% paraformaldehyde/PBS for 15 min and stained with 50 mg/mL filipin (F9765, Sigma-Aldrich) in PBS for 30 min. The cells were then washed with PBS and observed with the same fluorescence microscope system as above with 330–385 nm band-pass excitation and 420–460 nm band-pass emission filters. Images were taken from 20 locations per sample, and signal intensity was obtained from 5 cells per field of view by placing a 33.3×33.3 μm-sized square region of interest (ROI) inside each cell.

Cell culture imaging
Coverslips holding cultured cells were mounted in a stainless-steel chamber (SCC12-D35-SET, Tokai Hit, Fujinomiya, Japan) containing HEPES-buffered saline (HBS; in mM, 20 HEPES, 115 NaCl, 5.4 KCl, 1 M MgCl₂, 2 CaCl₂, 10 glucose, pH 7.4). A Ca indicator, Fura-2, was loaded to cells by incubation with 2.5 μM Fura-2-AM (Dojindo, Kumamoto, Japan) in HBS at 37°C for 10 min followed by three washes with HBS. Time-lapse imaging was performed with the fluorescence microscope described above. Fura-2 imaging was performed as described (Adachi et al., 2019) with a modification in the baseline correction method: asymmetrically reduced penalized least squares smoothing method (Baek et al., 2015) built in the TI Workbench software (Inoue, 2018) was used for baseline correction. Threshold for Ca transient detection was set as 6× standard deviation of the baseline. Oval ROIs were set on all cells identified by the Fura-2 staining in each field of view.

Gene transfection and YC3.60 imaging
Plasmid DNA encoding YC3.60 (Nagai et al., 2004) was introduced into hippocampal glial culture by electroporation using the Amaxa Basic Nucleofector kit for Primary Mammalian Glial Cells (Lonza Japan, Tokyo, Japan) according to the manufacturer’s protocol with modifications. In brief, cultured astrocytes were stripped from dishes with 0.025% trypsin for 10 min at 37°C. 1.0×10⁶ cells were centrifuged at 80 g for 10 min at RT. Collected cells were diluted in the nucleofection solution with 3 μg plasmid DNA and electroporated with the T-020 setting of the electroporator (Nucleofector 2b, Lonza Japan), followed by a recovery step with 500 μL equilibrated culture medium. Transfected astrocytes were divided into 12 equal portions and placed on PLL-coated round coverslips (12 mm in diameter). Equilibrated culture medium was added gently just after. Two–five days after transfection, cells at 60–80% confluency were used for Ca imaging. During this set of imaging experiments culture medium containing 25 mM HEPES (pH 7.40) was used as an extracellular medium rather than HBS because of the long recording periods (6 h). YC3.60 imaging was performed with the fluorescence microscope system described above with a 10× objective (UPlanApo, NA. 0.40, Olympus). YC3.60 was excited with light passed through a 426–496 nm band-pass filter, and image pairs of alternating emission wavelength (467–496 nm and 515–560 nm for CFP and YFP, respectively) were taken every 5 s. The fluorescence intensity ratio (FYFP/FCFP) was calculated at each time point in each ROI by dividing fluorescence intensity of YFP by that of CFP after background subtraction. Baseline determination was done as described for Fura-2 imaging, and threshold for event detection was set to 2× standard deviation of the baseline.

Cholesterol quantification
Cholesterol was quantified with Amplex™ Red Cholesterol Assay Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer’s protocol. Cells were washed twice with ice cold PBS and then lysed in 200 μL reaction buffer. 50 μL of each sample was added to 50 μL assay solution containing 300 μM Amplex Red.
reagent, 2 U/mL HRP and 2 U/mL cholesterol oxidase. After preincubation at 37°C for 30 min, fluorescence was measured using excitation at 560 nm and emission at 590 nm with a microplate reader (Synergy H1, Wakenyaku Co., Ltd., Kyoto, Japan). Relative amount of cholesterol was calculated using a standard curve obtained with the cholesterol standard solution.

**Real-time PCR**

Total RNA was isolated from hippocampal glial culture plated in a 6-well plate using ISOGEN II RNA extraction kit (NIPPON GENE, Tokyo, Japan) according to the manufacturer’s protocol. cDNA was constructed by reverse transcription using 200–300 ng isolated RNA, and real-time PCR was conducted using THUNDERBIRD SYBR qPCR kit (Toyobo, Osaka, Japan) according to the manufacturer’s protocol with StepOnePlus Real-Time PCR System (Applied Biosystems, Bedford, MA, USA). Primers used were: mouse *ggfa* forward, 5’-TTGCTGGAGGGCGAAGAAAA-3’; reverse, 5’-TGCTTTTGCCCCCTCGGAT-3’; mouse *β*-actin forward, 5’-GCTCCTAGCACCATGAAGA-3’; reverse, 5’-ACTCCTGCTTGCTGATCCAC-3’.

**Cell viability assay**

Cell viability assay was performed using Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell, Heidelberg, Germany) according to the manufacture’s protocol. Cells were washed twice with binding buffer, and stained with FITC-Annexin V, ethidium homodimer III and Hoechst 33,342 for 30 min at RT. Images were taken as described in the “Immunohistochemistry” section. Twenty images were taken per sample and positive cell number in each image was averaged.

**Ca imaging in brain slice**

Hippocampal slices, 400 μm thickness, were prepared from 4- to 8-week-old Mlc1-tTA:tetO-TC-Nano50 female mice (Kanemaru et al., 2014) using a standard method (Samios and Inoue, 2014) and used within 12 h after preparation. Continuous superfusion of extracellular solution (artificial cerebrospinal fluid; ACSF; in mM, NaCl 124; KCl 2.5; NaH2PO4 1.25; NaHCO3 26; MgCl2 2; CaCl2 2; glucose 20; bubbled with 95% O2 and 5% CO2 gas mixture) at 3–5 mL/min in imaging chamber on the microscope stage is necessary for acute brain slice imaging. Because continuous superfusion of slices with cholesterol-containing ACSF was technically difficult, hippocampal slices were incubated in still ACSF containing 250 μM cholesterol or vehicle (water, 1:10 vol:vol) in ACSF, for 1–2 h in slice incubation chambers (Dorris et al., 2014), then transferred to imaging chamber on the microscope stage. Time-lapse imaging was performed with an in-house two-photon microscope (Inoue, 2018) mounted on an upright microscope (BX51, Olympus) with a 20× water immersion objective (XLUMPlanFl, N.A. 0.95, Olympus). Brain slices were continuously superfused with ACSF. An excitation laser of wavelength 860 nm (titanium-sapphire pulse laser, Mai Tai DeepSee, Spectra-Physics, Santa Clara, CA, USA) was used. The emission was divided with a 500–550 nm band-pass filter for the CYP and YFP signals, respectively. Data were obtained every 5 s and converted to the relative change in fluorescence, FYFP/FCFP, with TI Workbench, then analyzed with the AQuA software (Wang et al., 2019) using the built-in cytosolic GCaMP6f ex vivo preset parameter with modifications: minimal pixel size, 25; seed growing threshold, 2; correct baseline trend, 2. Ca events of which duration (period above 10% of peak amplitude) was shorter than 20 s were discarded.

**Lipid droplet staining**

Cells were fixed with 4% paraformaldehyde/PBS for 15 min and stained with 1 mg/mL Nile red (N-1142, Thermo Fisher Scientific, Tokyo, Japan) in PBS for 30 min. After staining, cells were washed with PBS for 3 times and observed using a confocal microscope (FV-300, Olympus) equipped on an inverted microscope (IX-70, Olympus) with a 60× water immersion objective (UPlanSApo, N.A. 1.2, Olympus). Nile red was excited using a 561 nm diode-pumped solid-state laser (BS-YCA-010-040, Melles Griot, Rochester, NY, USA), and the fluorescence was detected through a 565 nm long-pass filter. To quantify dot like structures, Nile red images were processed with a watershed segmentation algorithm (Vincent and Soille, 1991) equipped in TI Workbench, and particles consisting of more than 4 pixels of intensity larger than 300 (a.u.) were taken into account.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

For comparison between two groups, Mann-Whitney U test included in the ALGLIB library (www.alglib.net) implemented in TI Workbench was used unless otherwise indicated. For multiple groups,
Kruskal-Wallis test and post hoc Bonferroni corrected Mann-Whitney U test was performed with R (version 4.0.3; Ihaka and Gentleman, 1996) unless otherwise mentioned. Sample numbers were described in figure legends. *p* values less than 0.001, 0.01, 0.05 were indicated by ***, ** and *, respectively. Box and whisker plots indicate median as the horizontal line, interquartile range as the box and 10 and 90% ranges as the whiskers.