Cholesterol-dependent balance between evoked and spontaneous synaptic vesicle recycling

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Cholesterol is a prominent component of nerve terminals. To examine cholesterol’s role in central neurotransmission, we treated hippocampal cultures with methyl-β-cyclodextrin, which reversibly binds cholesterol, or mevastatin, an inhibitor of cholesterol biosynthesis, to deplete cholesterol. We also used hippocampal cultures from Niemann-Pick type C1-deficient mice defective in intracellular cholesterol trafficking. These conditions revealed an augmentation in spontaneous neurotransmission detected electrically and an increase in spontaneous vesicle endocytosis judged by horseradish peroxidase uptake after cholesterol depletion by methyl-β-cyclodextrin. In contrast, responses evoked by action potentials and hypertonicity were severely impaired after the same treatments. The increase in spontaneous vesicle recycling and the decrease in evoked neurotransmission were reversible upon cholesterol addition. Cholesterol removal did not impact on the low level of evoked neurotransmission seen in the absence of synaptic vesicle SNARE protein synaptobrevin-2 whereas the increase in spontaneous fusion remained. These results suggest that synaptic cholesterol balances evoked and spontaneous neurotransmission by hindering spontaneous synaptic vesicle turnover and sustaining evoked exo-endocytosis.

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Cholesterol is a major lipid component of cellular membranes and regulates the degree of membrane fluidity. The presence of cholesterol is thought to be important for proper synapse structure and function in the brain. Experiments in which cholesterol levels are altered have revealed several roles for cholesterol in neurones including promotion of synaptogenesis, maintenance of synapse organization, and enablement of synaptic vesicle (SV) fusion and endocytosis (Hering et al. 2003; Pfrieger, 2003; Salaun et al. 2004; Rohrbough & Broadie, 2005). During development, glia-derived cholesterol enhances the formation of synapses (Mauch et al. 2001; Pfrieger, 2003; Goritz et al. 2005). In neuroendocrine cells, cholesterol depletion disrupts syntaxin clusters and decreases evoked catecholamine release (Chamberlain et al. 2001; Lang et al. 2001; Gil et al. 2005; Salaun et al. 2005). In addition, cholesterol depletion with methyl-β-cyclodextrin (MCD) inhibits clathrin-dependent endocytosis in multiple preparations (Rodal et al. 1999; Subtil et al. 1999). In nerve terminals, cholesterol interacts with several SV proteins (Thiele et al. 2000). In addition, cholesterol is a prominent component of SV membranes (Deutsch & Kelly, 1981) and has been proposed as a spatial organizer of synaptic vesicle recycling (Jia et al. 2006).

To further elucidate the role of cholesterol in regulation of neurotransmitter release, we used three complementary approaches to manipulate cholesterol levels in hippocampal synapses. In the first approach, we employed MCD, which binds cholesterol and extracts it from the membrane. By treating hippocampal cultured neurones with this compound, we could effectively remove cholesterol from exposed membranes in a relatively rapid fashion. The second approach utilized mevastatin, an inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Treatment of cultured hippocampal neurones with mevastatin led to an inhibition of cholesterol synthesis, resulting in a reduction in overall neuronal cholesterol content. The third approach took advantage of a naturally occurring disorder known as Niemann-Pick type C1, which is characterized by a loss of function of the Niemann-Pick type C1 protein (NPC1). NPC1-deficient cells exhibit a defect in the movement of cholesterol from late endosomes and lysosomes (Kobayashi et al. 1999; Vanier & Millat, 2003). In neurones, NPC1 deficiency results in a
decreased concentration of cholesterol at distal axons due to a decrease in the efficiency of cholesterol trafficking (Karten et al. 2002, 2003), which allowed us to observe the effect of decreases in synaptic cholesterol.

Under these experimental conditions, we observed a substantial increase in spontaneous SV fusion, coupled to enhanced endocytosis, and a concomitant decrease in evoked neurotransmission and vesicle recycling. Differential regulation of evoked and spontaneous recycling is consistent with earlier findings that spontaneously fusing SVs may be distinct from those that fuse in response to presynaptic action potentials (Sara et al. 2005). Although the mechanisms underlying spontaneous fusion are not well understood, these observations suggest that synaptic cholesterol is a crucial component of the machinery that prevents spontaneous fusion of SVs at rest.

Methods

Cell culture

The hippocampus was dissected and dissociated from postnatal day 0–3 (P0–3) Sprague-Dawley rats or NPC1-deficient mice (Jackson Laboratory, BALB/cNctr-NPC1m1N/J) as previously described in Kavalali et al. (1999). Rats and mice were rapidly killed by decapitation after sedation by chilling on an ice-cold metal plate. Dissociated cells were plated on zero thickness 12 mm glass coverslips and stored at 37°C with 5% CO2 in a humidified incubator. Synaptobrevin-2-deficient dissociated hippocampal cultures (courtesy of Dr Thomas C. Südhof) were prepared following previously published protocols (Schoch et al. 2001). Banker-style glia-free hippocampal cultures were prepared from hippocampi from embryonic day 18 (E18) Sprague-Dawley rats following published protocols (Goslin et al. 1998).

Hippocampal slice preparation

After Nembutal (20 mg kg−1; Abbott Laboratories, IL, USA) anaesthesia, mice were rapidly killed by decapitation, hippocampi were removed, and transverse slices (400 μm) were cut in oxygenated, ice-cold dissection (low Ca2+–high Mg2+ artificial cerebrospinal fluid; ACSF) solution using a Vibratome (St Louis, MO, USA) and incubated at 34°C for 30 min. Slices were kept at room temperature for at least 60 min in ACSF containing (mm): 124 NaCl, 5 KCl, 12 NaH2PO4, 26 NaHCO3, 10 d-glucose, 2 CaCl2 and 1 MgCl2, gassed with 95% O2 and 5% CO2. After removal of the CA3 region, slices were transferred to the recording chamber, perfused with oxygenated ACSF. All the handling and killing procedures for animals were approved by the Institutional Animal Care and Use Committee of U.T. Southwestern Medical Center.

Electrophysiology

Hippocampal cultures. A modified Tyrode solution was used for all experiments (except where noted otherwise) that contained (mm): 140 NaCl, 4 KCl, 2 MgCl2·6H2O, 10 glucose, 10 Hepes and 2 CaCl2 (pH 7.4, osmolarity 300 mosmol l−1). Pyramidal cells were whole-cell voltage clamped at −70 mV with borosilicate glass electrodes (3–5 M2). Electrode solutions contained (mm): 105 caesium methanesulphonate, 10 CsCl, 5 NaCl, 10 Hepes, 20 TEA.Cl hydrate, 4 Mg-ATP, 0.3 GTP, 0.6 EGTA and 10 Lidocain N-ethyl bromide, sodium-channel blocker (QX-314). For spontaneous mEPSCs, recordings were performed in the modified Tyrode solution containing 1 μm tetrodotoxin (TTX) and 50 μm picrotoxin (PTX). For Ca2+-buffered spontaneous mEPSCs recordings, cells were incubated for 30 min with 1 μm BAPTA-AM in Ca2+-free modified Tyrode solution before recording mEPSCs in a Ca2+-free modified Tyrode solution containing 1 μm TTX and 50 μm PTX. For ionomycin experiments, mEPSCs were recorded from treated cultures for at least 2 min before perfusing an ionomycin solution for 3 min following the removal of ionomycin by washing with the spontaneous mEPSC solution to prevent excessive insertion of the ionophore. The ionomycin solution consisted of the modified Tyrode solution containing 1 μm ionomycin, 1 μm TTX and 50 μm PTX. Sucrose recordings were performed by infusing Tyrode solution containing 500 mm sucrose and 1 μm TTX with 50 μm PTX for 30 s. Evoked response experiments were performed using field stimulation with platinum electrodes at 20 mA for 1 ms per action potential in the Tyrode solution containing 50 μm PTX.

Hippocampal slices. Electrophysiological recordings were carried out in the whole-cell voltage-clamp configuration on the CA1 pyramidal neurons. Patch pipettes had resistance of 3–6 MΩ when filled with pipette solution containing (mm): 110 potassium gluconate, 20 KCl, 10 NaCl, 10 Hepes, 0.6 EGTA, 4 Mg-ATP, 0.3 GTP, 10 QX-314 and buffered to pH 7.2–7.3 with CsOH (280–290 mosmol l−1). Recordings were obtained with an Axopatch-200B patch-clamp amplifier (Molecular Devices, Union City, CA, USA). Signals were low-pass filtered at 2 kHz and digitized at 10 kHz.

Drug treatments

Methyl-β-cyclodextrin. Elevated K+ Tyrode solution used for MCD treatments contained (mm): 106 NaCl, 20 KCl, 2 MgCl2·6H2O, 10 glucose, 10 Hepes, 2 CaCl2 (pH 7.4, osmolarity 280 mosmol l−1), and 15 mm MCD (Sigma; approximately 20 mg ml−1; average methyl substitution, 10.5–14.7) with 10 μm NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoline-7-sulphonamide) and 50 μm AP5 (D,L-2-amino-5-phosphonovaleric acid).
NBQX and AP5 were added to these solutions to prevent recurrent activity and toxicity due to excessive glutamate signalling during the treatments. For 20 mM K+ alone treatments, cultures were incubated in an elevated K+ Tyrode solution containing (mM): 126 mM NaCl, 20 KCl, 2 MgCl₂·6H₂O, 10 glucose, 10 Hepes, 2 CaCl₂ (pH 7.4, osmolarity 300 mosmol l⁻¹) with 10 μM NBQX and 50 μM AP5. For MCD alone treatments, cultures were incubated in a Tyrode solution containing (mM): 131 mM NaCl, 4 KCl, 2 MgCl₂·6H₂O, 10 glucose, 10 Hepes, 2 CaCl₂ (pH 7.4, osmolarity 280 mosmol l⁻¹) with 15 mM MCD, 1 μM TTX, 10 μM NBQX and 50 μM AP5. Hippocampal cultures (10–15 days in vitro, DIV) were incubated for 30 min in the treatment solution at room temperature (22–25°C). After treatment, the cells were washed thoroughly, and then experiments were performed.

**Mevastatin treatment.** Hippocampal cultures (12–15 DIV) were treated with mevastatin (4 μM, Sigma) and mevalonate (0.25 mM, Sigma) in a serum-free medium supplemented with double B-27 supplement (Invitrogen) at 37°C for 6 h in a humidified incubator with 5% CO₂.

**Cholesterol addition.** In order to produce MCD: cholesterol complexes (molar ratio, 9.78 : 1), a 5% MCD solution was heated to 80°C and 30 mg of cholesterol dissolved in 9 ml of chloroform: methanol (1 : 2) was added drop-wise until all of the solution was dissolved. The solution was crystallized and re-dissolved in 5 ml of distilled water and stored at −20°C (adapted from Klein et al. 1995). For cholesterol addition, cells were incubated at room temperature for 1 h in 0.61 mM of complexed cholesterol (about 6 mM MCD saturated with cholesterol) with 10 μM NBQX, and 50 μM AP5 in either a 20 mM K+ Tyrode solution (depolarization) or 4 mM K+ Tyrode solution with 1 μM TTX (no depolarization). The solution was thoroughly washed away and experiments were performed.

**Filipin staining**

Cultures were fixed with 4% paraformaldehyde (ice-cold) for 30 min and incubated overnight with filipin III, 0.125 mg ml⁻¹ (diluted from 0.4 mg ml⁻¹ DMSO : H₂O (1 : 4), Sigma). Coverslips were thoroughly washed and mounted. Images were taken with a confocal microscope (Nikon D-Eclipse C1) and analysed using MetaFluor software (Universal Imaging Corp., Downingtown, PA, USA). At least three coverslips (n = 3) were imaged, using five images per coverslip.

**Trypan Blue staining – cell viability**

After MCD treatments, cultures were washed for 10 min with modified Tyrode solution. Then a 0.4% Trypan Blue solution (Sigma) was added to the cells at a final concentration of 0.2% (200 μl modified Tyrode solution : 200 μl Trypan Blue 0.4%) for 10 min. Cells were washed again for 10 min and DIC images (at least 5 per coverslip) were acquired with a CCD camera (Roper Scientific, Trenton, NJ, USA) under bright field illumination. To calculate the per cent of viable neurones, the number of blue (non-viable) and non-blue (viable) cells in a 0.01 mm² region were counted, and the number of viable neurones were divided by the total number of neurones per area (× 100). Then the values for the areas of one coverslip were averaged.

**Electron microscopy**

For high K+ HRP uptake, treated cells were incubated with horseradish peroxidase (10 mg ml⁻¹ HRP, Sigma) for 2 min in a modified Tyrode solution with 47 mM K⁺, 10 μM NBQX and 50 μM AP5, then washed twice with buffer. The cells were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C then washed with Tris-Cl (100 mM, pH 7.4). The cells were then incubated with 3,3’-diaminobenzidine (0.1% DAB, Sigma) in Tris-Cl buffer and H₂O₂ (0.02%) for 15 min. After washing with distilled water, cells were incubated in 1% OsO₄ for 30 min at room temperature then stained en bloc with 2% aqueous uranyl acetate for 15 min, dehydrated in ethanol, and embedded in Poly/Bed 812, polyciences Inc, Warrington, PA, USA for 24 h. Sections (60 nm) were post-stained with uranyl acetate and lead citrate and viewed with a JEOL 1200 EX transmission microscope. For the spontaneous HRP uptake, the procedure was the same as above except that the cells were treated with 25 mg ml⁻¹ HRP for 15 min in a modified Tyrode solution with 1 μM TTX, 10 μM NBQX and 50 μM AP5. For structural analysis, rat hippocampal cultures were treated as described except cells were not incubated with HRP or DAB solutions.

**Statistical analysis**

The Kolmogorov-Smirnov test (K-S test) was used for statistical analysis of all cumulative mEPSC amplitude distributions. The significance level for this test was set to P < 0.0001. ANOVA was used for statistical analysis of all multiple comparison experiments. The Student’s t test (two-tailed) was used for pair-wise comparisons.

**Results**

**Cholesterol depletion impairs evoked neurotransmission**

To deplete cholesterol acutely, we treated hippocampal cultures with a 15 mM MCD solution for 30 min in a depolarizing medium (20 mM K⁺) at room temperature...
and measured neurotransmission after removal of MCD as well as the treatment medium. MCD binds cholesterol reversibly and is commonly used to deplete membrane cholesterol acutely from both leaflets of the bilayer (Steck et al. 2002). Neurones treated with MCD and depolarization are predicted to have decreased amounts of cholesterol in the plasma membrane, in spontaneously fusing vesicles, and vesicles that fuse in a Ca\(^{2+}\)-dependent manner. Under this condition, the removal of cholesterol from SV membranes will depend on the relative accessibility of respective membrane compartments to extracellular MCD. We quantified the reduction in global cholesterol levels in this and other cholesterol manipulations using filipin staining, which labels free cholesterol. For cultures treated with MCD, the filipin fluorescence intensity was reduced by 27.7 ± 0.1% compared with the fluorescence values of non-treated cultures (no treatment, n = 4; 20 mM K\(^+\) with MCD, n = 3, P < 0.001).

To assess the viability of cells after MCD treatment, we incubated cells with Trypan Blue, which is a dye with a negatively charged chromophore that only reacts with damaged membranes, and calculated the fraction of viable cells. We did not detect a significant change in cell viability after MCD treatment (non-treated cultures: 87.1 ± 3.7% versus MCD-treated cultures: 82.7 ± 0.9%, calculated from at least 2 coverslips with 5–20 images per coverslip, not significant (n.s.), P > 0.3). In addition, we assessed the integrity of the cell membranes after treatment with MCD by comparing the membrane resistances of the cells to control values from untreated cells. The membrane resistances were 2.20 ± 0.31 GΩ for non-treated cultures and 2.00 ± 0.39 GΩ for MCD-treated cultures, indicating no significant breach of neuronal membrane integrity after MCD treatment (n.s., P > 0.4).

To characterize excitatory transmission after cholesterol depletion, we stimulated MCD-treated cultures with a hypertonic sucrose solution (+500 mosmol l\(^{-1}\), 30 s) or field potentials and measured excitatory postsynaptic currents (EPSCs) using whole-cell voltage-clamp methods. Hypertonic sucrose mobilizes a specific set of vesicles, referred to as the readily releasable pool, in a Ca\(^{2+}\)-independent manner (Rosenmund & Stevens, 1996). The ability of hypertonic sucrose to trigger release was significantly lower after treatment with MCD (75% reduction) compared with no treatment (no treatment, n = 18; 20 mM K\(^+\) with MCD, n = 18, P < 0.01) (Fig. 1A and B). Treatment with 20 mM K\(^+\) alone for 30 min did not significantly alter the hypertonic sucrose response, indicating that depolarizing medium was not the cause of the decrease observed after MCD treatment in 20 mM K\(^+\) solution. Considering the possible effects of vesicle fusion during the 20 mM K\(^+\) with MCD treatment, cultures were treated with MCD alone (in a 4 mM K\(^+\) solution with tetrodotoxin, TTX, to prevent action potentials). The MCD alone treatment should presumably allow depletion of only the exposed membranes (plasma membrane and spontaneously recycling vesicles). Treatment with MCD alone resulted in a 68% reduction in the charge transfer induced by hypertonic sucrose compared with non-treated cultures (no treatment, n = 18; MCD alone, n = 16, P < 0.05).

Using field stimulation to evoke Ca\(^{2+}\)-dependent neurotransmitter release, we observed a similar decrease in the EPSCs of MCD-treated cultures (73% reduction) compared with non-treated cultures (no treatment, n = 7; 20 mM K\(^+\) with MCD, n = 8, P < 0.01) (Fig. 1C and D). Consistent with the hypertonic sucrose results, treatment with 20 mM K\(^+\) alone did not affect the amplitude of the evoked EPSCs, while treatment with MCD alone reduced the amplitude by 57% (no treatment, n = 7; MCD alone, n = 12, P < 0.05). These results are in agreement with those reported earlier from other secretory preparations after cholesterol depletion (Chamberlain et al. 2001; Lang et al. 2001; Zamir & Charlton, 2006).

After MCD treatment, the reduction in the hypertonic sucrose response and the EPSC amplitudes were reversible by the re-addition of cholesterol using MCD : cholesterol complexes. Re-addition of cholesterol after 20 mM K\(^+\) treatment with MCD caused a 1.3-fold increase in cholesterol levels as measured by filipin fluorescence (only 7% less than control) (Fig. 1).

**Cholesterol depletion augments spontaneous fusion rate**

In the next set of experiments, we characterized the effect of cholesterol depletion on spontaneous fusion events by recording spontaneous miniature EPSCs (mEPSCs) after MCD treatment. The frequency of mEPSCs increased 5-fold in cultures depleted with MCD (no treatment, n = 28; 20 mM K\(^+\) with MCD, n = 31, P < 0.001) (Fig. 2A and B), while the amplitudes of the events were not significantly different indicating that the properties of postsynaptic glutamate receptors were not significantly altered by these manipulations (Fig. 2C). Treatment with 20 mM K\(^+\) alone did not affect the frequency or the amplitudes of the mEPSCs. Cultures treated with MCD alone had a 3-fold higher frequency of mEPSCs; however, this increase was not significantly different from the non-treated cultures (no treatment, n = 28; MCD alone, n = 24, n.s., P > 0.05). The increased frequency after MCD treatment was reversed by the re-addition of cholesterol from MCD : cholesterol complexes (Fig. 2A and B).

The substantial increase in the spontaneous fusion rate seen in cholesterol-depleted cultures prompted us to determine whether the high mEPSC frequency would persist over time, or alternatively, rapidly diminish suggesting depletion of a vesicle pool that sustains this
form of release. To determine the longevity of the increase in the spontaneous fusion rate, we recorded mEPSCs for 20 min and compared non-treated and MCD-treated frequencies at the end of the experiment. We found that the frequency of mEPSCs in MCD-treated cells was still increased 5-fold compared with control cells at 20 min (at 20 min, \( n = 3, \ P < 0.05 \)) (Fig. 2D). This finding is consistent with the constant spontaneous recycling of a pool of vesicles to maintain this high fusion rate.

When mEPSC recordings were performed on cultures treated in 20 mM K\(^+\) with MCD, then loaded for 30 min with BAPTA-AM (a fast Ca\(^{2+}\) buffer), the frequency of spontaneous events was increased 10-fold compared with BAPTA-AM-loaded, non-treated cultures (no treatment, BAPTA-AM, \( n = 7 \); 20 mM K\(^+\) with MCD, \( n = 6, \ P < 0.05 \)) (Fig. 3A–C). For non-treated cultures, the rate of spontaneous events was reduced after incubation with BAPTA-AM; however, the reduction was not significant (no treatment, no BAPTA-AM, \( n = 3 \); no treatment, BAPTA-AM, \( n = 7, \ P > 0.05 \)). Cultures treated with MCD alone were also increased (6-fold); however, this increase was not significant (no treatment, BAPTA-AM, \( n = 7 \); MCD alone, \( n = 4, \ P > 0.05 \)). Thus, the increased frequency observed after MCD treatment is not
dependent on Ca\(^{2+}\) or leakiness of neuronal membranes after cholesterol removal.

To examine the Ca\(^{2+}\) sensitivity of spontaneous neurotransmission seen in MCD-treated neurones, we perfused cultures with the Ca\(^{2+}\) ionophore ionomycin to increase the cytoplasmic Ca\(^{2+}\) concentration. Before ionomycin perfusion, the basal mEPSC frequency was 3.5-fold higher in neurones from cultures treated in 20 mM K\(^{+}\) with MCD compared with those treated with 20 mM K\(^{+}\) alone (20 mM K\(^{+}\) alone, \(n = 4\); 20 mM K\(^{+}\) with MCD, \(n = 6\), \(P < 0.05\)) (Fig. 3D and E). After ionomycin perfusion, the average fold increase in the frequency of spontaneous events was 2- and 5-fold for neurones from cultures treated with 20 mM K\(^{+}\) alone and with 20 mM K\(^{+}\) with MCD, respectively (20 mM K\(^{+}\) alone, \(n = 4\); 20 mM K\(^{+}\) with MCD, \(n = 6\), \(P > 0.05\)) (Fig. 3F). Spontaneous synaptic vesicle fusion is a Ca\(^{2+}\) -sensitive process albeit to a reduced degree. Therefore, taken together with our observation that spontaneous release is dramatically augmented after cholesterol removal, the increase in the effectiveness of ionomycin to trigger release is consistent with an increase in the number of vesicles available for spontaneous fusion.

**Altered neurotransmission after cholesterol depletion is not dependent on the presence of glial cells**

Next, we asked whether the effect of cholesterol depletion we see in the dissociated hippocampal cultures is specific to neurones or caused by an indirect effect of cholesterol removal from glial cells. To address this issue, we examined excitatory transmission after cholesterol depletion in the absence of glia using Banker-style hippocampal cultures (Goslin *et al.* 1998). These cultures are plated with embryonic day 18 (E18) hippocampal neurones without serum to prevent glial growth and incubated in a glia-enriched medium. Cultures were treated with 20 mM K\(^{+}\) alone and 20 mM K\(^{+}\) with MCD, and then evoked and spontaneous neurotransmission were measured. The 20 mM K\(^{+}\) with MCD treatment resulted in a 94 and 85% decrease in the hypertonic sucrose response and
field-stimulated EPSCs, respectively (Fig. 4A–D) (sucrose: 20 mM K+ alone, n = 6; 20 mM K+ with MCD, n = 6, P < 0.005; EPSCs: 20 mM K+ alone, n = 6; 20 mM K+ with MCD, n = 3, P < 0.05). The frequency of mEPSCs was also increased in the absence of glia (20 mM K+ alone, n = 6; 20 mM K+ with MCD, n = 9, P < 0.05) (Fig. 4E–G), indicating that the effect of cholesterol depletion by MCD on spontaneous and evoked fusion is not dependent on glial cells.

Cholesterol depletion leads to decreased evoked uptake and increased spontaneous uptake of HRP

To determine whether acute cholesterol depletion caused structural differences at the presynaptic terminal, we analysed electron micrographs from MCD-treated neurons (Fig. 5A). In 20 mM K+ with MCD-treated cultures, the number of docked vesicles was normal compared with the control (data not shown); however, the total number

Figure 3. Ca2+ dependence of the increased spontaneous event frequency after cholesterol depletion

A–C, mEPSC recorded after BAPTA-AM loading (1 µm for 30 min) in MCD-treated cultures. A, sample traces. B, summary graph shows a 10-fold increase in the frequency of mEPSCs for cultures treated in 20 mM K+ with MCD then loaded with BAPTA-AM before recording compared with BAPTA-AM-loaded, non-treated cultures. The frequency of mEPSCs in non-treated cultures loaded with BAPTA-AM was 60% of the frequency of non-treated cultures not loaded with BAPTA-AM. The frequency of mEPSCs in cultures treated with MCD alone had a 6-fold increase in frequency; however, the difference was not significant from the frequency of BAPTA-AM-loaded, non-treated cultures. This demonstrates that the increase in the frequency of mEPSCs after MCD treatment is not due to an increase in Ca2+ levels/influx. C, the distributions of mEPSC amplitudes were not affected by the BAPTA-AM treatment as determined by the K-S test (P > 0.0001). D, no treatment, no BAPTA-AM, n = 3; no treatment, BAPTA-AM, n = 7; MCD alone, n = 4; and 20 mM K+ with MCD, n = 6). D–F, ionomycin responses from MCD-treated cultures. D, sample traces of ionomycin perfusion (1 µm for 3 min) (above). Detailed view of the traces before ionomycin and after ionomycin for each treatment are shown below. E, histogram of the average frequency over 10 s intervals of both the 20 mM K+ with MCD and the 20 mM K+ alone treatments before and after ionomycin perfusion. Cultures treated in 20 mM K+ with MCD had a 3-fold higher mEPSC frequency before ionomycin treatment than cultures treated with 20 mM K+ alone. F, summary graph of the fold increase in mEPSC frequency after ionomycin perfusion. The average fold increase in the frequency of mEPSCs after ionomycin perfusion was 2.5- and 5-fold for cultures treated with 20 mM K+ alone and 20 mM K+ with MCD, respectively; however, the difference is not significant. Horizontal black bar indicates the presence of ionomycin, 1 culture, 20 mM K+ alone, n = 6; 20 mM K+ with MCD, n = 3. Error bars represent the s.e.m. *P < 0.05.
of vesicles per synapse was decreased by 44% in cultures treated with MCD (no treatment, n = 94; 20 mM K\(^+\) with MCD, n = 108, \(P < 0.001\)) (Fig. 5B). The significant loss of vesicles after MCD treatment in 20 mM K\(^+\) in a brief, 30 min interval suggests that some vesicles could not be retrieved after fusion during MCD treatment. In contrast, the total number of vesicles per synapse in cultures treated with MCD alone was only reduced by 8% (no treatment, n = 94; MCD alone, n = 68, n.s., \(P > 0.05\)), indicating that depolarization during MCD incubation is needed for the loss of vesicles.

To monitor SV endocytosis under these conditions, we analysed electron micrographs from MCD-treated cells after either evoked or spontaneous horseradish peroxidase (HRP) uptake. The evoked uptake of HRP was performed by maximally stimulating cultures with a buffer containing 47 mM K\(^+\) and HRP for 2 min (Fig. 5C). The number and the corresponding per cent of HRP-positive vesicles were decreased by 61 and 64\%, respectively, in cultures treated 20 mM K\(^+\) with MCD (no treatment, n = 11; 20 mM K\(^+\) with MCD, n = 6, \(P < 0.01\)) (Fig. 5D and E). For cultures treated with MCD alone, the number and the corresponding per cent of HRP-positive vesicles were reduced by 30 and 42\%, respectively, compared with non-treated cultures (no treatment, n = 11; MCD alone, n = 8, n.s., \(P > 0.05\)). The decrease in the number and overall per cent of HRP-positive vesicles is consistent with the decreased responses observed with hypertonic sucrose and field stimulation (Fig. 1).

The spontaneous uptake of HRP was performed in a buffer with a normal K\(^+\) (4 mM K\(^+\)) concentration in the presence of TTX to prevent action potentials. While the percentage of vesicles that took up HRP with maximal stimulation is decreased after MCD treatment, the number of vesicles that took up HRP spontaneously was increased 1.6-fold and the per cent of HRP-positive

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**Figure 4. MCD effect on neurotransmission is not dependent on the presence of glial cells**

A and B, hypertonic sucrose stimulation after MCD treatment in the absence of glia. A, sample traces. B, summary graph showing that the average charge transfer during the first 10 s of the 30 s sucrose response is decreased 94\% after treatment with MCD compared with cultures treated with 20 mM K\(^+\) alone. Horizontal bar represents the presence of hypertonic sucrose; 1 culture, 20 mM K\(^+\) alone, n = 6; 20 mM K\(^+\) with MCD, n = 6. C and D, field stimulation evoked responses after MCD treatment in the absence of glia. C, sample traces. D, summary graph showing an 85\% reduction in the average evoked EPSC amplitude for cultures treated with MCD compared with cultures treated with 20 mM K\(^+\) alone. Arrow represents timing of the stimulation; 1 culture, 20 mM K\(^+\) alone, n = 6; 20 mM K\(^+\) with MCD, n = 3. E–G, mEPSCs after MCD treatment in the absence of glia. E, sample traces. F, summary graph shows a 3-fold increase in the frequency of mEPSCs for cultures treated with MCD compared with cultures treated with 20 mM K\(^+\) alone. G, the distributions of mEPSC amplitudes were not affected by these treatments as determined by the K-S test (\(P > 0.001\)). 1 culture; 20 mM K\(^+\) alone, n = 6; 20 mM K\(^+\) with MCD, n = 9. Error bars represent the s.e.m. *\(P < 0.05\), **\(P < 0.005\).

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vesicles was increased 2.5-fold compared with synapses from non-treated cultures (no treatment, \( n = 47 \); 20 mm K\(^+\) with MCD, \( n = 72 \), \( P < 0.05 \)) (Fig. 5F–H). This outcome suggests that in 15 min there were more vesicles recycling spontaneously in MCD-treated synapses compared with controls. This increase could be either due to an elevation in the number of spontaneously fusing vesicles as suggested above (Figs 2D and 3D–F) or an increase in their rate of recycling. Earlier work in non-neuronal cell lines showed that cholesterol depletion impairs clathrin-mediated endocytosis while not affecting clathrin-independent pathways (Subtil et al. 1999; Rodal et al. 1999). Taken together with this previous study, our findings suggest that spontaneous SV endocytosis operates through a clathrin-independent mechanism.

The 44% decrease in the number of vesicles per synapse after treatment in 20 mm K\(^+\) with MCD (Fig. 5A and B) suggests that some fused vesicles were unable to endocytose. In agreement with this premise, we detected several stranded endocytic structures in images from neurones treated in 20 mm K\(^+\) with MCD (Fig. 5I and K) compared with the images from neurones treated with 20 mm K\(^+\) alone (Fig. 5I and J).

**Cholesterol depletion-dependent increase in spontaneous fusion rate does not require synaptobrevin-2**

The differential effect of cholesterol depletion on the evoked and spontaneous synaptic vesicle recycling suggested cholesterol depletion was hindering evoked fusion by a mechanism that does not impair spontaneous fusion. To better understand the mechanisms underlying this phenomenon, we repeated the same manoeuvres in dissociated hippocampal neurones obtained from mice that lack a major synaptic vesicle SNARE, synaptobrevin-2 (also called VAMP-2). Synaptobrevin-2-deficient (Syb2\(^{-/-}\)) synapses manifest a severe reduction in evoked exocytosis and endocytosis (Schoch et al. 2001; Deak et al. 2004). In initial experiments, we detected a significant decrease in the response to hypertonic sucrose after cholesterol depletion (Fig. 1). This result can be explained by the dispersion of syntaxin clusters, thus impairment of SNARE-mediated fusion, seen after cholesterol depletion in other systems (Lang et al. 2001; Churchward et al. 2005). To test this prediction, we measured the effect of cholesterol depletion on the low level of hypertonic sucrose-evoked neurotransmission seen in the absence of synaptic vesicle SNARE protein synaptobrevin-2. The response to hypertonic sucrose in non-treated synaptobrevin-2-deficient neurones was 80% less than the response from non-treated wild-type (WT) neurones (WT, \( n = 8 \); Syb2\(^{-/-}\), \( n = 7 \), \( P < 0.05 \)). Wild-type neurones treated with MCD had an 80% decrease in the response to hypertonic sucrose compared with non-treated wild-type neurones (WT, \( n = 8 \); WT, MCD, \( n = 5 \), n.s., \( P > 0.8 \)), while the response to hypertonic sucrose stimulation in synaptobrevin-2-deficient neurones treated with MCD were not decreased compared with non-treated synaptobrevin-2-deficient neurones (13-fold, Syb2\(^{-/-}\), \( n = 7 \), Syb2\(^{-/-}\), MCD, \( n = 4 \), \( P < 0.001 \)) (Fig. 6A and B). Thus, these experiments revealed no further reduction in hypertonic sucrose-driven release in synaptobrevin-2-deficient synapses, suggesting that removal of cholesterol impairs evoked release primarily by affecting SNARE-dependent fusion. In contrast, under the same conditions, we could detect a significant increase in the frequency of mEPSCs after MCD treatment in both the wild-type (2-fold, WT, \( n = 7 \); WT, MCD, \( n = 5 \), \( P < 0.005 \)) and synaptobrevin-2-deficient neurones (13-fold, Syb2\(^{-/-}\), \( n = 7 \); Syb2\(^{-/-}\), MCD, \( n = 4 \), \( P < 0.001 \)). In non-treated cultures, the frequency of mEPSC events in synaptobrevin-2-deficient neurones was 97% less than in wild-type neurones (WT, \( n = 7 \); Syb2\(^{-/-}\), \( n = 7 \), \( P < 0.001 \)). Even with the increase in frequency after MCD treatment, the frequency in synaptobrevin-2-deficient neurones was still 65% lower than the spontaneous fusion rate in non-treated wild-type neurones (WT, \( n = 7 \); Syb2\(^{-/-}\), MCD, \( n = 4 \), \( P < 0.05 \)) (Fig. 6C–E).

So while the response to hypertonic sucrose stimulation was not affected by MCD treatment in synaptobrevin-2-deficient neurones, we could still detect an increase in the spontaneous fusion rate in after MCD treatment without a significant alteration in the amplitudes of individual mEPSCs. This suggests that cholesterol may act downstream of synaptobrevin-2 function to restrain spontaneous fusion.

**Inhibition of cholesterol biosynthesis mimics the effect of acute cholesterol removal**

In the next set of experiments, we asked whether impairing cholesterol synthesis in neurones would mimic the results obtained after acute depletion of membrane cholesterol with MCD. For this purpose, we used mevastatin, a ‘statin’ compound that inhibits HMG-CoA reductase (the rate-limiting enzyme in cholesterol synthesis) in a cholesterol-free medium. To quantify the relative reduction in cholesterol, we treated cells with mevastatin for 6 h and then labelled with filipin. The level of filipin fluorescence was reduced by 36.0 ± 0.1% compared with cells that were not treated with mevastatin (no mevastatin, \( n = 4 \); mevastatin, \( n = 3 \), \( P < 0.001 \)).

Treatment with mevastatin for 6 h significantly decreased the hypertonic sucrose response and the
Figure 5. Cholesterol depletion decreases the number of vesicles per synapse and effects depolarization-evoked and spontaneous HRP uptake differentially

A and B, electron micrographs (EMs) of MCD-treated hippocampal cultures. A, representative synapses. B, summary graph showing a 44% decrease in the average number of vesicles per synapse for cultures treated in 20 mM K+ with MCD compared with the non-treated cultures. Cultures treated with MCD alone only showed an 8% decrease in the number of vesicles per synapse, indicating that treatment with MCD with stimulation is necessary for the decrease in vesicles. No treatment, n = 94; MCD alone, n = 68; and 20 mM K+ with MCD, n = 108. C–E, EMs of MCD-treated cultures loaded with HRP using 47 mM K+ depolarization for 2 min. C, representative synapses (white arrows indicate HRP-positive (HRP+) vesicles). D, summary graph showing a 61% decrease in the average number of HRP+ vesicles per synapse for cultures treated in 20 mM K+ with MCD compared with the non-treated cultures. Cultures treated with MCD alone had a 30% decrease in the number of HRP+ vesicles per synapse compared with the non-treated cultures. E, summary graph showing a 64% decrease in the average number of HRP+ vesicles per synapse for cultures treated in 20 mM K+ with MCD compared with the non-treated cultures. Cultures treated with MCD alone had a 42% decrease in the per cent of HRP+ vesicles per synapse compared with the non-treated cultures. No treatment, n = 11; MCD alone, n = 8; and 20 mM K+ with MCD, n = 6. F–H, EMs of MCD-treated...
field stimulation-evoked EPSC amplitude 80% and 70%, respectively, compared with non-treated cultures (sucrose: no mevastatin, \( n = 7 \); mevatatin, \( n = 12 \), \( P < 0.01 \); EPSCs: no mevastatin, \( n = 5 \); mevatatin, \( n = 5 \), \( P < 0.05 \)) (Fig. 7A–D). Interestingly, after mevastatin treatment, we observed the same upward trend in the frequency of the spontaneous events (3-fold increase) (no mevastatin, \( n = 24 \); mevatatin, \( n = 13 \), \( P < 0.001 \)) (Fig. 7E and F) with no effect on the amplitude of the events, indicating that postsynaptic receptors were not significantly affected by this manipulation (Fig. 7G). Both the decreased evoked responses (Fig. 7A–D) and increased frequency (Fig. 7E and F) after mevastatin treatment were reversed by the addition of cholesterol from MCD: cholesterol complexes. After cholesterol addition, the filipin fluorescence was increased 1.4-fold compared with mevastatin-treated cultures, which is 11.1 ± 0.1% less than the fluorescence levels in non-treated cells (no mevastatin, \( n = 4 \); mevastatin, \( n = 3 \); mevastatin + cholesterol, \( n = 3 \), \( P < 0.05 \)).

cultures loaded with HRP spontaneously. F. representative synapse sections (white arrows indicate HRP+ vesicles). G. summary graph showing a 1.6-fold increase in the average number of HRP+ vesicles per synapse for cultures treated in 20 mM K+ with MCD. H. summary graph showing a 2.5-fold increase in the average per cent of HRP+ vesicles per synapse for cultures treated in 20 mM K+ with MCD. No treatment, \( n = 47 \); and 20 mM K+ with MCD, \( n = 72 \). I–K. EMs of MCD-treated hippocampal cultures depicting stranded endocytic structures. I. representative images. J. magnified images of synapses treated with 20 mM K+ alone. K. magnified images of synapses treated with 20 mM K+ with MCD. Error bars represent the S.E.M. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \); scale bar, 100 nm.
The mevastatin treatments were performed in the presence of 0.25 mM mevalonate, which is the product of the reaction that mevastatin inhibits. Mevalonate is the precursor molecule for the synthesis of cholesterol and isoprenoids. This concentration of mevalonate during mevastatin treatment can maintain the level of isoprenoids, while not generating sufficient levels of cholesterol (Alberts et al. 1980; Brown & Goldstein, 1980; Goldstein & Brown, 1990; Simons et al. 1998). To rule out the effect of mevalonate on evoked and spontaneous neurotransmission, cultures were treated with mevastatin in the presence or absence of mevalonate and the lack of mevalonate did not result in a loss of the effect seen with mevastatin and mevalonate (data not shown).

**Figure 7. Inhibition of cholesterol synthesis with mevastatin mimics acute depletion**

A and B, hypertonic sucrose stimulation after 6 h mevastatin treatment. A, sample traces. B, summary graph showing that the average charge transfer during the first 10 s of the 30 s sucrose response is decreased 80% after treatment with mevastatin for 6 h compared with non-treated cultures. The addition of cholesterol after mevastatin treatment rescued the depleted hypertonic sucrose responses to values not significantly different from non-treated cultures. Horizontal bar represents the presence of hypertonic sucrose. At least 2 cultures; no mevastatin, n = 7; mevastatin, n = 12; mevastatin + cholesterol, n = 3. C and D, field stimulation evoked responses after 6 h mevastatin treatment. C, sample traces. D, summary graph depicting a 70% reduction in the average evoked EPSC amplitude for cultures treated with mevastatin compared with non-treated neurones. The addition of cholesterol after mevastatin treatment rescued the reduced EPSC amplitudes to values not significantly different from non-treated cultures. Arrow represents timing of the stimulation. At least 2 cultures; no mevastatin, n = 5; mevastatin, n = 5; mevastatin + cholesterol, n = 4. E–G, mEPSCs after 6 h mevastatin treatment. E, sample traces. F, summary graph shows a 3-fold increase in the frequency of mEPSCs for cultures treated for 6 h with mevastatin compared with untreated neurones. The increased frequency was reduced to non-treated frequency levels after incubation with MCD : cholesterol complexes. G, the distributions of mEPSC amplitudes were not affected by these treatments as determined by the K-S test (P > 0.0001). At least 2 cultures; no mevastatin, n = 24; mevastatin, n = 13; mevastatin + cholesterol, n = 4. Error bars represent the s.e.m. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001.

Alterations in synaptic transmission in a mouse model of Niemann-Pick type C1 disease parallel the effects of acute cholesterol manipulations

To examine whether genetic alterations in cholesterol homeostasis can also affect evoked and spontaneous neurotransmission we took advantage of a natural mutation that results in loss of function of the Niemann-Pick type C1 (NPC1) protein and ultimately disrupts the normal trafficking of cholesterol from the late endosome–lysosome pathway (Kobayashi et al. 1999; Vanier & Millat, 2003). There are several mouse models of this disease, one of which is a model with a complete loss of the NPC1 protein (BALB/cNctr-NPC1m1N/J) (Loftus et al. 1997). Loss of NPC1 presents with weight loss and...
motor deficits and results in lethality by 2 months of age (Mukherjee & Maxfield, 2004). Cholesterol levels in the whole brain are only slightly decreased (Li et al. 2005) but the cellular distribution of the remaining cholesterol is altered with an accumulation of cholesterol in the soma and a loss of cholesterol at distal axons (Karten et al. 2002) which may contribute to defective vesicle trafficking in presynaptic nerve terminals (Karten et al. 2006).

Neurotransmission evoked by hypertonic sucrose and field stimulation in hippocampal cultures from NPC1-deficient brains showed a significant reduction compared with similar recordings from wild-type cultures (65 and 77%, respectively) (sucrose: WT, \( n = 10 \); NPC1, \( n = 9 \), \( P < 0.01 \); EPSCs: WT, \( n = 4 \); NPC1, \( n = 4 \), \( P < 0.05 \)) (Fig. 8A–D). NPC1-deficient cultures also had a significant increase in the frequency of spontaneous

![Figure 8: Altered cholesterol trafficking in Niemann Pick C1-deficient mice causes abnormalities in neurotransmission mimicking the effect of acute cholesterol depletion](image-url)

**Figure 8.** Altered cholesterol trafficking in Niemann Pick C1-deficient mice causes abnormalities in neurotransmission mimicking the effect of acute cholesterol depletion

A–G, electrophysiological recordings from NPC1-deficient (NPC1) and wild-type littermate (WT) hippocampal cultures. A and B, hypertonic sucrose stimulation. A, sample traces. B, summary graph showing an 80% decrease in the average charge transfer during the first 10 s of the 30 s sucrose response in NPC1 neurones compared with WT neurones. The incubation of NPC1 neurones with MCD : cholesterol complexes resulted in an increased response to hypertonic sucrose that was not significantly different from WT neurones. Horizontal bar represents the presence of hypertonic sucrose; 4 cultures; WT, \( n = 10 \); NPC1, \( n = 9 \); NPC1 + cholesterol, \( n = 5 \).

C and D, field stimulation evoked responses. C, sample traces. D, summary graph depicting a 65% decrease in the average evoked EPSC amplitude for NPC1 neurones compared with WT neurones. The addition of cholesterol to NPC1 neurones rescued the reduced EPSCs to values not significantly different from WT neurones. Arrow represents timing of the stimulation; 4 cultures; WT, \( n = 4 \); NPC1, \( n = 4 \); NPC1 + cholesterol, \( n = 4 \).

E–G, mEPSCs from NPC1 and WT cells. E, summary graph of the frequency of mEPSCs for NPC1 neurones compared with WT neurones. The increased frequency in NPC1 neurones was reduced to the WT frequency levels after incubation with MCD : cholesterol complexes. G, the distributions of mEPSC amplitudes were not different under all conditions as determined by the K-S test (\( P > 0.0001 \)). 4 cultures; WT, \( n = 16 \); NPC1, \( n = 17 \); NPC1 + cholesterol, \( n = 9 \). H–J, electrophysiological recordings from NPC1 and WT hippocampal slices. H, sample mEPSC traces. I, summary graph of the frequency of mEPSCs showing a 3.4-fold increase in the frequency of mEPSCs in the NPC1 neurones. J, the distributions of mEPSC amplitudes were not different as determined by the K-S test (\( P > 0.0001 \)). WT, \( n = 6 \); NPC1, \( n = 5 \).
events (2.5-fold) with no effect on the amplitude of these events (WT, n = 16; NPC1, n = 17, P < 0.01) (Fig. 8E–G). Since the NPC1-deficient phenotype might be altered by culturing the neurones, we measured the rate of spontaneous events in hippocampal slices obtained from the same mice and found a similar increase (3.4-fold) in the frequency of mEPSCs (WT, n = 6; NPC1, n = 5, P < 0.005) (Fig. 8H–J). The addition of cholesterol to the NPC1-deficient hippocampal cultures rescued the evoked responses and the frequency of spontaneous mEPSCs to a level not significantly different from the wild-type values (Fig. 8).

The consistency of the findings from dissociated hippocampal neuronal cultures and intact hippocampal slices from the NPC1-deficient mice argues for a crucial role of neuronal cholesterol homeostasis in setting the balance between evoked and spontaneous fusion. Furthermore, this fundamental abnormality in synaptic transmission can be detected irrespective of whether cholesterol levels are altered acutely (MCD or mevastatin) or chronically (NPC1 mutants).

Discussion
In this study, using three complementary experimental settings, we investigated the role of cholesterol homeostasis in synaptic vesicle recycling. MCD-mediated cholesterol depletion during sustained depolarization revealed that SV cholesterol, along with plasma membrane cholesterol, is a key player in decreasing the probability of spontaneous SV fusion. The increase in spontaneous uptake of HRP and the persistence of the high level of spontaneous release seen after cholesterol removal also suggest that the potentiation of spontaneous fusion is coupled to an increase in endocytosis. Taken together, acute MCD-mediated cholesterol removal results in an overall augmentation of spontaneous vesicle recycling rather than an increase in spontaneous fusion alone. The experimental results obtained with acute MCD application were also corroborated by the manipulation of neuronal cholesterol levels by the mevastatin-mediated inhibition of cholesterol biosynthesis or by impairment of cholesterol trafficking in NPC1-deficient neuronal cultures. The enhanced spontaneous vesicle recycling seen after the decrease in cholesterol levels is consistent with earlier results from our laboratory which suggested that vesicles giving rise to spontaneous neurotransmission are distinct from those that fuse in response to action potential firing (Sara et al. 2005; Virmani et al. 2005). This earlier finding implies that most SVs resident within the readily releasable pool are actively prevented from fusing spontaneously. The current work implicates the basal level of synaptic cholesterol level as a major impediment for spontaneous fusion and vesicle recycling. However, once cholesterol is removed spontaneous fusion and recycling can be increased dramatically. This increase may be in part due to increased spontaneous synaptic vesicle recycling. However, it may also involve the recruitment of vesicles, which would normally fuse solely in response to action potentials, to the spontaneous recycling pathway. This premise is consistent with the mixing of evoked and spontaneous vesicle recycling pools that we have detected earlier in synaptobrevin-2-deficient synapses (Sara et al. 2005).

In contrast to its stimulatory effect on spontaneous neurotransmission, acute removal of cholesterol resulted in a substantial decrease in evoked SV fusion triggered by action potential-induced Ca\(^{2+}\) influx or hypertonic sucrose application. The parallel impairment of both hypertonic sucrose-triggered and Ca\(^{2+}\)-evoked synaptic responses suggests that cholesterol removal has a direct effect on evoked SV fusion and retrieval machinery, rather than on voltage-gated Ca\(^{2+}\) entry. However, these results cannot exclude an effect of cholesterol removal on action potential generation, as recently documented in crayfish neuromuscular junction (Zamir & Charlton, 2006). Nevertheless, the reduction in hypertonic sucrose-triggered fusion, which does not require action potential generation, argues for a direct impairment in the SV fusion machinery or a decrease in the number of vesicles that are available for evoked fusion. Interestingly, application of the Ca\(^{2+}\) ionophore ionomycin caused a robust increase in release after cholesterol removal. We believe this effect of ionomycin is consistent with the apparent Ca\(^{2+}\) sensitivity of spontaneous synaptic vesicle fusion (Sara et al. 2005). Furthermore, both the increase in spontaneous vesicle recycling and the decrease in evoked fusion seen after cholesterol removal in all conditions (MCD-treated, mevastatin-treated and NPC1-deficient) were readily reversible after the re-addition of cholesterol by incubation with MCD : cholesterol complexes. This acute cholesterol-dependent rescue of the alterations in synaptic transmission suggests that cholesterol levels, rather than secondary effects of cholesterol reduction, regulate the balance between spontaneous and evoked synaptic transmission.

The reciprocal regulation of spontaneous and evoked neurotransmission by cholesterol we document here supports a role for cholesterol-rich membranes in the spatial organization of vesicle trafficking within a synapse (Jia et al. 2006). This proposal is also bolstered by the common requirement for the vesicular SNARE protein synaptobrevin-2 and cholesterol for maintenance of evoked fusion. In our experiments, the deficit in evoked neurotransmission seen after cholesterol removal was partly attributable to disruption of synaptobrevin-2-mediated fusion because the low level of hypertonic sucrose-evoked release seen in hippocampal cultures derived from synaptobrevin-2-deficient mice was unaffected by cholesterol removal. Impairment of syntaxin clusters at the surface membrane that
are critical for SNARE-mediated fusion may underlie the reduction in evoked release, because the deficit in evoked release caused by impairment in surface membrane syntaxins is likely to be occluded in the absence of synaptobrevin-2 (Lang et al. 2001). However, as the magnitude of neurotransmission is substantially diminished in the absence of synaptobrevin-2, possible synaptobrevin-2-independent changes may not be as easily detectable on the synaptobrevin-2 null background. In addition, a decrease in SV endocytosis may also contribute to the reduction in evoked neurotransmission, as suggested by the decreased uptake of HRP and the clear reduction in the number of SVs in electron micrographs after acute cholesterol removal. The endocytotic defects may emerge upon cholesterol removal from SVs. The treatments we performed in this study typically alter global cholesterol levels, therefore we cannot exclude the possibility that some of the alterations we detected in synaptic transmission originated from removal of cholesterol from non-synaptic membranes.

An intriguing finding that emerges from this study is the observation of cholesterol-dependent inhibition of spontaneous SV recycling. The substantial increase observed in the spontaneous fusion rate after cholesterol removal was coupled to an increase in spontaneous endocytosis, which is detected by the increase in the fraction of HRP-positive SVs. The increase in spontaneous fusion after cholesterol depletion was also detectable, albeit to a reduced degree compared with wild-type, in the absence of synaptobrevin-2, suggesting that the mechanism that underlies this increase in fusion is partially dependent on vesicular SNAREs. Mutations that impair sphingolipid synthesis in vesicular SNARE-deficient yeast mutants also lead to a significant rescue of constitutive fusion, which is thought to be analogous to spontaneous fusion at the synapse (David et al. 1998). The analogous findings from these two systems imply that reciprocal interaction of membrane lipids and vesicular SNAREs in the regulation of constitutive and/or spontaneous fusion may be phylogenetically conserved.

The current observations suggest separate roles for the different membrane lipids regulating synaptic vesicle trafficking. Recent evidence indicates that impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking by hindering both spontaneous and evoked neurotransmission (Di Paolo et al. 2004). In contrast, our results suggest that cholesterol normally inhibits spontaneous recycling although it is necessary for the maintenance of evoked vesicle turnover. In future experiments it will be interesting to examine potential interactions between these two major membrane lipid-signalling pathways in the regulation of synaptic vesicle trafficking.

Our results indicate that proper maintenance of synaptic function requires tight regulation of cholesterol levels. More specifically, in order to restrain the extent of uncontrolled spontaneous neurotransmitter release, significant levels of synaptic cholesterol need to be maintained, particularly within SV membranes. These findings suggest a critical role for cholesterol as an endogenous modulator of neurotransmission in the central nervous system. Impairments in synaptic transmission, especially the large increase in spontaneous neurotransmission we observed here, might well form the potential basis for the neurological symptoms and neurodegeneration seen in patients with Niemann-Pick disease. Similar alterations in sterol homeostasis may also underlie other brain disorders. Therefore a detailed analysis of neuronal function, particularly synaptic transmission, after genetic manipulations of key signalling elements and enzymes in the sterol synthesis pathway (Bjorkhem & Meaney, 2004; Kotti et al. 2006), may reveal valuable information about the mechanisms underlying presynaptic function and their role in the pathophysiology of central nervous system disorders.

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