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Cholesterol modulates open probability and desensitization of NMDA receptors

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Key points

- NMDA receptors (NMDARs) are tetrameric cation channels permeable to calcium; they mediate excitatory synaptic transmission in the CNS and their excessive activation can lead to neurodegeneration.
- Although these receptors are in direct contact with plasma membrane, lipid–NMDAR interactions are little understood.
- Using cultured rat cerebellar granule cells, we show that acute and chronic pretreatments resulting in cell cholesterol depletion profoundly diminish NMDAR responses and increase NMDAR desensitization, and also that cholesterol enrichment potentiates NMDAR responses; however, cholesterol manipulation has no effect on the amplitude of AMPA/kainate receptor responses.
- Diminution of NMDAR responses by cholesterol depletion is the result of a reduction of the ion channel open probability, whereas the increase in receptor desensitization is the result of an increase in the rate constant of entry into the desensitized state.
- These results demonstrate the physiological role of membrane lipids in the modulation of NMDAR activity.

Abstract NMDA receptors (NMDARs) are glutamate-gated ion channels that mediate excitatory neurotransmission in the CNS. Although these receptors are in direct contact with plasma membrane, lipid–NMDAR interactions are little understood. In the present study, we aimed at characterizing the effect of cholesterol on the ionotropic glutamate receptors. Whole-cell current responses induced by fast application of NMDA in cultured rat cerebellar granule cells (CGCs) were almost abolished (reduced to 3%) and the relative degree of receptor desensitization was increased (by seven-fold) after acute cholesterol depletion by methyl-β-cyclodextrin. Both of these effects were fully reversible by cholesterol repletion. By contrast, the responses mediated by AMPA/kainate receptors were not affected by cholesterol depletion. Similar results were obtained in CGCs after chronic inhibition of cholesterol biosynthesis by simvastatin and acute enzymatic cholesterol degradation to 4-cholesten-3-one by cholesterol oxidase. Fluorescence anisotropy measurements showed that membrane fluidity increased after methyl-β-cyclodextrin pretreatment. However, no change in fluidity was observed after cholesterol enzymatic degradation, suggesting that the effect of cholesterol on NMDARs is not mediated by changes in membrane fluidity. Our data show that diminution of NMDAR responses by cholesterol depletion is the result of a reduction of the open probability, whereas the increase in receptor desensitization is the result of an increase in the rate constant of entry into the desensitized state. Surface NMDAR population, agonist affinity, single-channel conductance and open time were not altered in cholesterol-depleted CGCs. The results of our experiments show that cholesterol is a strong endogenous modulator of NMDARs.

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Abbreviations CGC, cerebellar granule cell; DIV, days *in vitro*; ECS, extracellular solution; GFP, green fluorescent protein; k_b, k_u, k_o, k_c, k_d and k_r, rate constants of binding (k_b), unbinding (k_u), opening (k_o), closing (k_c), desensitization (k_d) and resensitization (k_r); k_{MK}, blocking rate constant of MK-801; MβCD, methyl-β-cyclodextrin; MEM, minimum essential medium; MK-801, (+)D-methyl-l0,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; NMDAR, NMDA receptor; P_o, peak open probability; PBS, phosphate-buffered saline; TMA-DPH, *N*,*N*,*N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium *p*-toluenesulphonate.

Introduction

NMDA receptors (NMDARs) are glutamate-gated ion channels that mediate synaptic plasticity and memory formation, and also play an important role in several neurological and psychiatric diseases. Numerous endogenous and synthetic compounds modulate the function of NMDARs either from the extracellular or the intracellular side of the plasma membrane (Ogden & Traynelis, 2011). The transmembrane domain of an NMDAR is probably a target for amphiphilic modulatory compounds that are assumed to access NMDARs via the plasma membrane (e.g. arachidonic acid, lysophospholipids, tetrahydroisoquinolines, or 24(S)-hydroxycholesterol) (Miller *et al.* 1992; Casado & Ascher, 1998; Ogden & Traynelis, 2013; Paul *et al.* 2013; Linsenbardt *et al.* 2014).

Plasma membranes contain more than 1000 molecular species of lipids that are unevenly distributed (Ohvo-Rekila *et al.* 2002). Cholesterol, as one of the major components in membranes of most mammalian cells, constitutes 10–45% of plasma membrane lipid molecules (Yeagle, 1985). It forms cholesterol-rich domains, which are often identified with the detergent-resistant membrane fraction (Simons & Ikonen, 1997). Dendritic spines and especially postsynaptic membranes were reported to belong to the detergent-resistant membrane fraction (Hering *et al.* 2003). Correspondingly, numerous ionotropic receptors (e.g. NMDA, AMPA, nicotinic acetylcholine and $GABA_A$ receptors) colocalize mainly with the detergent-resistant membrane fraction (Bruses *et al.* 2001; Hering *et al.* 2003; Abulrob *et al.* 2005; Dalskov *et al.* 2005). Cholesterol-rich domains can be disrupted by cholesterol depletion induced either by the chronic effect of statins (drugs inhibiting cholesterol biosynthesis) (Endo, 2010) or acutely by cyclodextrins (cyclic molecules capable of binding cholesterol to their cavities) (Christian *et al.* 1997). Cholesterol depletion potentiates or inhibits the activity of numerous voltage-dependent and ligand-gated ion channels (Levitan *et al.* 2014). Acute cholesterol depletion inhibits long-term potentiation in rat hippocampal slices (Frank *et al.* 2008) and both acute and chronic cholesterol depletion have a neuroprotective effect against NMDA-induced excitotoxicity in cultured neurons (Zacco *et al.* 2003; Abulrob *et al.* 2005; Bosel *et al.* 2005; Ponce *et al.* 2008). However, the effect of cholesterol on the function of glutamate-gated ion channels is poorly understood.

The present study aimed to determine the effect of changes in the membrane cholesterol on the function of ionotropic glutamate receptors. We found that cholesterol depletion of cultured cerebellar granule cells (CGCs) induced by methyl-β-cyclodextrin (MβCD) or by simvastatin pretreatment reduces the amplitude of NMDAR responses by decreasing the open probability of NMDAR channels and increases the rate and extent of receptor desensitization. The amplitude of AMPA/kainate receptor responses was not influenced by cholesterol manipulation in CGCs. Our data reveal that plasma membrane cholesterol has a vital role for the proper function of the NMDARs.

Methods

Ethical approval

All animal protocols were conducted in accordance with the Protection of Animals Against Cruelty Act No. 246/1992 (Czech Republic), with the EU legislation, conform to the principles of UK regulations on animal experimentation and were approved by the Animal Welfare Board of Institute of Physiology, Academy of Sciences of the Czech Republic.

Cultures of cerebellar granule cells

Wistar rats (of either sex, postnatal day 6–8) were decapitated under ether anaesthesia and primary cultures from their cerebella were prepared as described in Prybylowski *et al.* (2005). CGCs were cultured at a density of 1,000,000 CGCs cm^{-2} in Basal Medium Eagle (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (v/v), 25 mM KCl, 2 mM glutamine (Sigma, St Louis, MO, USA) and 2% B-27 supplement (v/v; Life Technologies). After 5 days *in vitro* (DIV), the medium was exchanged for low-potassium medium [Minimum Essential Medium (MEM) with Earle's salts (containing 5 mM potassium and 2 mM glutamine; Life Technologies) supplemented with 10 μM cytosine arabinofuranoside,

5 mg ml^{-1} glucose (Sigma) and 1% ITS supplement (v/v; Life Technologies)].

Cholesterol depletion, repletion and enrichment

We used MβCD, cholesterol oxidase or simvastatin treatment to deplete CGCs of cholesterol and cholesterol/MβCD complex treatment to replete or enrich CGCs with cholesterol (Christian *et al.* 1997; Zacco *et al.* 2003).

For acute cholesterol depletion by MβCD, DIV 6–7 CGCs were exposed to low-potassium medium supplemented with 5 mM MβCD (mean molecular weight 1310 g mol^{-1}; Aldrich, St Louis, MO, USA) and incubated at 37°C in 5% CO_2 for 1–60 min as indicated.

Chronic cholesterol depletion by simvastatin was carried out by incubating DIV 5 CGCs in low-potassium medium supplemented with 100 nM simvastatin (Sigma) for 4 days prior to patch-clamp measurements (Zacco *et al.* 2003).

Enzymatic degradation of cholesterol was carried out by incubating CGCs in medium supplemented with 10 U ml^{-1} cholesterol oxidase for 60 min at 37°C in 5% CO_2 prior to patch-clamp measurements.

For cholesterol repletion, DIV 6–7 CGCs were first acutely depleted of cholesterol for 30 min and then subjected to 3.4/20 mM cholesterol/MβCD complex (Sigma). Cholesterol/MβCD complex, also called cholesterol–water soluble, contains 4.8% cholesterol (w/w) and 95.2% MβCD (w/w). Cholesterol/MβCD complex was either dissolved in extracellular solution (ECS; in mM: 160 NaCl, 2.5 KCl, 10 Hepes, 10 glucose, 0.3 $CaCl_2$ and 0.1 EDTA, pH 7.3) and applied on a patched neuron at room temperature or dissolved in low-potassium medium and CGCs were repleted at 37°C in 5% CO_2.

For cholesterol enrichment, DIV 6–7 CGCs were exposed to low-potassium medium supplemented with 3.4/20 mM cholesterol/MβCD complex and incubated at 37°C in 5% CO_2 for 10 or 60 min as specified.

Control CGCs were incubated at 37°C in 5% CO_2 in low-potassium medium supplemented with 5 mM sucrose to mimic the osmolarity of MβCD or 20 mM to mimic the osmolarity of cholesterol/MβCD.

Manipulation of the CGC cholesterol content was terminated by washing the CGCs in extracellular solution 2 (ECS2) containing (in mM): 160 NaCl, 2.5 KCl, 10 Hepes, 10 glucose, 2 $CaCl_2$ and 1 $MgCl_2$ (pH 7.3).

Electrophysiology

The patch-clamp technique was used to record whole-cell responses using an Axopatch 200 B amplifier (Molecular Devices, Sunnyvale, CA, USA). Electrophysiology recordings were carried out on DIV 6–7 CGCs (on DIV 9 for simvastatin pretreated CGCs) at room temperature (22–24°C) between 3 and 20 min after cholesterol manipulation. Patch pipettes (4–6 MΩ resistance) were pulled from borosilicate glass (BioMedical Instruments, Zoellnitz, Germany) and filled with intracellular solution composed of (in mM) 125 gluconic acid, 15 CsCl, 10 Hepes, 1 $CaCl_2$, 3 $MgCl_2$, 10 BAPTA and 2 ATP-Mg^{2+} salt (pH 7.2). Fast application of ECS containing agonists, cholesterol/MβCD complex and/or (+)D-methyl-l0,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801; Sigma) was carried out via the set of parallel tubes moved by a stepper motor. The exchange time (30 ± 10 ms; $n = 6$) for drug application was determined from the step (10–90%) in the response when a patched CGC was exposed to 100 μM NMDA dissolved in ECS and subsequently to 100 μM NMDA dissolved in ECS diluted (50:50) by water with sucrose. Glycine (10 μM, unless stated otherwise) was applied 2 s before and during the application of NMDA. The holding potential was –60 mV unless stated otherwise. Series resistance (<10 MΩ) was compensated to 90%. Receptor responses were low-pass filtered (2 kHz eight-pole Bessel filter) and digitally sampled at 10 kHz. Recording was conducted using pClamp 10.1 software (Molecular Devices).

Patch-clamp data were analysed using pClamp 10.1 software. Dose–response curves for glycine were acquired from the amplitudes of NMDAR responses at various concentrations of glycine. Amplitudes (I) were plotted against the concentration of glycine and fitted with the logistic equation: $I = I_{max}/(1 + [EC_{50}/(c + c_0)]^h)$, where I_{max} is the maximal response, EC_{50} is the concentration eliciting the half-maximal response, h is the Hill coefficient, c is the concentration of added glycine and c_0 is the background glycine concentration in the application solution. Dose–response curves for NMDA were found by fitting the appropriate data by the same equation without any background NMDA concentration.

The analysis of single-channel open times was carried out by approximating individual openings by a step function. Openings shorter than 0.5 ms were excluded from the analysis to allow for the Bessel filter (2 kHz) rise time (Colquhoun & Sakmann, 1985).

Measurements of plasma membrane fluidity

Changes in the plasma membrane fluidity were assessed by fluorescence anisotropy measurements using *N*,*N*,*N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium *p*-toluenesulphonate (TMA-DPH; Sigma) fluorescent probe as described in Illinger *et al.* (1995). Cerebella from postnatal day 8 rats were trypsinized, dissociated to single cells, centrifuged and resuspended in ECS2 solution (control cells) or ECS2 solution containing cholesterol oxidase (10 U ml^{-1}; Sigma) or 5 mM MβCD and incubated at 37°C for 3 or 60 min. Subsequently,

the cell suspension was centrifuged and the pellet was resuspended in fresh ECS2 containing 1 μM TMA-DPH. Steady-state fluorescence anisotropy was measured with a PC1 spectrofluorimeter (ISS, Champaign, IL, USA) using 355 nm excitation and detection of emission at 430 nm.

Immunocytochemical labelling and confocal microscopy

For immunocytochemistry experiments, CGCs were transfected on DIV 5 by a calcium phosphate technique described in Prybylowski *et al.* (2002). Briefly, 12 mM glass coverslips with CGCs were placed in 0.5 ml of MEM (Life Technologies). Then, we added 30 μl of DNA/Ca^{2+} mixture containing 3 μg of DNA encoding green fluorescent protein (GFP)-tagged GluN2B (a generous gift from Dr S. Vicini, Georgetown University School of Medicine, Washington, DC, USA; the construct is described in Luo *et al.* 2002). After 60 min of incubation at 37°C in 5% CO_2, cells were washed twice with MEM and maintained in low-potassium medium.

Immunocytochemical labelling was carried out on DIV 7. Labelling of surface NMDARs in transfected CGCs started with washing the transfected neurons with phosphate-buffered saline (PBS) and incubating them in blocking solution composed of PBS and 0.2% bovine serum albumin (MP Biomedicals, Carlsbad, CA, USA) at room temperature for 5 min. Subsequently, cells were exposed to primary polyclonal rabbit anti-GFP antibody (dilution 1:1000) (Millipore, Billerica, MA, USA) diluted in blocking solution for 10 min. CGCs were washed twice with PBS and exposed for 10 min to the goat anti-rabbit IgG secondary antibody conjugated with fluorescent dye Alexa Fluor 647 (Life Technologies) diluted in blocking solution. Neurons were washed twice with PBS and fixed with 4% paraformaldehyde (Sigma) in PBS (w/v) for 20 min. Subsequently, intracellular NMDARs were labelled. CGCs were permeabilized by 0.25% Triton X-100 (Sigma) for 5 min, blocked with blocking solution containing 0.1% Triton X-100 for 1 h and exposed to primary rabbit anti-GFP antibody for 30 min, followed by washing and 30 min of treatment with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; Life Technologies). Finally, CGCs were mounted with ProLong Antifade reagent (Life Technologies). An SPE confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to take z-stacks of images visualizing surface NMDARs (red emission of Alexa Fluor 647) and total NMDARs (green emission of Alexa Fluor 488 and GFP). ImageJ software (NIH, Bethesda, MD, USA) was used to analyse and compare the fluorescence of surface and total cellular NMDARs as described in Lichnerova *et al.* (2014).

Mass spectrometry assessment of cholesterol content in CGCs

Mass spectrometry measurements were carried out on DIV 6. Five million CGCs on the 25 mm coverslip were subjected to MβCD or cholesterol/MβCD pretreatment, washed three times by ECS2 and collected in deionized water. Special attention was paid to ensure minimal cell loss during the pretreatment and washing. The cholesterol concentration was assessed using a QTRAP 5500 mass spectrometer (AB Sciex, Framingham, MA, USA) in accordance with the method described in Liebisch *et al.* (2006).

Kinetic analysis of NMDAR responses

To analyse the effect of cholesterol on NMDARs, we used a five-state kinetic model (Lester & Jahr, 1992). Gepasi software (http://www.gepasi.org) was used to obtain the values of rate constants of desensitization and resensitization. First, the kinetic scheme of NMDAR activation was defined. Subsequently, electrophysiology NMDAR responses were fitted with the time course of open state in the kinetic scheme. The rate constants of desensitization and resensitization in the kinetic scheme were allowed to vary so that their optimal values were found.

Statistical analysis

The results are reported as the mean ± SEM. Student's *t* test was used to compare datasets. $P < 0.05$ was considered statistically significant.

Results

MβCD pretreatment selectively diminishes NMDAR responses

The present study aimed to determine the role of membrane cholesterol in the function of ionotropic glutamate receptors. We used MβCD pretreatment to manipulate cell cholesterol. MβCD is a cyclic glucose oligomer with a hydrophobic cavity that is able to bind lipids (especially cholesterol) and make them water soluble (Christian *et al.* 1997). Rapid application of NMDA (100 μM) in the presence of glycine (10 μM) was used to compare NMDAR responses in control and MβCD pretreated cultured CGCs. Because the responses of control CGCs show relatively strong run-down (up to 50% within 5 min) and MβCD has to act for several minutes, we did not perform experiments that compare NMDA-induced responses before and after MβCD application. Instead, CGCs were incubated in MβCD at 37°C and patch-clamp

recordings were carried out afterwards. Similarly, control CGCs were incubated in sucrose-supplemented medium for 1–60 min prior to patch-clamp recordings (see Methods). The amplitudes of NMDAR responses in control CGCs were independent of the duration of the sucrose pretreatment and therefore the data were pooled.

Incubation of CGCs in a medium supplemented with 5 mM MβCD resulted in two macroscopic changes of NMDAR responses. First, the amplitude of NMDAR responses decreased with the duration of MβCD pretreatment. NMDAR response amplitudes in control CGCs were 1180 ± 100 pA ($n = 21$). Figure 1*A* and *B* shows that NMDAR responses were significantly reduced to $52 \pm 8\%$ ($n = 4$, $P = 0.002$) already after 1 min of MβCD pretreatment, and were diminished to $3 \pm 1\%$ after 60 min of MBCD pretreatment ($n = 11$, $P = 2 \times 10^{-10}$). Second,

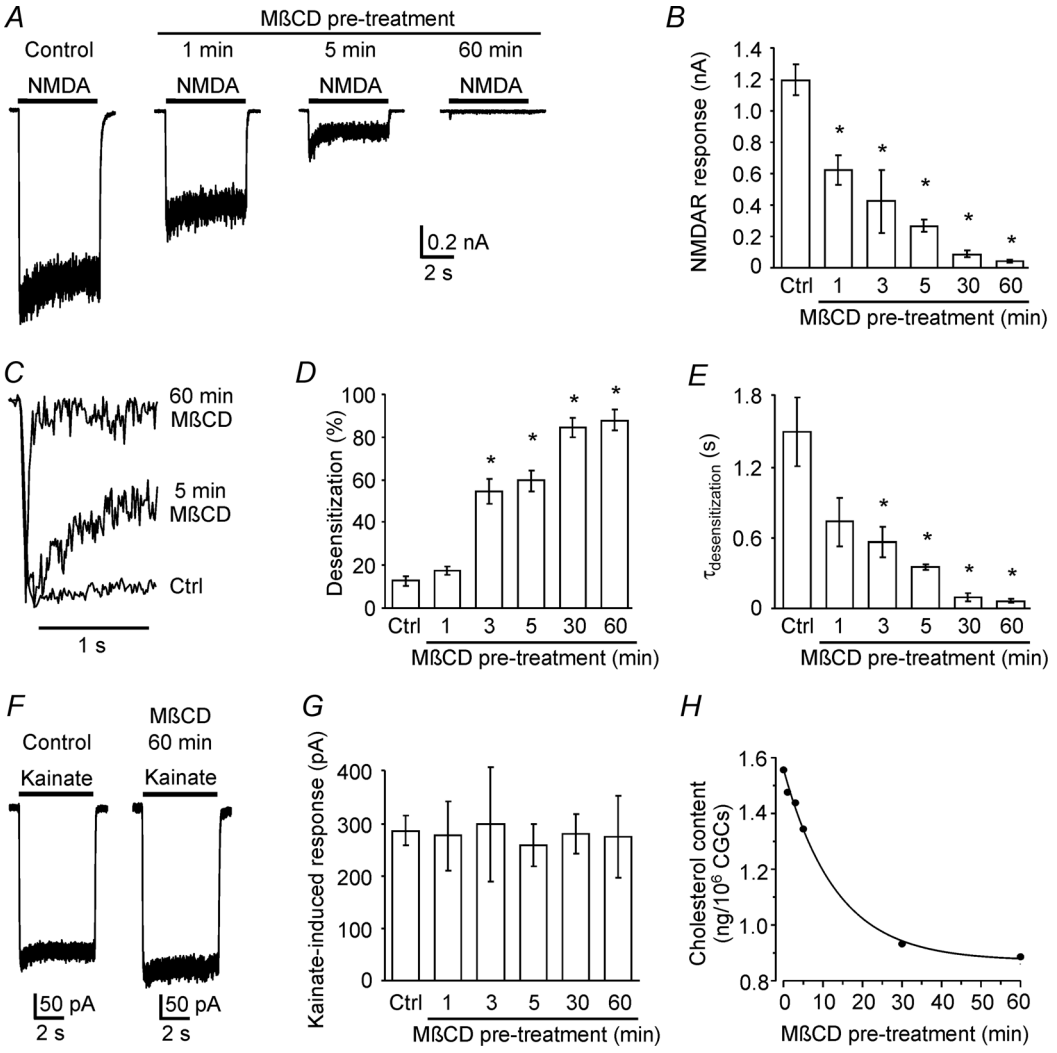

Figure 1. MβCD pretreatment diminishes amplitudes and alters the desensitization of NMDA-induced responses but does not affect kainate-induced responses
A, CGC responses to the application of 100 μM NMDA in the presence of 10 μM glycine. The responses were recorded from four different CGCs under control conditions and after 1, 5 and 60 min of 5 mM MβCD pretreatment. *B*, mean amplitudes of responses to 100 μM NMDA for control CGCs and for CGCs pretreated with 5 mM MβCD for 1, 3, 5, 30 and 60 min. The means were calculated from four to 21 CGCs. *C*, control response and responses after 5 and 60 min of MβCD pretreatment (same traces as in *A*) are superimposed and normalized with respect to their amplitudes to show differences in their desensitization. *D*, NMDAR desensitization expressed as (1 – steady state/peak amplitude) × 100% after different durations of MβCD pretreatment. The means were calculated from four to 21 CGCs. *E*, time constant of desensitization of NMDAR responses as a function of MβCD pretreatment duration. *F*, CGC responses to 100 μM kainate under control conditions and after 60 min of MβCD pretreatment. *G*, mean amplitudes of responses to 100 μM kainate. The means were calculated from four to 19 CGCs. *H*, total cholesterol content in CGCs after 0–60 min of MβCD (5 mM) pretreatment. Data points represent the mean of two independent mass spectroscopy measurements. $^*P < 0.05$ (relative to control).

the macroscopic receptor desensitization changed. The relative degree of desensitization observed during a 5 s NMDA application was 13 ± 2% ($n = 21$) in control CGCs and 88 ± 5% ($n = 7$, $P = 2 \times 10^{-7}$) in CGCs pretreated with MβCD for 60 min (Fig. 1*C* and *D*). The onset of desensitization was fitted by a single exponential function with $\tau = 1500 \pm 300$ ms in controls and the time constant decreased with the duration of MβCD pretreatment, reaching 64 ± 16 ms ($n = 7$, $P = 0.0009$) in CGCs pretreated with MβCD for 60 min (Fig. 1*C* and *E*). By contrast, responses induced by 100 μM kainate, a concentration that activates both AMPA and kainate receptors, were not affected by MβCD pretreatment for up to 60 min (Fig. 1*F* and *G*). The amplitudes of kainate-induced responses were 290 ± 30 pA and 270 ± 80 pA for control ($n = 19$) and 60 min of MβCD pretreated ($n = 10$) CGCs, respectively ($P = 0.89$). Similar effects of MβCD pretreatment on the amplitude and desensitization of NMDAR responses were observed in cultured rat hippocampal neurons and in human embryonic kidney 293 cells expressing GluN1/GluN2B receptors. The variability in the amplitude of NMDAR responses in these cells (often more than 10-fold) made it difficult to study their amplitudes. By contrast, the amplitude of NMDAR responses in CGCs is much less variable and therefore we decided to use CGCs in the present study.

Figure 1*H* shows the mass spectrometry analysis of the cholesterol content in CGCs. In control cells, the cholesterol content was 1.56 ng per million CGCs. Pretreatment with MβCD (5 mM) for 60 min resulted in cholesterol depletion to 0.89 ng per million CGCs. These results indicate that NMDARs are quite sensitive to cholesterol because a short MβCD pretreatment (e.g. 1 min) has only a small effect on the cell cholesterol content (Fig. 1*H*) but a profound effect on the amplitude of NMDAR responses (Fig. 1*B*).

Next, we analysed the effect of chronic cholesterol depletion on NMDARs (Fig. 2). CGCs were cultured for 4 days in the presence of simvastatin (100 nM), which is the inhibitor of cholesterol biosynthesis used in human medicine to reduce blood plasma cholesterol (Endo, 2010). NMDAR responses recorded from simvastatin pretreated CGCs were significantly smaller (reduced to 53%, $P = 0.003$) (Fig. 2*A* and *B*) than those recorded in control CGCs on the same DIV. Kainate-induced responses were not significantly affected (12% increase, $P = 0.48$) (Fig. 2*C* and *D*) after a 4-day simvastatin pretreatment. Our data show that the incubation of CGCs in either MβCD or simvastatin induces significant changes in the function and/or surface expression of NMDARs but no change in AMPA/kainate receptors.

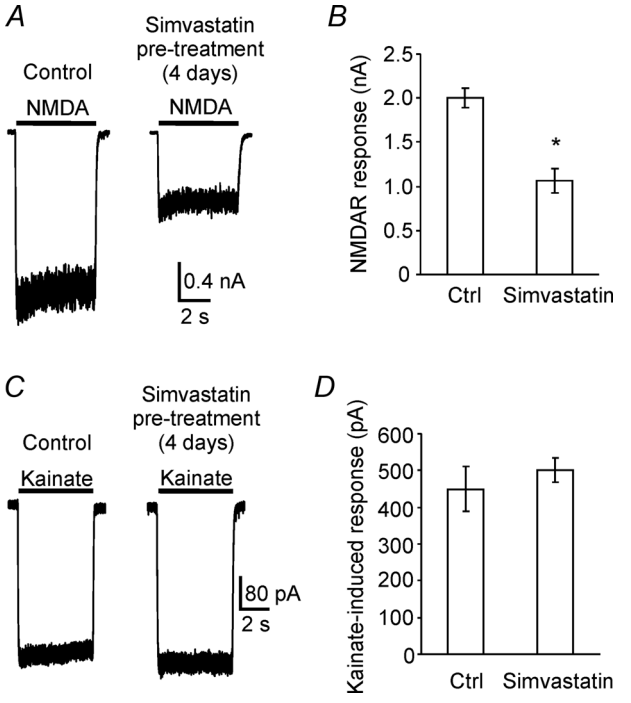

Figure 2. Chronic inhibition of cholesterol biosynthesis by simvastatin induces qualitatively the same effects on NMDA and AMPA/kainate receptors as acute MβCD pretreatment
A, responses of CGCs to the application of 100 μM NMDA. Control CGC (left) and CGC cultured with simvastatin (100 nM, 4 days, right). Both cells were measured on DIV 9 (see Methods). *B*, mean amplitudes of responses to 100 μM NMDA for control and simvastatin (100 nM, 4 days) cultured CGCs. The mean amplitude of control responses is higher than in Fig. 1*B*, where the measurement was carried out on DIV 6–7 ($n = 5$ for each of the means). *C*, responses of control and simvastatin (100 nM, 4 days) cultured CGCs to the application of 100 μM kainate. *D*, mean amplitudes of responses to 100 μM kainate for control and simvastatin (100 nM, 4 days) cultured CGCs ($n = 5$ for each of the means). $^*P < 0.05$ (relative to control).

Cholesterol repletion reverses the effects of MβCD pretreatment on NMDAR responses

Cyclodextrin treatment depletes the plasma membrane of cholesterol and, to a lesser extent, other membrane constituents, such as sphingomyelin, phosphatidylcholine and glycosphingolipids (Ohvo-Rekila *et al.* 2002; Ottico *et al.* 2003). To confirm the specific role of cholesterol for NMDAR function, we tested whether cholesterol repletion may reverse MβCD-induced changes in the NMDAR responses. Figure 3*A* shows a small current response to NMDA in a CGC that was incubated in MβCD (5 mM) for 30 min prior to patch-clamp recording. Within 400 s of a coapplication of NMDA and 3.4/20 mM cholesterol/MβCD complex at room temperature, the steady-state current gradually increased from 10 to 800 pA. A subsequent response to NMDA made in the absence of cholesterol/MβCD had an amplitude (1010 pA) and desensitization (13%) similar to the responses of control CGCs (Fig. 1*A*). Figure 3*B* shows that,

in MβCD-pretreated CGCs (30 min), the application of NMDA (600 s) without concurrent cholesterol repletion had no significant effect on the amplitude of the NMDAR-mediated current. The analysis of the rate of recovery (Fig. 3*C–F*) of NMDAR responses after 30 min of MβCD pretreatment indicates that the amplitude of NMDAR responses, the relative degree of desensitization and the time constant of desensitization onset recovered to control values within 30 min of cholesterol/MβCD pretreatment at 37°C. These results show that cholesterol plays an important role in controlling NMDAR channel function.

Our subsequent experiment aimed to test the effect of cholesterol enrichment on NMDAR responses in intact CGCs (Fig. 4). The amplitude of responses to 100 μM NMDA in CGCs pretreated with 3.4/20 mM cholesterol/MβCD complex at 37°C for 10 min was 1590 $\pm$ 140 pA ($n = 11$), which is significantly higher compared to control CGCs ($P = 0.02$) (Fig. 4*A* and *B*). Desensitization parameters of NMDAR responses in these cells were not affected: 13 $\pm$ 4% desensitization ($n = 8$, $P = 0.95$) with the time constant of desensitization onset of 1450 $\pm$ 300 ms ($n = 8$, $P = 0.89$) (Fig. 4*C* and *D*). Longer cholesterol enrichment (60 min) did not induce any further increase in NMDAR responses. By contrast to NMDARs, the amplitude of responses induced by 100 μM kainate after 3.4/20 mM cholesterol/MβCD pretreatment for 10 or 60 min were not significantly different from control kainate-induced responses ($P = 0.30$ and 0.81, respectively) (Fig. 4*E* and *F*). Mass spectrometry indicated that cholesterol enrichment increased CGC cholesterol content from 1.56 ng per million CGCs in control to 2.41 ng million^{-1} CGCs after 10 min of pretreatment with 3.4/20 mM cholesterol/MβCD complex at 37°C.

Cholesterol affects membrane proteins by direct and indirect mechanisms (Paila & Chattopadhyay, 2009). Of the indirect mechanisms, changes in the membrane physical properties have been proposed as a mechanism to explain the modulation of NMDAR responses after lysophospholipid and arachidonic acid application (Casado & Ascher, 1998). To investigate whether diminution of the NMDAR responses after cholesterol

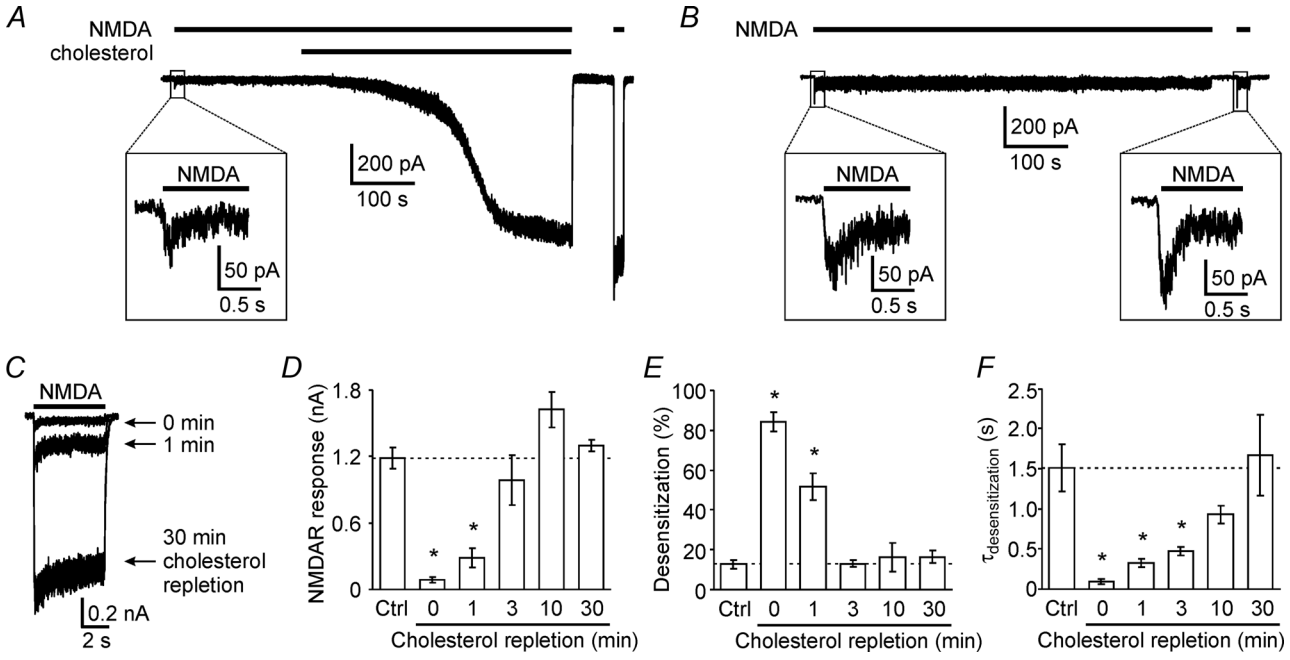

Figure 3. Cholesterol repletion restores the amplitude and desensitization of NMDAR responses in MβCD-pretreated CGCs to control values
A, response of 30 min MβCD-pretreated CGC to 100 μM NMDA before and during room-temperature cholesterol repletion with 3.4/20 mM cholesterol/MβCD complex. The initial response to NMDA (40 pA amplitude) is shown using an expanded scale in the inset. The cholesterol repletion induced a strong increase in response, which was maintained after cholesterol/MβCD washout. *B*, response of 30 min MβCD-pretreated CGC to 100 μM NMDA showing no significant increase without cholesterol repletion. *C*, typical responses to 100 μM NMDA of three CGCs pretreated with MβCD for 30 min and subsequently repleted with cholesterol (using 3.4/20 mM cholesterol/MβCD pretreatment at 37°C before electrophysiology measurements). The repletion lasted 0 (no repletion), 1 and 30 min, respectively. *D–F*, amplitudes of 100 μM NMDA-induced currents, the degree of desensitization and the desensitization time constant from CGCs pretreated for 30 min in MβCD followed by 0 (no repletion), 1, 3, 10 and 30 min of cholesterol repletion (using cholesterol/MβCD pretreatment at 37°C). Control CGC values were added to each graph (first columns) for comparison. The means were calculated from four to 21 CGCs. *$P < 0.05$ (relative to control).

depletion is caused by changes in membrane physical properties, we used enzymatic cholesterol degradation by cholesterol oxidase, which converts cholesterol to 4-cholesten-3-one (Fig. 5). We estimated changes in plasma membrane fluidity in CGCs by measuring fluorescence anisotropy of the TMA-DPH fluorescent probe. TMA-DPH dissolves in the extracellular leaflet of the plasma membrane and so an increase in plasma membrane fluidity is accompanied by a lower TMA-DPH fluorescence anisotropy (Illinger *et al.* 1995). In control CGCs, the anisotropy was 0.291 ± 0.002 (three independent experiments, each with nine measurements) and it was not significantly changed after 60 min of pretreatment by 10 U ml^{-1} cholesterol oxidase at 37°C (0.287 ± 0.003; three experiments, each with nine measurements; $P = 0.24$) (Fig. 5*A*). By contrast, similar pretreatment with 5 mM MβCD (60 min) reduced the anisotropy to 0.255 ± 0.003 (three experiments, each with nine measurements; $P = 1 \times 10^{-13}$). The effect of 5 mM MβCD on anisotropy was significant (0.279 ± 0.002; three experiments, each with nine measurements; $P = 2 \times 10^{-5}$) already after 3 min of 5 mM MβCD pretreatment. These data correspond to that observed after cholesterol manipulation in isolated plasma membranes and confirm that sterols stiffen lipid membranes (Gimpl *et al.* 1997). Next, we analysed the effect of cholesterol oxidase on NMDAR responses. After 60 min of CGC pretreatment with 10 U ml^{-1} of cholesterol oxidase at 37°C, the responses induced by 100 μM NMDA were significantly reduced to 36 ± 6% of control ($P = 2 \times 10^{-5}$) and the desensitization increased to 48 ± 6%, which is an extent similar to that for 3 min of MβCD pretreatment (Fig. 5*B* and *C*). These data show that the 4-cholesten-3-one has an effect on membrane fluidity similar to that of cholesterol, although it is unable to replace cholesterol at the NMDARs. These data indicate that the cholesterol modulation of NMDARs is not carried out by changing the plasma membrane fluidity.

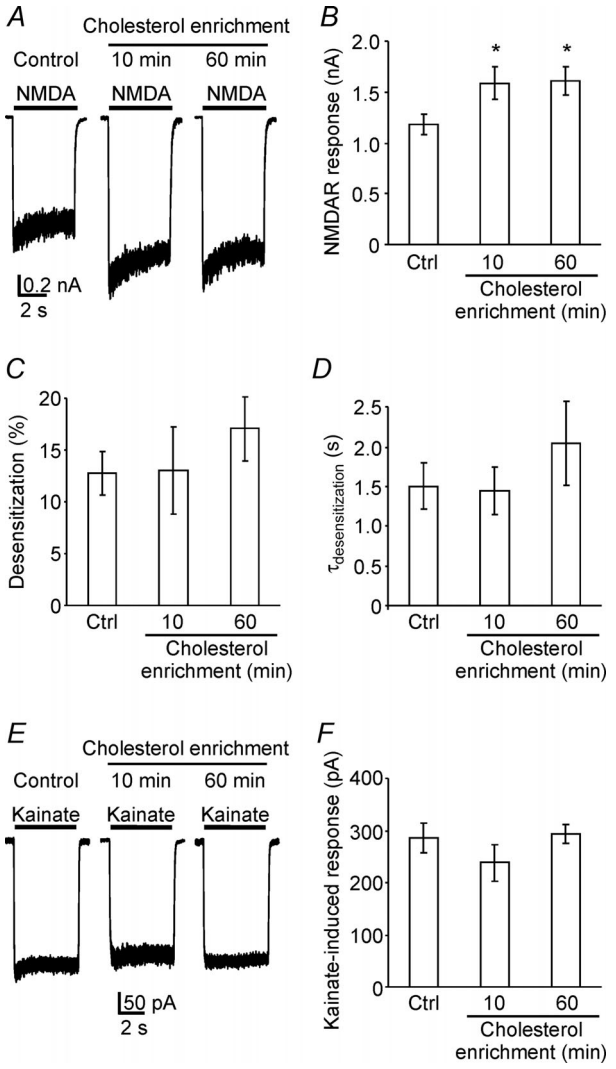

Figure 4. Native NMDARs are not saturated with cholesterol
A, typical responses of control and cholesterol-enriched CGCs to the application of 100 μM NMDA. Cholesterol enrichment was carried out by pretreating the CGCs with 3.4/20 mM cholesterol/MβCD for 10 and 60 min. *B*, mean amplitude of NMDAR responses to 100 μM NMDA is significantly increased in cholesterol-enriched cells compared to control CGCs. *C* and *D*, extent and time constant of desensitization are not significantly changed by cholesterol enrichment. *E*, 100 μM kainate-induced responses in control CGCs and CGCs pretreated with 3.4/20 mM cholesterol/MβCD for 10 and 60 min. *F*, amplitudes of responses induced by application of 100 μM kainate are not significantly changed by cholesterol enrichment. *$P < 0.05$ (relative to control).

Peak open probability (P_o) of NMDAR channels is reduced by cholesterol depletion

In the subsequent experiments, we aimed to determine the molecular mechanism of cholesterol-induced changes in NMDAR properties. We have considered cellular mechanisms (altered surface expression of NMDARs) and/or change in the receptor properties (probability of channel opening, single-channel conductance, ion channel open time and NMDAR affinity for agonists). As an experimental tool, we used an open channel blocker (MK-801) to compare NMDAR properties in control and 5 min cholesterol-depleted CGCs (5 mM MβCD pretreatment).

Figure 6*A* shows the response of control and cholesterol-depleted CGCs to a coapplication of 10 μM NMDA and 10 μM MK-801. Although, in both cases, the NMDAR current gradually decreased as a result of the blocking effect of MK-801, the time course of this decrease was quite different [half decay time of control CGCs was 0.60 ± 0.15 s ($n = 7$) compared to 2.3 ± 0.4 s ($n = 7$) for cholesterol-depleted CGCs]. These data indicate that peak open probability (P_o) of NMDAR

channels, mean open time or agonist affinity may be altered by cholesterol depletion.

To estimate P_o, we used the method introduced by Jahr (1992) that determines the P_o as the ratio of NMDARs opened at the peak of the response to the total number of NMDARs in the plasma membrane. We quantified the total number of NMDARs by the method described in Rosenmund *et al.* (1995), which utilizes NMDAR responses recorded in the presence of MK-801 (Fig. 6*A*). The blocking rate constant of MK-801 is $k_{MK} = 25\ \mu M^{-1}\ s^{-1}$ (Jahr, 1992). In the presence of 10 μM MK-801, an NMDAR opens cumulatively for a mean time of $t_b = 1/([\text{MK-801}] \times k_{MK}) = 4$ ms before it is blocked. Mean charge transfer conducted by a single NMDAR is: $q = t_b \times i$ (eqn 1), where i is the single-channel current (see below). The number of NMDARs in the plasma membrane is: $N = Q/q$ (eqn 2), where Q is the charge transfer conducted by all NMDARs activated in the whole-cell configuration in the presence of MK-801. Analysis of the responses to 10 μM NMDA and 10 μM MK-801 in control and in 5 min cholesterol-depleted CGCs (Fig. 6*A*) showed the charge transfer of 83.6 ± 6.9 pA s ($n = 7$) and 92.4 ± 10.2 pA s ($n = 7$), respectively.

We assessed single-channel properties for receptors activated by a low concentration of NMDA (0.5 μM) in the whole-cell configuration (Fig. 7*A* and *B*). This aimed to exclude changes in the receptor properties induced by patch excision (Lester & Jahr, 1992; Rosenmund *et al.* 1995; Clark *et al.* 1997; Korinek *et al.* 2010). Single-channel current amplitudes measured at –60 mV in control and cholesterol-depleted CGCs (5 min of 5 mM MβCD pre-treatment) were similar, with values of 4.58 ± 0.16 pA ($n = 5$) and 4.28 ± 0.23 pA ($n = 5$) ($P = 0.34$), respectively.

The total number of the surface NMDARs estimated using eqs (1) and (2) was 4570 ± 380 receptors in control and 5390 ± 560 receptors in 5 min cholesterol-depleted CGCs (not significantly different, $P = 0.27$) (Fig. 6*B*), showing that the diminution of NMDAR responses upon cholesterol depletion is not a result of their internalization. This finding is supported by immuno-cytochemistry experiments in which we estimated the ratio of fluorescently labelled surface to total NMDARs (Fig. 6*C*). This ratio was normalized with respect to control CGCs and its value after cholesterol depletion (5 min of 5 mM MβCD pretreatment) was 1.09 ± 0.13, which is not significantly different from control ($P = 0.57$) (Fig. 6*D*).

To determine the value of P_o, we assessed the number of receptors opened at the peak of the response as the ratio of the peak current measured at the saturating concentration of NMDA (1 mM) and a single NMDAR channel current. The peaks of responses to 1 mM NMDA were 2180 ± 180 pA ($n = 6$) in control and 640 ± 90 pA ($n = 6$) in 5 min cholesterol-depleted CGCs, indicating that 460 ± 40 and 150 ± 20 channels were open in control and in cholesterol-depleted CGCs, respectively. P_o, calculated as the ratio of the number of receptors opened at the peak to the total number of surface receptors, was 10.0 ± 1.2% in control CGCs. The value of P_o in CGCs after 5 min of 5 mM MβCD pretreatment was significantly reduced to 2.8 ± 0.5% ($P = 0.006$).

To check other parameters that may be altered by cholesterol depletion, open-time analysis and dose–response analysis were performed. Single-channel open times were measured in the whole-cell configuration at a membrane potential of –60 mV using 0.5 μM NMDA. Figure 7*C* shows representative open-time histograms from control and cholesterol-depleted CGCs. Single exponential fits provided time constants (τ_{open}) of 1.29 ± 0.13 ms (control CGCs, $n = 7$) and 1.12 ± 0.10 ms (5 min of 5 mM MβCD; $n = 8$; not statistically different from control, $P = 0.35$). These time constants are similar to those measured in CGCs by Clark *et al.* (1997).

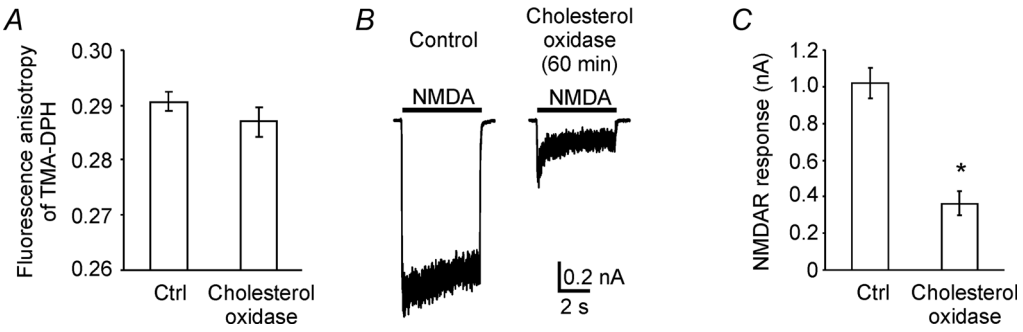

Figure 5. Enzymatic degradation of cholesterol to 4-cholesten-3-one does not change plasma membrane fluidity but reduces NMDAR responses
A, fluorescence anisotropy measurement of TMA-DPH dye was used to assess the fluidity of CGC plasma membranes. No significant difference in the mean anisotropy of fluorescence was seen after 60 min of pre-treatment with 10 U ml^{-1} cholesterol oxidase at 37°C. *B*, typical responses to 100 μM NMDA of control CGC and CGC after 60 min of pretreatment with 10 U ml^{-1} cholesterol oxidase. *C*, mean amplitudes of CGC responses induced by 100 μM NMDA. Significant reduction of amplitude induced by 60 min of cholesterol oxidase pre-treatment is shown ($n = 8$ for each of the means). *$P < 0.05$ (relative to control).

Dose–response analysis in Figure 8 shows that the EC_{50} values for glycine or NMDA were not significantly different in control and in cholesterol-depleted (5 min of 5 mM MβCD) CGCs. Glycine: $EC_{50} = 0.46 \pm 0.09$ μM and 0.44 ± 0.06 μM for control ($n = 6$) and 5 min cholesterol-depleted CGCs ($n = 6$), respectively, ($P = 0.87$). NMDA: $EC_{50} = 41.4 \pm 2.6$ μM and 41.8 ± 7.1 μM for control ($n = 5$) and cholesterol-depleted ($n = 5$) CGCs, respectively, ($P = 0.97$).

Cholesterol depletion induces an increase in the rate constant of desensitization

As shown above, cholesterol depletion results in apparent changes in NMDAR desensitization (Fig. 1*C*, *D* and *E*). To analyse this effect, we used a five-state kinetic scheme introduced by Lester & Jahr (1992), where the rate constants k_b, k_u, k_o, k_c, k_d, and k_r determine the transitions between individual NMDAR states (Fig. 9*A*). More advanced kinetic schemes based on single-channel

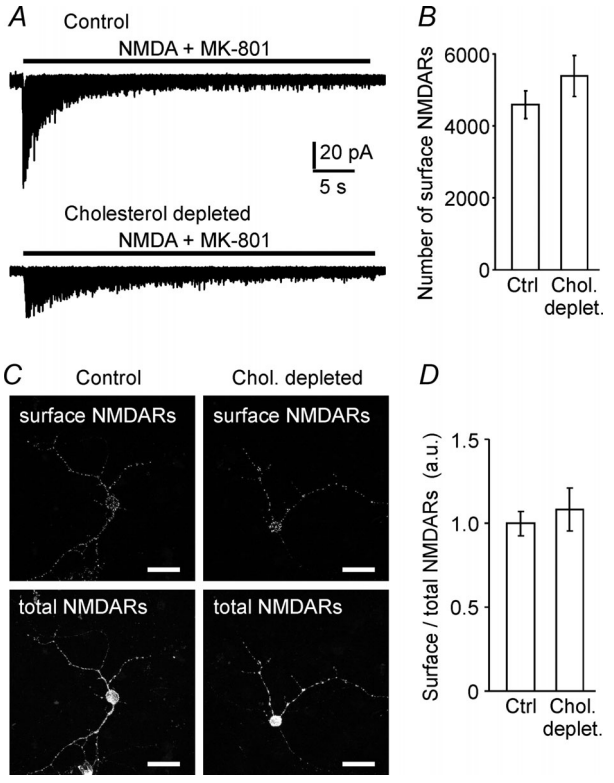

Figure 6. Comparison of the number of NMDARs in the plasma membrane of control and cholesterol-depleted CGCs
A, responses of control and cholesterol-depleted (5 min of 5 mM MβCD pretreatment) CGCs to a coapplication of 10 μM NMDA and 10 μM MK-801. *B*, charge transfer measured during the NMDA and MK-801 coapplication was used to calculate the number of NMDARs in the plasma membrane. The mean number of surface NMDARs in control and cholesterol-depleted CGCs is shown ($n = 7$ for each column). *C*, confocal fluorescence microscopy of immunocytochemically labelled surface (top) and total (bottom) NMDARs in control and cholesterol-depleted (5 min of 5 mM MβCD) CGCs. Scale bars = 20 μm. *D*, ratio of fluorescence originating from surface receptors to fluorescence from total receptors in control and cholesterol-depleted (5 min of 5 mM MβCD) CGCs. Column height was normalized with respect to control CGCs ($n = 21$ for each column).

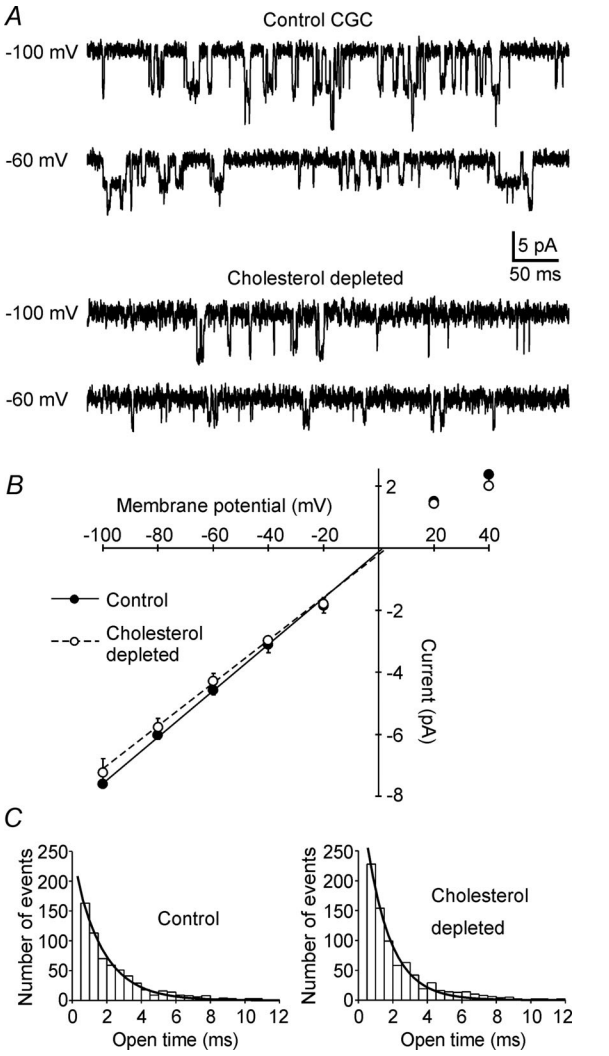

Figure 7. Cholesterol depletion does not alter single-channel conductance and open time
A, single-channel openings elicited by 0.5 μM NMDA in control and cholesterol-depleted (5 min of 5 mM MβCD pretreatment) CGCs in the whole-cell configuration at a membrane potential of –100 and –60 mV. Control CGCs have a higher probability of channel opening, which induces occasional double-channel openings. *B*, current–voltage relationships for NMDARs in control and 5 min cholesterol-depleted CGCs. The data points at negative membrane potentials were linearly fitted to determine conductances: 75.0 ± 3.7 pS (control CGCs, $n = 5$) and 69.0 ± 3.1 pS (cholesterol-depleted CGCs, $n = 6$), $P = 0.25$. *C*, open-time distributions of single-channel events measured in control and cholesterol-depleted (5 min of 5 mM MβCD) CGCs. Data were recorded in the whole-cell configuration at –60 mV using 0.5 μM NMDA, the distributions were fitted with single exponential functions.

measurements were proposed (Popescu & Auerbach, 2003); however, the five-state scheme describes basic processes of agonist binding, NMDAR opening and desensitization on the whole cell level, where thousands of receptors are activated simultaneously. As a result of a low concentration of calcium in our application solution, the kinetic scheme does not need to have the calcium-dependent desensitized state (Cais *et al.* 2008). Because the time course of desensitization is determined by the rate constants of desensitization (k_d) and resensitization (k_r) (Fig. 9*A*), we focused on finding their values for various times of MβCD-induced cholesterol depletion. To achieve this, CGC responses to 100 μM NMDA (Fig. 1*A*) were fitted using the kinetic scheme. The rate constants that are not affected by cholesterol depletion were assessed prior to fitting: the values of the rate constants of NMDA binding and unbinding $k_b = 1.16\ \mu M^{-1}\,s^{-1}$ and $k_u = 20\ s^{-1}$ were found from the deactivation of NMDAR responses and from the NMDA EC_{50} value (Cais *et al.* 2008). The rate constant k_c is the reciprocal value of the mean τ_{open}, $k_c = 830\ s^{-1}$. The rate constants k_d, k_r and k_o were allowed to vary during fitting. Figure 9*B* shows examples of responses to 100 μM NMDA fitted with the kinetic scheme. The estimated values of k_d and k_r as a function of the duration of MβCD pretreatment indicate that the value of k_d increased by more than 200-fold from $0.33 \pm 0.09\ s^{-1}$ in control to $75 \pm 26\ s^{-1}$ in 60 min cholesterol-depleted CGCs, whereas the k_r values were unaffected (Fig. 9*C*). The value of k_o decreased from $91 \pm 1\ s^{-1}$ in control to $8.4 \pm 3.6\ s^{-1}$ in 60 min cholesterol-depleted CGCs. These results suggest that cholesterol depletion-induced changes in the k_d explain macroscopic changes in both the rate and extent of desensitization.

Discussion

In the present study, we show the regulatory effect of endogenous cholesterol on the function of ionotropic glutamate receptors. In the model of cultured CGCs, both acute and chronic cholesterol depletion diminished the amplitudes of NMDAR responses and an enrichment of the cell cholesterol content potentiated the amplitudes of responses. The amplitudes of kainate-induced responses were not influenced by cholesterol manipulation. Our data show that the open probability and desensitization of NMDAR channels are strongly influenced by changes in cell cholesterol content.

Our MK-801 experiments indicate that changes in the open probability of NMDARs are an important factor underlying the cholesterol modulatory effect at NMDAR. The open probability of NMDAR channels is in the range 1–50% for recombinant receptors depending on the receptor subunit composition (Erreger *et al.* 2005; Yuan *et al.* 2009). Native NMDARs show values of P_o in the range 4–27% (Jahr, 1992; Rosenmund *et al.* 1995). In agreement with these studies, we found $P_o = 10\%$ in control CGCs and this value dropped in cholesterol-depleted CGCs. Cholesterol is not evenly distributed in plasma membranes of intact cells with a higher concentration in cholesterol-rich domains. These domains were reported to be enriched two-fold in cholesterol compared to bulk plasma membrane (Pike *et al.* 2002). Because

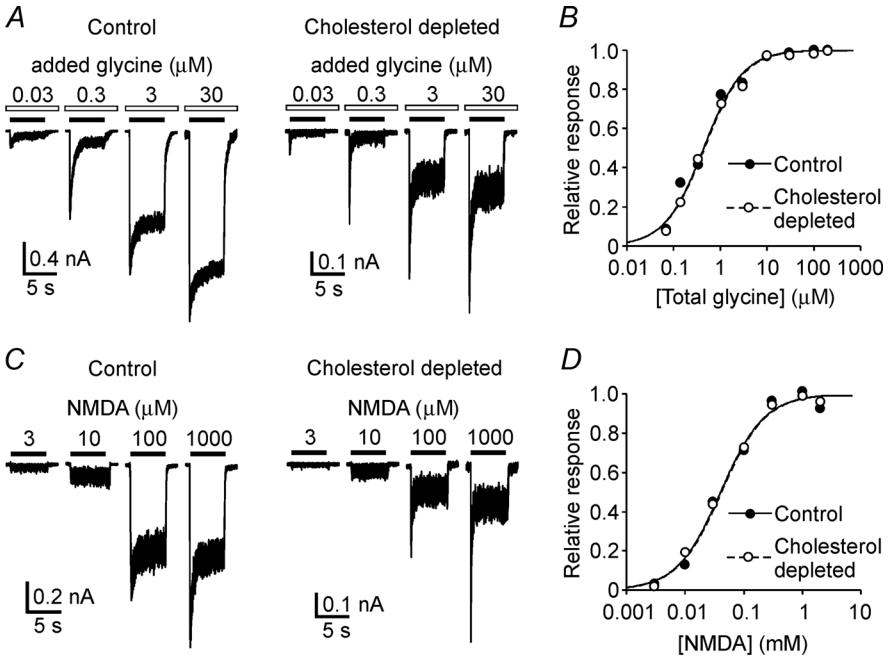

Figure 8. The affinity of glycine and NMDA for NMDARs is not changed by cholesterol depletion
A, responses of control and cholesterol-depleted (5 min of 5 mM MβCD pretreatment) CGCs to 1000 μM NMDA (full bars) applied at various concentrations of glycine (open bars). *B*, dose–response curves for glycine are almost identical for control and cholesterol-depleted CGCs. The *x*-axis shows the total concentration of applied glycine, which includes the background concentration of 0.04 μM glycine (see Methods) ($n = 6$ for each curve). *C*, responses of control and 5 min cholesterol-depleted CGCs to various concentrations of NMDA at 10 μM glycine. *D*, dose–response curves for NMDA. Fits to the measured data are overlapping ($n = 5$ for each curve).

NMDARs are localized both in and out of cholesterol-rich domains (Hering *et al.* 2003; Frank *et al.* 2004; Abulrob *et al.* 2005), our results suggest that, under physiological conditions, the properties of individual NMDARs (P_o and desensitization) may differ substantially depending on the local cholesterol content.

P_o is influenced by the rate constants controlling the transition between agonist-bound closed and open states and those of receptor desensitization. In control CGCs, entry into the desensitized state is much slower than the opening of an NMDAR ion channel. Therefore, desensitization has only a small effect on the amplitude of control responses. In agreement with a previous study (Lester & Jahr, 1992), the results of simulations of NMDAR responses (using the kinetic scheme and rate constants defining transitions between individual states; see Results) predict that, in control CGCs, the rate constants of desensitization reduce the amplitude of NMDAR responses by only 2.7%. However, under conditions of increased desensitization (e.g. at physiological temperature), the entry of NMDAR into the desensitized state competes with the receptor opening and reduces significantly the amplitude of responses (Cais *et al.* 2008). Similarly, we show that cholesterol depletion increases the rate constant of entry into the desensitized state k_d (Fig. 9*C*). Moreover, analysis of macroscopic responses indicates that cholesterol depletion reduces the rate constant of receptor opening (k_o). Simulation of responses revealed that, in strongly cholesterol-depleted CGCs (60 min MβCD), the increase in k_d induces a three-fold diminution of P_o and the decrease in k_o is responsible for a 10-fold diminution of P_o. Both effects together combine to account for a 30-fold decrease of response amplitude in 60 min cholesterol-depleted CGCs compared to control CGCs (Fig. 1*A* and *B*). Therefore, fast desensitization contributes substantially but not dominantly to the reduction of NMDAR response amplitudes in cholesterol-depleted CGCs.

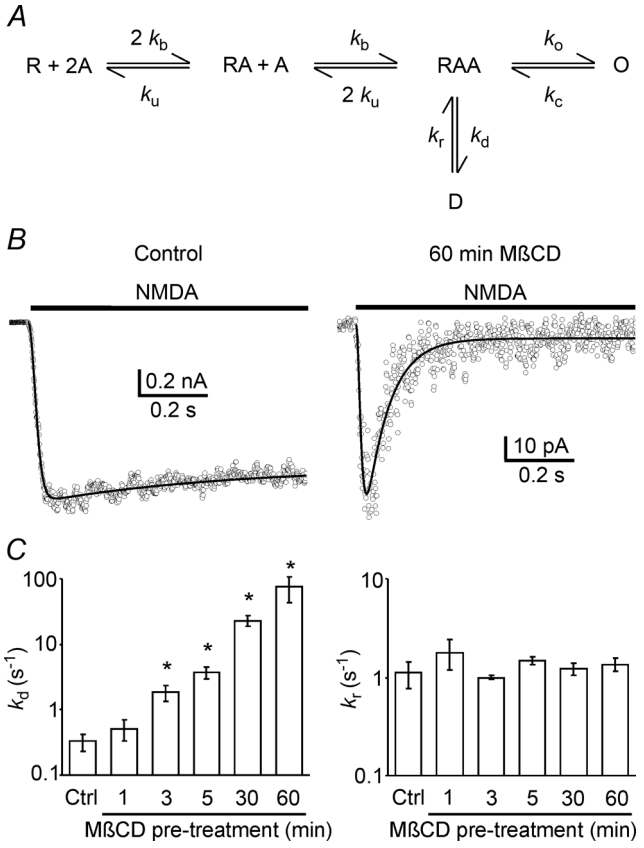

Figure 9. A steep increase in the rate constant of desensitization underlies changes in NMDAR desensitization induced by cholesterol depletion
A, kinetic scheme of NMDA receptor activation. Agonist molecules (A) bind to NMDAR (R) and allow entry into the open state (O) or the desensitized state (D). The rate constants k_b, k_u, k_o, k_c, k_d and k_r characterize the transitions between individual states. This scheme was used to determine the rate constants of desensitization k_d and resensitization k_r. *B*, two examples of NMDAR responses (control and 60 min cholesterol-depleted CGCs) and their fits with the time course of the open state in the kinetic scheme (smooth curve). The values of the rate constants were used for both curves: $k_b = 1.16\ \mu M^{-1}\ s^{-1}$, $k_u = 20\ s^{-1}$, $k_c = 830\ s^{-1}$. Fitting revealed the values of $k_o = 89.6\ s^{-1}$, $k_d = 0.29\ s^{-1}$ and $k_r = 0.90\ s^{-1}$ for the control CGC (left) and $k_o = 11.9\ s^{-1}$, $k_d = 66\ s^{-1}$ and $k_r = 0.98\ s^{-1}$ for the depleted CGC (right). *C*, mean values of the rate constants of desensitization and resensitization determined by fitting the responses of control and cholesterol-depleted CGCs. Vertical axes are in the logarithmic scale. $^*P < 0.05$ (relative to control).

Results reported previously indicate that, besides cholesterol, NMDAR channel activity can be potentiated by other 5(6)-unsaturated steroids: pregnenolone sulphate and 24(S)-hydroxycholesterol (Horak *et al.* 2004; Paul *et al.* 2013; Linsenbardt *et al.* 2014). Even though the site of action of these compounds at the NMDAR has not yet been determined, the pharmacological data indicate that pregnenolone sulphate and 24(S)-hydroxycholesterol bind to independent modulatory sites (Paul *et al.* 2013; Linsenbardt *et al.* 2014). Several oxysterols derived from 24(S)-hydroxycholesterol were also shown to be strong NMDAR potentiators (Paul *et al.* 2013), whereas our experiments involving the enzymatic degradation of cholesterol showed that another cholesterol metabolite, 4-cholesten-3-one, was unable to replace cholesterol with respect to its potentiating effect on NMDARs. The fact that these molecules, which are closely related to cholesterol, differ in their effects on NMDAR indicates that the cholesterol modulatory effect on NMDARs is partially specific.

Other types of ion channels are modulated by cholesterol (Levitan *et al.* 2014). Surprisingly, even though cholesterol depletion by MβCD diminishes the amplitude of both NMDAR and nicotinic acetylcholine receptor responses, it is underlain by different effects. In the case of NMDARs, cholesterol depletion induced the reduction of channel P_o and no change in the NMDAR surface

expression, whereas, in the case of nicotinic acetylcholine receptors, a small intraburst increase in P_o and enhanced internalization resulted in reduced surface expression (Borroni *et al.* 2007). A complex model of cholesterol modulation of acetylcholine receptors was proposed that includes 15 molecules of cholesterol (three per subunit) bound to the receptor (Brannigan *et al.* 2008). Another mechanism of cholesterol modulation of ion channels was described for $GABA_A$ receptors. Cholesterol depletion and enrichment both diminish their responses by lowering the receptor affinity for the agonist (Sooksawate & Simmonds, 2001). In the case of NMDARs, the affinity of agonists was the same in control and cholesterol-depleted CGCs.

Regarding the subunit composition, cerebellar neurons in acutely prepared brain slices are known to express GluN2C subunit, which is characterized by a low conductance (33 pS at 1 mM Ca^{2+}) (Farrant *et al.* 1994). However, our cultivation media did not contain neuregulin-β, which is necessary for the expression of GluN2C in the cerebellum (Ozaki *et al.* 1997). The high conductance NMDARs reported in both the present study and previous studies (Clark *et al.* 1997), as well as the partial (59%) sensitivity of our NMDARs to ifenprodil (a selective inhibitor of GluN2B containing receptors; data not shown), indicates that GluN2A and GluN2B containing receptors were dominant in our CGC cultures.

Our experiments show that changes in the cell cholesterol content affect NMDAR channel activity and also that changes in the brain cholesterol content occurring during ontogeny or pathological states may affect NMDAR-mediated brain functions. Out of all the human organs, the brain has the highest content of cholesterol and its concentration rises by more than 250% within the first few years of life. However, during ageing, its levels decline in certain anatomical regions of the brain (Soderberg *et al.* 1990; Dietschy & Turley, 2004). Under pathological conditions, cholesterol accumulation in the brain has been shown for various forms of dyslipidimia and lipidoses (e.g. Niemann–Pick type C disease) (Zervas *et al.* 2001; Distl *et al.* 2003). Therefore, the results of the present study indirectly indicate a possible role of brain cholesterol changes in the process of synapse maturation and in neurological and psychiatric symptoms.

Excessive activation of NMDARs leads to excitotoxic cell death, which can be prevented by NMDAR antagonists (Traynelis *et al.* 2010). In accordance with this, several studies showed unanimously that cholesterol depletion by statins or cyclodextrins has a neuroprotective effect against NMDA-induced excitotoxic death in neuronal cultures (Zacco *et al.* 2003; Abulrob *et al.* 2005; Bosel *et al.* 2005; Ponce *et al.* 2008). Statins reduce plasma and brain cholesterol content (Cibickova, 2011) and some studies have reported their ameliorating effect on brain damage after stroke, as well as in the prevention of Alzheimer's disease and dementia in general (Schreurs, 2010). Our results indicate a possible link between the effect of cholesterol at NMDARs and the neuroprotective effect of cholesterol-lowering drugs.

In the present study, we show a key role of endogenous cholesterol for NMDAR channel function in neurons. Our data indicate a new cholesterol-based mechanism of modulation of NMDAR channel P_o and desensitization, which demonstrates the physiological role of membrane lipids in the regulation of the activity of ionotropic glutamate receptors.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

All experiments were performed at the Institute of Physiology, Czech Academy of Sciences. MK, VV, JB, JK, AB, TS, MH and LV designed the experiments. MK, VV, JB, KL, MK, BK and AB collected, analysed and interpreted the data. MK, VV, JB, KL, MK, BK, JK, AB, TS, MH and LV wrote and revised the manuscript. All authors approved the final version of the manuscript. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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