Cholesterol perturbing agents inhibit NMDA-dependent calcium influx in rat hippocampal primary culture

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Abstract The present study was carried out to investigate the potential involvement of cholesterol-rich membrane microdomains in the mobilization of calcium induced by NMDA-receptors (NMDA-R). We herein provide evidence that agents interfering with plasma membrane cholesterol (namely, filipin and methyl-β-cyclodextrin (Cdex)) inhibit the NMDA-stimulated influx of calcium in hippocampal cells in culture. Filipin-treated cells maintained their morphology and were able to respond with a calcium influx to high K^+ challenge, whereas Cdex altered both cellular parameters. These results suggest that the NMDA-R can be located in cholesterol-rich membrane microdomains or alternatively that the mechanisms coupling their dynamics in the post-synaptic membrane are dependent on the integrity of the microdomains.
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1. Introduction

Lipid microdomains rich in sphingolipids and cholesterol, also known as lipid rafts, have been proposed as biochemically and functionally distinct subregions of plasma membrane [1]. Rafts are enriched in specific proteins including receptors, signal transducer molecules and regulative elements of cell trafficking and architecture as well as cell recognition [2]. Despite the understanding of membrane, microdomain function is one of the major current topics in cell biology, only recently the presence of microdomains in the membrane of neurons has unequivocally been demonstrated [3,4]. Up to now, a growing number of regulative proteins, such as growth factor-receptors, ionic channels, adhesion proteins and elements of SNARE complex, have been proposed as putative raft components [5]. Lipid rafts seem to also contribute to the maintenance of dendritic morphology and at least four chemical-gated channels, $GABA_B$ receptor [6], the α7 nicotinic acetylcholine receptor [7], NMDA and AMPA receptors [8], apparently require cholesterol to maintain their localization in somatic spines.

However, the presence/absence of some molecules, particularly post-synaptic receptors, in the brain rafts is still under debate. The different methodologies used in raft preparation and, even more, the difficulty of obtaining homogeneous populations of neurons or subcellular fractions represent the major causes of the discrepancies that are present in reports by different authors. Thus, Suzuki et al. [3] claimed that NMDA-receptors (NMDA-R) are not localized in the post-synaptic rafts. More recently, an immunofluorescent approach has proved that various glutamate receptors (AMPA and NMDA) and post-synaptic scaffold proteins are associated with rafts in neurons [8].

Glutamate is the most important excitatory aminoacid present in the nervous system. The NMDA-R, that belongs to glutamate receptor family, plays a critical role in excitotoxicity due to its remarkable calcium permeability [9,10]. A crucial role in excitotoxic neuronal death is played by glutamate-mediated calcium overload that, depending on the free intracellular calcium concentration and the severity of the injury, leads to necrosis or apoptosis [9,11]. Glutamate neurotoxicity has been implicated in the neuronal damage found in cerebral ischemia, as well as in the pathogenesis of different neurodegenerative diseases, including amyotrophic lateral sclerosis and Huntington's, Alzheimer's, and Parkinson's diseases [10,12].

Hence, our aim was to investigate the relationship between NMDA-R and lipid rafts in hippocampal cell in cultures. It is worth noting that hippocampal neuronal cultures are an appropriate substrate to study the effects of activation of these receptors, NMDA-R being widely represented in hippocampus, particularly in area CA1. Therefore, we treated hippocampal neurons in culture with filipin and methyl-β-cyclodextrin (Cdex), two agents that, acting on cholesterol with different mechanism, have been utilized to disarray rafts [13]. The localization of NMDA-R into the neuron membrane and the NMDA-evoked calcium influx have been evaluated and compared in control and cholesterol-depleted cells.

2. Materials and methods

2.1. Cell culture and drugs

Primary cultures of rat hippocampal neurons were prepared as described previously [14]. Fetuses were removed on embryonic day 17

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from maternal rats anesthetized with ether and killed by decapitation. After removal of meninges, the hippocampi were collected in Hank's balanced salt solution (HBSS) and mechanically fragmented. Hippocampal cells were washed in HBSS and re-suspended in Neurobasal medium supplemented with 0.5 mM L-glutamine, 2% B-27 supplement, 5 U/ml penicillin and 5 μg/ml streptomycin (referred as complete medium, CM). Aliquots of $2–3 \times 10^4$ cells were placed in 35 mm Petri dishes coated with poly-L-lysine (5 μg/ml) and maintained at 37 °C in humidified air with 5% CO_2. Every 4 days, 0.5 ml of medium was removed and replaced by the same volume of fresh CM.

NMDA was purchased from Tocris Cookson Ltd. (UK); filipin and Cdex were from Sigma (St. Louis, MO). All compounds were stored as stock solutions and applied by directly dropping in the bath.

2.2. Drug application

For immunofluorescence microscopy, cells were treated with 1 mg/ml Cdex or 2 μg/ml filipin for 120 min before cell fixation. For Fura-2AM experiments, four different procedures were carried out. In detail, the incubation times before the application of NMDA were the following: (i) cells were pre-incubated with Cdex or filipin for 60 min in bath solution, Fura 2 was added and the incubation continued for further 60 min (120 min of total drug incubation time); (ii) each drug was applied together with Fura 2 (60 min of total incubation time); (iii) each drug was applied only 10 min before the end of the incubation time with Fura 2; (iv) each drug and NMDA were applied simultaneously to untreated cells. Agents were added by directly dropping into the bath solution. Drugs were present during NMDA stimulation for the whole duration of the experiments. In the experiments with high K^+, cells were treated as described above and stimulated by adding a solution containing a concentration of K^+ sufficient to rise to 8 mM the basal concentration (1 mM).

2.3. Immunofluorescence microscopy

Cells were grown, as described above, on glass coverslips pre-coated with polylysine and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. For localization of NMDA-R and GM1 ganglioside, samples were incubated at 37 °C for 30 min with monoclonal antibodies to Pan-NMDA-R NR1 (Calbiochem) or polyclonal antibodies to GM1 (Calbiochem). Cells were then incubated with Texas Red-conjugated anti-rabbit IgG (Alexa Fluor 594, Molecular Probes) or FITC-conjugated anti-mouse IgG (Alexa Fluor 488, Molecular Probes) for detection of GM1 ganglioside and NMDA-R subunit NR1, respectively. After washing, all samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope. Images were captured by a color chilled 3CCD camera (Hamamatsu, Japan). Normalization and background subtraction were performed for each image. Figures were obtained by the OPTI-LAB (Graftek, France) software for image analysis.

2.4. Fura-2AM experiments

Experiments were started 13–15 days after plating. Optical fluorimetric recordings with Fura-2AM were used to evaluate the intracellular calcium concentration ($[Ca^{2+}]_i$). Fura-2AM stock solutions were obtained by adding 50 μg of Fura-2AM to 50 μl of 75% DMSO plus 25% pluronic acid. Cells were bathed for 60 min at room temperature with 5 μl of stock solution diluted in 1 ml of extracellular solution (in mM: 125 NaCl, 1 KCl, 5 CaCl$_2$, 1 MgCl$_2$, 8 glucose, and 20 HEPES, pH 7.35) for a final Fura concentration of 5 μM. This solution was then removed and replaced with extracellular solution, and the dishes were quickly placed on the microscope stage. To measure fluorescence changes, a Hamamatsu (Shizouka, Japan) Argus 50 computerized analysis system was used, recording every 6 s the ratio between the values of light intensity at 340 and 380 nm stimulation. The basal level of $[Ca^{2+}]_i$ was estimated as approximately 80 nM using the calibration standard kit (Molecular Probes), equivalent to a ratio value of about 0.8. To compare the results obtained with the different substances, we analyzed the respective mean areas under curve (AUC), that express the $[Ca^{2+}]_i$ in terms of ratio during a defined period of time (45 min in this work).

2.5. Cell viability assay

Cell survival was evaluated by the assay of the conversion of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma).

The cultures were incubated for 30 min at 37 °C with 0.5 mg/ml MTT in HBSS. The purple formazan salt was dissolved in dimethyl sulfoxide. The spectral photometric absorbance of the samples was determined at a wavelength of 570 nm. The amount of MTT conversion was displayed as a percentage of the absorbance measured in treated cells relative to the absorbance of control cells. Statistical comparisons were made using the non-parametric Mann–Whitney U-test.

3. Results

3.1. Morphological features of hippocampal neurons

To deplete lipids rafts, we treated hippocampal cultures at 13–14 days with Cdex (1 mg/ml) or filipin (2 mg/ml) for 2 h at 37 °C. Cholesterol-depleted cells showed a morphology very similar to that of control cells. However, the cell bodies of Cdex-treated cells appeared more rounded with a less extended neurite network. A no toxic effect of cholesterol-depleting agents on hippocampal cells was demonstrated by the viability assay carried out with the MTT method. The viability of Cdex- and filipin-treated-cells with respect to the control ones was 101 ± 12 and 97 ± 7, respectively. We used this method to assess the cell viability because there was no evidence of a cell membrane permeabilization by the study of basal level of intracellular calcium. The basal level of free intracellular calcium had a similar value (80 ± 11 nM, see below) in both control and cholesterol-depleted cells. Taken together, these results exclude, in our experimental conditions, a toxic effect of either filipin or Cdex in hippocampal cell cultures. We first analyzed the possible relationship between NMDA and lipid rafts in hippocampal cells after 13–14 days in vitro. We chose as a marker of lipids rafts the ganglioside GM1 that is indicated as one of the lipid raft component in the nervous cells [15]. Hence, we analyzed the double immunostaining of anti-NMDA-R (green) and anti-GM1 ganglioside (red), in the absence or in the presence of filipin and Cdex, which are known to induce cholesterol redistribution or efflux from plasma membrane, respectively [14]. In control cells, immunoreactivity was localized both in cellular bodies and in neurites as bright dot spots; the merge image of NMDA-R (green) and GM1 (red) staining revealed brown–yellow areas resulting from the overlap of green (Fig. 1(a)) and red (Fig. 1(b)) and corresponded to a nearly complete co-localization of the two markers. Notably, immunostaining of NMDA-R demonstrated a more diffuse localization of the receptors in cholesterol-depleted cells (Fig. 1(d) and (g)) with respect to controls (Fig. 1(a)). In cholesterol depleting agents-treated neurons, we observed a less evident GM1 labelling that seems to be confined in cell bodies (Fig. 1(e) and (h)). The fluorescence merge of NMDA-R and ganglioside GM1 in control cells (Fig. 1(c)) revealed a discrete co-localization of the two markers, indicating that subpopulations of NMDA-R were associated with GM1. After manipulation of cholesterol by either filipin or Cdex, a reduced level of the merge of the two antibodies was evident (Fig. 1(f) and (i)).

3.2. Cdex and filipin drastically prevent NMDA-induced intracellular calcium increase

To evaluate whether a link would exist between NMDA-R and cholesterol in hippocampal neurons, we treated cultured hippocampal neurons with Cdex or filipin, before measuring the NMDA-induced $[Ca^{2+}]_i$ increase. This was compared with the increase induced by NMDA alone. Bath application of 50 μM NMDA markedly raised $[Ca^{2+}]_i$, as shown in Fig. 2(a). The mean AUC value was 230.3 ± 38.1 (average of 95 cells from nine different experiments: $N = 95/9$). Cdex (1 mg/ml), applied 2 h
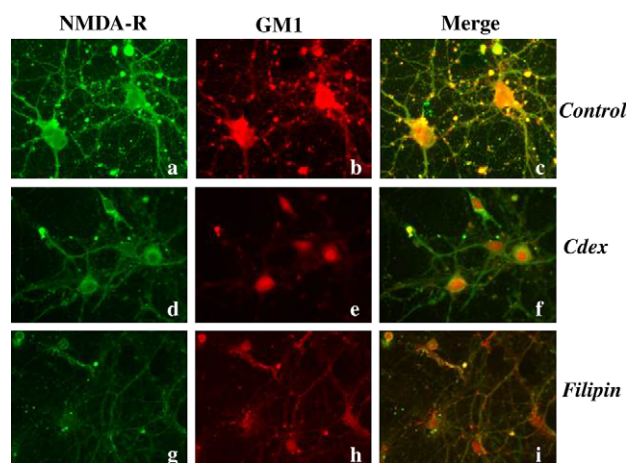
Fig. 1. Morphological effects induced by Cdex and filipin in hippocampal neurons. Immunofluorescence analysis of NMDA-R (green) and ganglioside GM1 (red) distribution in hippocampal neurons. Single and double (merge) staining are shown. (a–c) In control cells, merged image of (a) NMDA-R and (b) GM1 staining reveals their co-localization, as detected by (c) yellow staining, which is due to red and green overlapping. (d–f) Cdex-treated neurons show a typical hippocampal neurons morphology but lack the overlapping of (d) NMDA-R and (e) GM1, as observable in the (f) merged image. (g–i) Filipin-treated cells show a typical hippocampal neurons morphology but the localization pattern of (g) NMDA-R and (h) GM1 differs from control cells as observable in the (i) merged image.
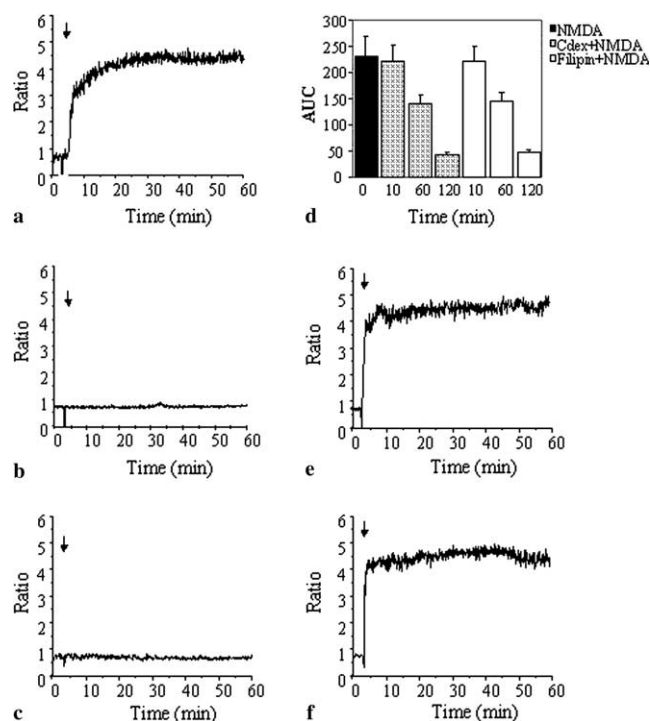

Fig. 2. Effects of Cdex and filipin on NMDA-induced $[Ca^{2+}]_i$ increase as measured by Fura-2AM. (a) Addition of NMDA (50 μM) (arrows) induces a fast and persistent $[Ca^{2+}]_i$ increase in hippocampal neurons. The scale on the left indicates the values of ratio (340/380). (b) Pre-treatment of cells with Cdex (1 mg/ml) for 2 h totally prevents the NMDA-induced $[Ca^{2+}]_i$ increase. (c) Application of filipin (2 μg/ml) for 2 h fully prevents NMDA-induced $[Ca^{2+}]_i$ increase. (d) Cdex and filipin prevent the NMDA-induced $[Ca^{2+}]_i$ increase in a time-dependent manner. The columns represent the $[Ca^{2+}]_i$ increase expressed as AUC. First column (black): application of 50 μM NMDA markedly raised $[Ca^{2+}]_i$. Second column: NMDA-induced $[Ca^{2+}]_i$ increase was not significantly influenced by pre-incubation with Cdex for 10 min, but partially reduced (third column) or fully prevented (fourth column) by 1 h or 2 h of Cdex pre-exposure, respectively. Similarly, NMDA-induced $[Ca^{2+}]_i$ increase was unaffected by 10 min (fifth column), partially reduced after 1 h (sixth column) and fully prevented after 2 h of filipin pre-incubation (seventh column). (e,f) Were co-applied with NMDA to untreated cells, either (e) Cdex (1 mg/ml) or (f) filipin (2 μg/ml) fails in influencing the NMDA-induced $[Ca^{2+}]_i$ increase.

before and then co-applied with high K$^+$, fully prevented the NMDA-induced $[Ca^{2+}]_i$ (Fig. 2(b), AUC = 41.6 ± 5.7, $N = 69/6$). Similarly, filipin (2 μg/ml), applied 2 h before and then co-applied with NMDA, fully prevented the NMDA-induced $[Ca^{2+}]_i$ increase (Fig. 2(c), AUC = 47.7 ± 4.5, $N = 64/6$). Interestingly, when Cdex or filipin was co-applied with NMDA they fully failed to prevent the NMDA-induced $[Ca^{2+}]_i$ increase (Fig. 2(e) and (f), respectively). When administered alone, both Cdex and filipin failed in influencing basal levels of $[Ca^{2+}]_i$ within 2 h of observation (data not shown).

The time-course of NMDA-induced $[Ca^{2+}]_i$ increase was tested in cells pre-exposed to Cdex or filipin for 10 min, 1 h and 2 h, before application of NMDA (Fig. 2(d)). As previously described, application of 50 μM NMDA markedly raised $[Ca^{2+}]_i$ (AUC = 230 ± 38.1; $N = 95/9$, Fig. 2(d) first column), whereas NMDA-induced $[Ca^{2+}]_i$ increase was not significantly influenced by pre-incubation with Cdex for 10 min (AUC = 220.49 ± 31.3; $N = 45/5$, Fig. 2(d), second column), it was partially reduced (AUC = 140.47 ± 16.4; $N = 39/5$) or fully prevented (AUC = 41.6 ± 5.7; $N = 69/6$) by 1 h or 2 h of Cdex pre-exposure, respectively (Fig. 2(d), third and fourth columns). Similarly, NMDA-induced $[Ca^{2+}]_i$ increase was unaffected by 5–10 min (AUC = 220.71 ± 28.7; $N = 41/6$, Fig. 2(d), fifth column), partially reduced after 1 h (AUC = 143.84 ± 17.3; $N = 42/6$) and fully prevented after 2 h (AUC = 47.7 ± 4.5; $N = 64/6$) of filipin pre-incubation (Fig. 2(d), sixth and seventh columns).

3.3. Cdex but not filipin prevents the high K$^+$-induced intracellular calcium increase

In order to verify whether a specificity between cholesterol depletion and NMDA-R activity would exist, we triggered the calcium entry by high potassium-dependent membrane depolarization. Thus, effects of Cdex or filipin were tested in high K$^+$-induced $[Ca^{2+}]_i$, a no specific model based on a diffused neuronal depolarization. In hippocampal neurons, bath application of K$^+$ (8 mM) markedly raised $[Ca^{2+}]_i$ (Fig. 3(a)). As shown in the figure, the mean AUC value was 59.9 ± 7.2 ($N = 63/6$). Cdex (1 mg/ml), applied 2 h before and then co-applied with high K$^+$, fully prevented the high K$^+$-induced $[Ca^{2+}]_i$ (Fig. 3(b), AUC = 40.85 ± 4.9, $N = 59/6$). On the contrary filipin 2 μg/ml, applied 2 h before and then co-applied with high K$^+$, did not influence the effects of high K$^+$ on $[Ca^{2+}]_i$ (Fig. 3(c), AUC = 63.8 ± 5.7, $N = 55/6$).

4. Discussion

Our paper provides evidence supporting the hypothesis that depletion of plasma membrane cholesterol plays a critical role in NMDA-mediated Ca^{2+} influx. Cholesterol-binding agents, such as Cdex and filipin, that efficiently remove or sequester cholesterol from the plasma membrane, respectively, inhibit in a time-dependent way the NMDA-R activity. Neither of the two cholesterol-binding agents inhibits the NMDA activity
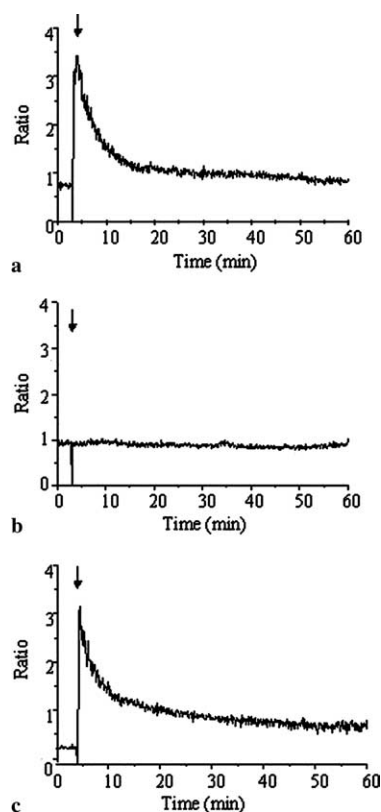
Fig. 3. Effects of Cdex and filipin on high KCl-induced $[Ca^{2+}]_i$ increase. (a) Increase of KCl concentration up to 8 mM (arrow) in the bath solution induces a fast transient calcium response in untreated hippocampal neurons. (b) Two hours of Cdex (1 mg/ml) pre-treatment blocks the KCl-induced $[Ca^{2+}]_i$ increase (arrow indicates the 8 mM KCl application). (c) Two hours of filipin (2 µg/ml) pre-exposure does not prevent KCl-induced (arrow) $[Ca^{2+}]_i$ increase.

when administered together with the glutamate agonist, demonstrating their lack of competition with the receptor or with the ligand. Moreover, the decrease of NMDA-induced calcium influx is paralleled by the loss of the co-localization of one marker of lipid rafts (GM1) and NMDA-R in both Cdex and filipin-treated cells. On the basis of this observation, we propose that NMDA-R is either located in lipid rafts, or its stability is maintained by molecules related to lipid rafts.

The property of Cdex and filipin to interfere with cholesterol in the plasma membranes made these two molecules particularly useful agents to study the function of rafts [13,16]. We herein report immunofluorescence evidence that both cholesterol-perturbing agents strongly affect the topology of GM1-enriched lipid microdomains in hippocampal cells in culture. Neurons treated with both drugs appeared viable also after 2 h of incubation. At variance, Sonnino' group published a paper indicating that long-term incubation of nervous cells in culture with Cdex, severely affects the membrane integrity leading to dramatic change in cell viability [17]. However, we observed that Cdex seems to induce a more drastic effect on cell membrane with respect to filipin concerning both morphological and functional features. Cells treated with Cdex still showed a Ca^{2+} basal level identical to that of control cells but partially lose the characteristic pyramidal shape of the control cells showing a more rounded cellular body, and failed in responding to the high K$^+$ stimulation. Conversely, when in-

cubated with the filipin concentration able to abolish NMDA-evoked response, the cells showed only slight change in their pyramidal shape and maintained their capability to respond to high K$^+$ stimulation. Calcium influx evoked by high K$^+$ challenge indicates that both the ionic membrane barrier and the machinery of the cell devoted to the mechanism of depolarization are not affected by filipin. Thus, filipin, but not Cdex, seems to induce only a mild perturbation at the cell membrane, likely limiting its effect to the cholesterol-rich microdomains. The reason for this difference probably relays on the different mode of action of the two cholesterol-perturbing agents. Cdex binds cholesterol and extracts the lipid from the bilayer [18], whereas filipin binds cholesterol in the membrane and prevents its correct localization in the rafts [19]. The pharmacological differential effects of filipin and Cdex on receptor signalling have already been reported in B cells and vascular endothelial cells [13,20]. Recently, Giocondi et al. [21] reported that Cdex is able to extract sphingomyelin from supported bilayers. Hence, we must be aware that the functional and biochemical modifications associated with a Cdex treatment of biological membranes are not solely due to cholesterol concentration changes.

We observed that GM1 and NMDA-R showed a good correlation in their localization in control neurons, indicating that a population of glutamate receptor is associated with the ganglioside. However, when cholesterol was perturbed we observed a more diffused localization of both antibodies and their co-localization appeared only in the cell body (Fig. 1). These data are in agreement with a recent work by Hering et al. [8], where the role of lipid rafts in receptor stability maintenance in dendritic spines was accurately investigated. The authors reported that the cholesterol depletion induces, in cultured neurons, a reduced density of dendritic spines and an enlargement of the remaining ones. In particular, NMDA-R subunit NR1 was concentrated in many small dendritic puncta in control neurons but in cholesterol-depleted ones NR1 clusters were fewer in number and larger in size [8]. A hypothesis could be that Cdex/filipin-dependent rafts destabilization leads to a disarrangement of the integrity of the platform where the cluster of NMDA-R is embedded. We cannot exclude, however, that the cholesterol-depletion can interfere with some scaffold proteins involved in the maintenance of NMDA-R function. Notably, glutamate-receptor interacting protein and PSD-95, two proteins that play a major role in the clustering of NMDA-R, both are located in post-synaptic rafts [22,23]. PSD-95 is a clustering molecule, which anchors NMDA-R that is an important determinant of the efficacy of neuronal transmission. Vinaie et al. [24] demonstrated that the cleavage of PSD-95 alters the integrity and localization of NMDA-R in isolated post-synaptic density. Finally, a further possibility is that a lipid microenvironment enriched in cholesterol can represent a particular condition specifically required for the channel properties of NMDA-R. It is well known that ion channels, and more generally, any membrane protein, may be regulated via differential packing of lipids and by the biophysical properties of their membrane microenvironment [25]. For example, removal of the potassium channel Kv2.1 (but not Kv4.2) from lipid rafts by cholesterol depletion significantly shifted the steady-state inactivation of Kv2.1, without altering the activation kinetics or voltage sensitivity [26]. Finally, although a common opinion is that acute cholesterol manipulation with agents such as

filipin and Cdex interests only the pool of cholesterol present in the plasma membrane, it is impossible to exclude that other pools are also interested. In particular, it is possible that the NMDA-R inhibition by Cdex and filipin was derived by an imbalance of the exo–endocytic vesicular transport circuits and consequently of the glutamate receptor recycling.

In conclusion, cholesterol modulates NMDA-R activity in a manner that has dramatic effects on neuronal excitability. Hence, having in mind that NMDA-R must be considered fundamental therapeutic targets, our observations can represent a useful starting point for the development of neuroprotective strategies that involve the handling of lipid rafts.

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