

The $\alpha 7$ nicotinic acetylcholine receptor function in hippocampal neurons is regulated by the lipid composition of the plasma membrane

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Non-technical summary The $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) are a therapeutic target for the treatment of neurological disorders such as Alzheimer's disease and schizophrenia, and drugs that potentiate $\alpha 7$ nAChRs through the regulation of desensitization are currently being developed. Here we show that changes to the lipid composition of the plasma membrane in rat hippocampal neurons, through either acute treatment with drugs that remove cholesterol and breakdown sphingomyelin or chronic treatment with synthesis inhibitors for cholesterol and sphingomyelin, result in significant changes in the desensitization of $\alpha 7$ nAChRs. These data provide evidence that the lipid composition of the plasma membrane is able to modulate the desensitization of $\alpha 7$ nAChRs, which will be important for the development of new therapeutic reagents.

Abstract The $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) play an important role in cellular events such as neurotransmitter release, second messenger cascades, cell survival and apoptosis. In addition, they are a therapeutic target for the treatment of neurological disorders such as Alzheimer's disease and schizophrenia, and drugs that potentiate $\alpha 7$ nAChRs through the regulation of desensitization are currently being developed. Recently, these channels were found to be localized into lipid rafts. Here we show that the disruption of lipid rafts in rat primary hippocampal neurons, through cholesterol-scavenging drugs (methyl- β -cyclodextrin) and the enzymatic breakdown of sphingomyelin (sphingomyelinase), results in significant changes in the desensitization kinetics of native and expressed $\alpha 7$ nAChRs. These effects can be prevented by cotreatment with cholesterol and sphingomyelin, and can be mimicked by treatment with cholesterol and sphingomyelin synthesis inhibitors (mevastatin and myriocin, respectively), suggesting that the effects on desensitization kinetics are indeed due to changes in the levels of cholesterol and sphingomyelin in the plasma membrane. These data provide new insights into the mechanism of desensitization of $\alpha 7$ nAChRs by providing evidence that the lipid composition of the plasma membrane can modulate the activity of the $\alpha 7$ nAChRs.

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Abbreviations $A\beta$, β -amyloid; GFP, green fluorescent protein; KCC2, K^+ - Cl^- transporter; M β CD, methyl- β -cyclodextrin; nAChR, nicotinic acetylcholine receptor; NKCC1, Na^+ - K^+ - $2Cl^-$ transporter; PAM, positive allosteric modulator; Ric, resistance to inhibitors of cholinesterase; SMase, sphingomyelinase

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are Cys-loop ligand-gated ion channels that are widely distributed throughout the nervous system (Jones *et al.* 1999; Gay *et al.* 2008), and are pentamers formed by combinations of $\alpha 2$ – $\alpha 9$ and $\beta 2$ – $\beta 4$ subunits. Of these, only the $\alpha 7$ – $\alpha 9$ subunits can form homomeric pentamers (Chen & Patrick, 1997). One important characteristic of the $\alpha 7$ nAChRs is their high permeability to Ca^{2+} , allowing this nAChR subunit to play an important role in cellular events such as neurotransmitter release (Sharma & Vijayaraghavan, 2003), second messenger cascades (Sharma & Vijayaraghavan, 2001), cell survival (Shimohama & Kihara, 2001) and apoptosis (Berger *et al.* 1998).

Studies investigating the localization of $\alpha 7$ nAChRs suggest that this subunit localizes into lipid rafts (Bruses *et al.* 2001; Oshikawa *et al.* 2003; Fernandes *et al.* 2010), which are areas of the cell membrane enriched in cholesterol and sphingolipids. They have been proposed to form microdomains that serve a role as organizational structures in signal transduction, a concept fuelled by observations made following the disruption of lipid rafts with cholesterol-scavenging molecules such as cyclodextrins (Simons *et al.* 1998; Bruses *et al.* 2001). Pharmacological disruption of lipid rafts through cholesterol-sequestering drugs results in a change in localization and an increase in mobility of $\alpha 7$ nAChRs in mammalian cell cultures (Oshikawa *et al.* 2003) and in chick ciliary neurons (Bruses *et al.* 2001; Fernandes *et al.* 2010). Some indirect effects of lipid raft disruption on $\alpha 7$ nAChR function have been reported, including the loss of inhibition of the calcium inhibitable isoform adenylate cyclase 6 on PC12 cells (Oshikawa *et al.* 2003). However, the role that $\alpha 7$ nAChR localization into lipid rafts exerts on channel function remains unknown.

In this study, we investigated the direct effects that lipid rafts and their components have on $\alpha 7$ nAChR function. We found that removal of the main components of lipid rafts (cholesterol and sphingomyelin) by acute treatment with cholesterol-sequestering agents, alone or in combination with the enzymatic breakdown of sphingomyelin, has profound effects by slowing the kinetics of desensitization (in part by increasing the rate of recovery from desensitization) and by increasing agonist affinity. Adding back cholesterol and sphingomyelin reversed the effects on desensitization. These effects are similar to those seen by the inhibition of *de novo* synthesis of both cholesterol and sphingomyelin, suggesting that the disruption of lipid rafts is responsible for the changes in $\alpha 7$ nAChR desensitization. The mechanism of desensitization of $\alpha 7$ nAChRs is not completely understood; however, therapeutic drugs that potentiate $\alpha 7$ responses through the removal of desensitization [type II $\alpha 7$ positive allosteric

modulators (PAMs), such as PNU-120596; Bertrand & Gopalakrishnan, 2007] are currently being developed to treat Alzheimer's disease and other neurological disorders (Young *et al.* 2008), highlighting the importance of understanding better the function of the $\alpha 7$ nAChRs.

Methods

Hippocampal neuron primary cultures

Primary cultures were prepared at embryonic day 18 using a modified version from Quitsch (2005). All procedures were approved and performed in compliance with NIEHS/NIH Humane Care and Use of Animals in Research protocols. Time pregnant Sprague Dawley rats were anesthetized with isoflurane and decapitated by guillotine. Briefly, hippocampi were mechanically dissociated after treatment for 30 min at 37°C with 0.05% trypsin EDTA (Gibco, Grand Island, NY, USA). Cells were plated in neurobasal medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% Glutamax (Gibco) at 3500 mm^{-2} on coverslips coated with poly-D-lysine (Sigma, St Louis, MO, USA) and grown in a humidified atmosphere of air containing 5% CO_2 at 37°C. Half the medium was replaced 24 h after plating and every 72 h thereafter with neurobasal media in which fetal bovine serum was substituted with 2% B27 (Gibco). Hippocampal neurons were transfected in Day in vitro 4 (DIV4) with $\alpha 7$ nAChR, Ric-3 (resistance to inhibitors of cholinesterase) and green fluorescent protein (GFP), or wild-type rat $\alpha 3$ and $\beta 2$ nAChR subunits and GFP, using a calcium phosphate procedure described by Pottorf *et al.* (2006). Cells were analysed 24–48 h after transfection Day in vitro 5–6 (DIV5–6), to minimize the possible contribution of endogenous channels which express at very low levels during this period (Arnaiz-Cot *et al.* 2008).

Manipulation of membrane lipids

For the acute manipulation of cholesterol and sphingomyelin, hippocampal neurons were treated for 15 min at 37°C with one of the following: PBS (control); 5 mM methyl- β -cyclodextrin (M β CD; Sigma), prepared in PBS and sterile filtered; 5 mM M β CD in combination with 0.5 U ml^{-1} sphingomyelinase (SMase; Sigma); 5 mM M β CD in combination with 2 mM water-soluble cholesterol (Sigma); or 5 mM M β CD in combination with 0.5 U ml^{-1} SMase, 2 mM cholesterol and 300 μM sphingomyelin (Sigma). Chronic manipulation of cholesterol and sphingomyelin were done using mevastatin (Sigma) and myriocin (Sigma), which inhibit the synthesis of cholesterol and sphingomyelin, respectively. Hippocampal neurons were incubated for 24–48 h at 37°C with 400 μM myriocin and 2 mM

mevastatin in serum-free media. After treatment, cells were immediately processed for cholesterol content or ACh-evoked current measurement.

Measurement of ACh-evoked currents

Coverslips containing transfected neurons were transferred to a chamber containing: 165 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 10 mM glucose, 5 mM Hepes, 1 μM atropine and 0.3 μM TTX; pH was adjusted to 7.3 with NaOH. Bath solution was perfused continuously through the chamber (1 ml volume) at 2 ml min^{-1} throughout the experiments. Neurons were visualized using a Nikon Eclipse TE300 microscope equipped for fluorescence detection. Borosilicate patch pipettes (3–6 M Ω) were filled with 120 mM CsCl, 2 mM MgCl_2 , 10 mM EGTA, 10 mM Hepes and ATP-regenerating compounds (5 mM ATP and 20 mM phosphocreatine); pH was adjusted to 7.3 with CsOH. Currents were recorded at -70 mV. Experiments were performed at room temperature (22°C). Whole-cell recordings were done using an Axopatch-200A amplifier connected to a Digidata 1322A and software (pCLAMP v. 10.1) from Axon Instruments. Currents were filtered at 1 kHz and digitized at 10 kHz with an output gain of one. Acetylcholine was applied using a synthetic quartz perfusion tube (0.7 mm i.d.) operated by a computer-controlled valve (AutoMate Scientific, Berkeley, CA, USA). The rate of solution exposure in these conditions was estimated to occur as fast as 1.5 ms. One second applications of 3 mM ACh were analysed using Clampfit 10. Data are plotted as means \pm SEM and was analysed using Prism 5 (GraphPad, La Jolla, CA, USA).

Dose–response measurements were done by applying different concentrations of ACh for 1 s followed by a 60 s wash. The concentrations used were 30, 100 and 300 μM , 1 and 3 mM ACh. To construct the dose–response curve, the responses were first normalized to the response of 1 mM ACh (responses with 3 mM were indistinguishable from 1 mM) and fitted using a log (agonist) *versus* normalized response with a variable slope equation on GraphPad Prism 5.

Recovery from desensitization was studied by applying 3 mM ACh for 10 s to induce near-complete (>90%) desensitization, and washing out ACh for various amounts of time (1.5, 2.5, 3.5, 5 and 10 s in control conditions; 1.5, 2, 2.5, 3 and 6 s for M β CD/SMase) followed by a 2 s application of ACh. The data are represented as a percentage of the initial response and were fitted using a one-phase decay exponential equation in GraphPad Prism 5.

Single-channel currents from outside-out patches were measured using the system described above, with the difference that currents were filtered at 2 kHz and digitized at 10 kHz, with an output gain of 20. Four second applications of 50 μM ACh were analysed using

Clampfit 10. Estimation of the single-channel amplitudes was done by using all-points histograms that were fitted with Gaussian distributions. Data are plotted as means \pm SEM and were analysed using GraphPad Prism 5.

Quantification of cholesterol levels

Hippocampal neurons were grown in poly-D-lysine-coated six-well plates at a density of 4×10^6 cells per well. Cells were treated with the corresponding drugs and washed with PBS and scraped off the wells on Day in vitro 7 (DIV7) using 2% Triton X-100 (Sigma) in PBS. Cells were homogenized with a glass homogenizer and stored at -20°C until needed. Cholesterol levels were measured using the Amplex Red Cholesterol Assay Kit as described by the manufacturer (Molecular Probes, Eugene, OR, USA). Cells were analysed for protein concentration in parallel using the Bicinchoninic acid assay (BCA) protein assay (Pierce, Rockford, IL, USA) and then cholesterol concentration was normalized to protein concentration first, and then to cholesterol concentration in the control samples. Cholesterol data are plotted as means \pm SEM and were analysed using GraphPad Prism 5.

Results

Disruption of lipid rafts affects the desensitization kinetics and acetylcholine sensitivity of neuronal $\alpha 7$ nAChRs

Cultured hippocampal neurons coexpressing wild-type rat $\alpha 7$ nAChR, Ric-3 and GFP, were exposed to a 1 s rapid application of ACh (3 mM) at a holding potential of -70 mV; this resulted in the rapid activation of an inward current that desensitized in the continued presence of agonist with a desensitization half-time of 27 ± 2 ms (Fig. 1A and D; Gay *et al.* 2008). These responses were completely blocked by the $\alpha 7$ nAChR-selective antagonist Methyllycaconitine (MLA) (10 nM, $n = 5$), indicating that they were due to the activation of the $\alpha 7$ nAChR.

The onset of desensitization, as previously described (Khiroug *et al.* 2002), was biphasic; the fast component ($\tau_{\text{fast}} = 18 \pm 1$ ms) comprised $81 \pm 2\%$, while the slow component ($\tau_{\text{slow}} = 382 \pm 41$ ms) comprised $12 \pm 1\%$ of the total desensitization (Fig. 1E; $n = 30$ cells).

As cholesterol can be either absorbed by receptor-mediated endocytosis or synthesized in the endoplasmic reticulum, we designed two approaches to disrupt lipid rafts. The first approach consisted of the acute removal of cholesterol from these microdomains through a cholesterol-sequestering agent and the enzymatic breakdown of sphingomyelin. Methyl- β -cyclodextrin has been shown to remove cholesterol from the plasma membrane of cultured cells (Klein *et al.* 1995). Moreover,

treatment of cells with the enzyme SMase, which hydrolyses sphingomyelin into phosphocholine and ceramide, has been shown to affect lipid rafts directly by decreasing sphingomyelin (Rogasevskaia & Coorssen, 2006; Szoke *et al.* 2010) and indirectly through the production of

ceramide, which has been shown to displace cholesterol from lipid raft membranes (Yu *et al.* 2005). To investigate the effects of lipid raft disruption on the function of $\alpha 7$ nAChR-mediated responses, cells were treated (for 15 min) with either 5 mM M β CD, or 5 mM M β CD in combination with 0.5 U ml⁻¹ SMase. Longer treatments or higher concentrations of either M β CD or SMase resulted in decreased cell viability.

Application of M β CD alone resulted in a significant decrease in current amplitude (514 ± 77 versus 1892 ± 297 pA in control conditions; $n = 13$ versus $n = 30$, respectively), accompanied by a change in desensitization kinetics in 13 of 18 cells tested (Fig. 1B). Although desensitization half-time (26 ± 2 versus 27 ± 2 ms in control conditions; Fig. 1D) and desensitization time constants ($\tau_{\text{fast}} = 23 \pm 2$ versus 18 ± 1 ms in control conditions; $\tau_{\text{slow}} = 265 \pm 61$ versus 382 ± 41 ms in control conditions) were not significantly affected by M β CD, the ratio of the fast (49 ± 3 versus $81 \pm 2\%$ in control conditions) and slow components (27 ± 3 versus $12 \pm 1\%$ in control conditions) changed significantly (Fig. 1E).

Co-application of SMase with M β CD resulted in a larger effect on desensitization kinetics than M β CD alone (Fig. 1C; 14 of 18 cells were affected); the desensitization half-time was significantly increased to 143 ± 41 ms (versus 27 ± 2 ms in control conditions; Fig. 1D). Furthermore, although the slow time constant was not significantly different (418 ± 52 versus 382 ± 41 ms in control conditions), the fast time constant was significantly slower (65 ± 18 versus 18 ± 1 ms in control conditions). Similar to the treatment with M β CD alone, the contribution of the fast and slow components to desensitization was significantly affected with both M β CD and SMase; the fast component was $43 \pm 5\%$ (versus $81 \pm 2\%$ in control conditions) and the slow component was $46 \pm 6\%$ (versus $12 \pm 1\%$ in control conditions; Fig. 1E). Interestingly, current amplitude was not significantly different (-1072 ± 175 versus -1892 ± 297 in control conditions; $n = 14$ versus $n = 30$, respectively). Furthermore, these changes in desensitization kinetics caused by the co-application of SMase and M β CD were present when cells were dialysed with the calcium chelator BAPTA (by adding it to the recording pipette), and by replacing external Ca²⁺ with Ba²⁺ (data not shown), suggesting that Ca²⁺ entry through the channel is not responsible for these effects.

To test whether lipid raft disruption alters the agonist sensitivity of the $\alpha 7$ nAChR, we treated the neurons with M β CD and SMase and constructed ACh dose–response curves by applying different concentrations of ACh. This disruption of lipid rafts resulted in a significant increase in potency ($EC_{50} = 70 \mu\text{M}$, $\log EC_{50} = 1.85 \pm 0.03$, Hill slope = 1.02 ± 0.10 ; $n = 7$) when compared with control conditions ($EC_{50} = 132 \mu\text{M}$, $\log EC_{50} = 2.12 \pm 0.03$, Hill slope = 1.39 ± 0.13 ; $n = 9$; Fig. 1F; $P < 0.0001$ for log

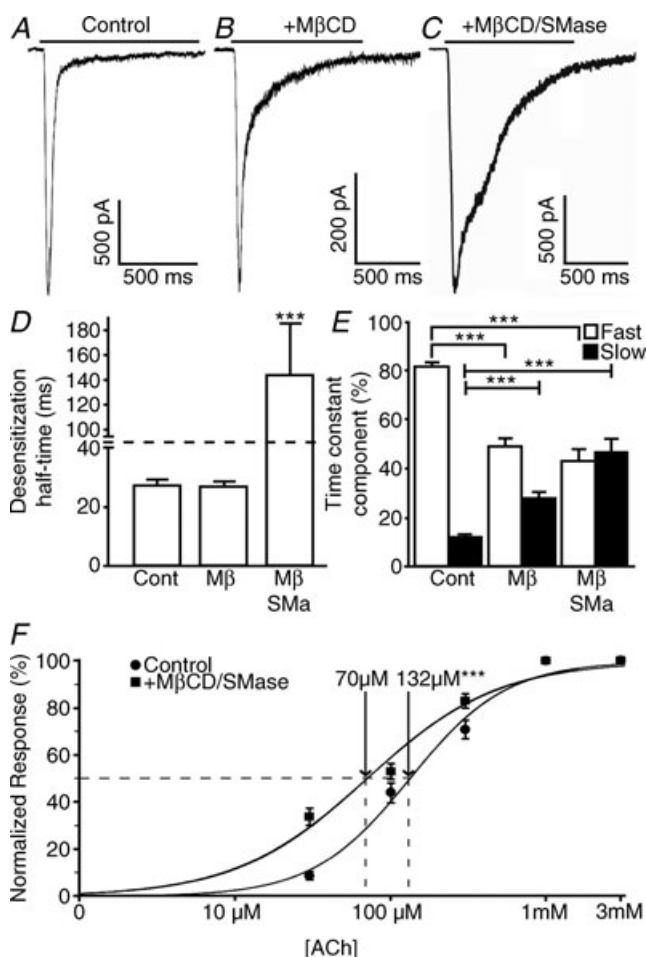


Figure 1. Disruption of lipid rafts affects the desensitization kinetics and acetylcholine sensitivity of neuronal $\alpha 7$ nAChRs

Cultured hippocampal neurons coexpressing wild-type rat $\alpha 7$ nAChR, Ric-3 and GFP were incubated for 15 min with PBS (control), 5 mM M β CD or 5 mM M β CD in combination with 0.5 U ml⁻¹ SMase and subjected to a 1 s application of 3 mM ACh. A–C, response to ACh application in control conditions (A), 5 mM M β CD (B) and 5 mM M β CD and 0.5 U ml⁻¹ SMase (C). D, M β CD treatment did not change the desensitization half-time; however, cotreatment of M β CD with SMase resulted in a fivefold increase in the desensitization half-time. E, desensitization time constants (τ) were calculated using Clampfit and fitted with a two-exponential equation. Although no difference was seen in τ values, the ratio of fast and slow desensitization was affected by both M β CD and M β CD/SMase cotreatment. F, neurons were subjected to consecutive 1 s applications of 30, 100 and 300 μM , 1 and 3 mM ACh. Responses were normalized to the response to 1 mM ACh. Dose–response curves were constructed and compared using GraphPad Prism 5. In D and E, plots show means \pm SEM and were subjected to a one-way ANOVA; *** $P < 0.0001$. In F, log EC_{50} was compared using a log (agonist) versus normalized response with variable slope fit in GraphPad Prism 5; *** $P < 0.0001$.

EC_{50} and Hill slope comparison between data sets). This suggests that changes in the lipid composition of the plasma membrane affect both the kinetics of desensitization as well as agonist potency of the $\alpha 7$ nAChR.

Effects of M β CD and SMase on $\alpha 7$ nAChR function are due to changes in the lipid composition of the plasma membrane

One possible drawback of using M β CD and sphingomyelinase to disrupt lipid rafts is that both drugs affect other cellular components. Besides sequestering cholesterol, M β CD can act as a non-specific chelator binding other phospholipids (Zidovetzki & Levitan, 2007), whereas SMase hydrolyses the membrane raft component sphingomyelin into ceramide, which can act as a second messenger altering cellular functions (Yu *et al.* 2005).

To determine whether the effects of M β CD and SMase on $\alpha 7$ nAChR desensitization kinetics were specifically caused by changes in the lipid composition of the plasma membrane, cells were treated with M β CD and SMase in the presence of water-soluble cholesterol and sphingomyelin.

Co-application of 2 mM cholesterol and 5 mM M β CD for 15 min resulted in the reversal of the effects of M β CD alone on both current amplitude (-1239 ± 162 versus -1892 ± 297 in control conditions; $n = 9$ versus $n = 30$, respectively) and desensitization kinetics (Fig. 2A and C); in the presence of cholesterol, the fast component was $75 \pm 2\%$ (versus $49 \pm 3\%$ in M β CD and $81 \pm 2\%$ in control conditions) and the slow component was $13 \pm 1\%$ (versus $27 \pm 3\%$ in M β CD and $12 \pm 1\%$ in control conditions; Fig. 2D).

Likewise, co-application of 2 mM cholesterol, 5 mM M β CD, 0.5 U ml^{-1} SMase and $300 \mu\text{M}$ sphingomyelin for 15 min resulted in the reversal of the effects of the co-application of M β CD and SMase on desensitization kinetics ($n = 9$; Fig. 2B–D). Desensitization half-time was significantly reduced from 143 ± 41 to 31 ± 3 ms (27 ± 2 ms in control conditions; Fig. 2C). The contributions of the fast and slow time constants to desensitization were also reversed to near control values, from 43 ± 5 to $68 \pm 4\%$ (for fast; not significantly different from control $81 \pm 2\%$), and from 46 ± 6 to $21 \pm 3\%$ (for slow; not significantly different from control $12 \pm 1\%$) respectively (Fig. 2D).

Effects of lipid raft disruption on neuronal $\alpha 7$ nAChR single-channel behaviour

Lipid raft disruption through cholesterol depletion using M β CD has been shown to affect single-channel behaviour

of a variety of channels (Balse *et al.* 2009; Tajima *et al.* 2010), while it has no effect on others (Sudarikova *et al.* 2009). Furthermore, mutagenesis studies have shown that lipid-exposed amino acids are critical for the gating mechanism of *Torpedo californica* nAChRs (Lee *et al.* 1994; Lasalde *et al.* 1996; Bouzat *et al.* 1998; Tamamizu *et al.* 1999, 2000).

To study the effect of lipid raft disruption on the single-channel behaviour of the $\alpha 7$ nAChR, outside-out patches from neurons coexpressing wild-type rat $\alpha 7$

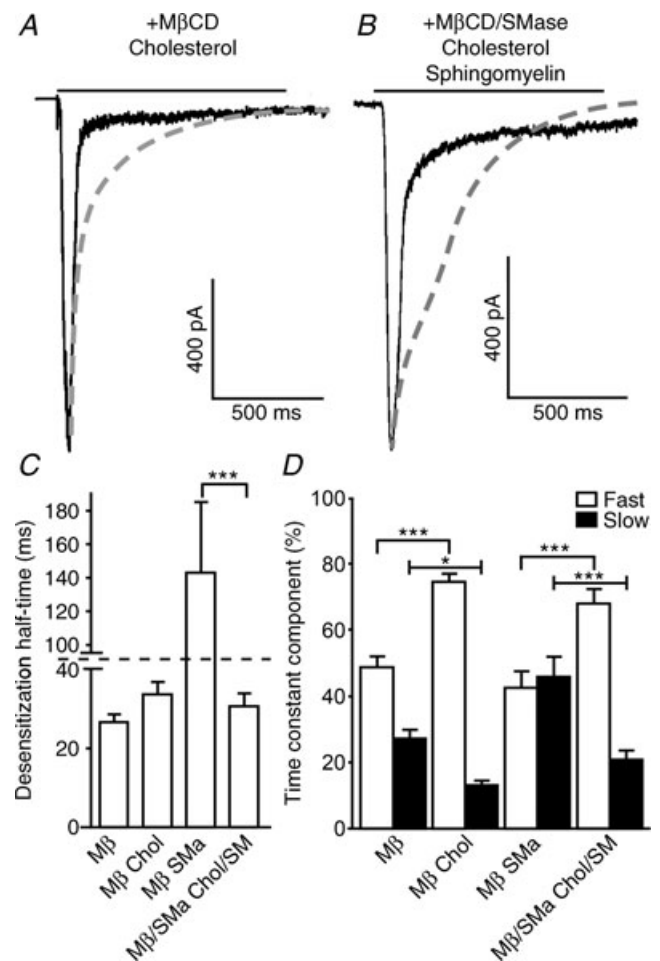


Figure 2. Cholesterol and sphingomyelin prevent the effects of lipid raft disruption through M β CD and SMase on desensitization kinetics of neuronal $\alpha 7$ nAChRs

Hippocampal neurons coexpressing wild-type rat $\alpha 7$ nAChR, Ric-3 and GFP were incubated for 15 min with M β CD either alone or in the presence of 2 mM cholesterol; likewise, cells were subjected to M β CD/SMase alone or in the presence of both 2 mM cholesterol and $300 \mu\text{M}$ sphingomyelin; and subjected to a 1 s application of 3 mM ACh. A–B, cholesterol and sphingomyelin are able to reverse most of the effects of M β CD and SMase (dashed lines) on $\alpha 7$ nAChR desensitization kinetics, including the increase of desensitization half-time, caused by the cotreatment with M β CD/SMase (C), and the changes that both M β CD alone and M β CD/SMase have on the ratio of fast and slow desensitization (D). Plots show means \pm SEM and were subjected to a one-way ANOVA; * $P < 0.05$; *** $P < 0.0001$.

nAChR, Ric-3 and GFP (McCormack *et al.* 2010) were treated with either PBS or M β CD and SMase, and exposed to a 4 s rapid application of ACh (50 μ M) at a holding potential of -70 mV.

For the $\alpha 7$ nAChR, agonist-evoked single-channel activity appears mainly as isolated brief openings with a mean open time of 0.33 ms, the frequency of which decreases with prolonged agonist application, as is expected due to desensitization (McCormack *et al.* 2010). The M β CD/SMase cotreated patches were characterized by significantly larger mean single-channel amplitude (-5.14 ± 0.04 versus -4.49 ± 0.05 pA in control conditions; $n = 2060$ and 6105 single-channel openings in control conditions and M β CD/SMase,

respectively; $P < 0.0001$; Fig. 3A–C) and increased channel activity (Fig. 3A and B). The M β CD/SMase cotreatment had no effect on the channel mean open times (0.34 ± 0.003 versus 0.33 ± 0.006 ms in control conditions; Fig. 3D); however, this cotreatment resulted in a significant decrease in the mean closed times ($2.7 \pm .089$ versus $7.8 \pm .351$ ms in control conditions; Fig. 3D). This indicates that the increase in single-channel activity is due to a large decrease in the mean closed time following M β CD/SMase cotreatment, which is consistent with our observations at the whole-cell level that lipid raft disruption reduces the rate of desensitization of neuronal $\alpha 7$ nAChRs (McCormack *et al.* 2010).

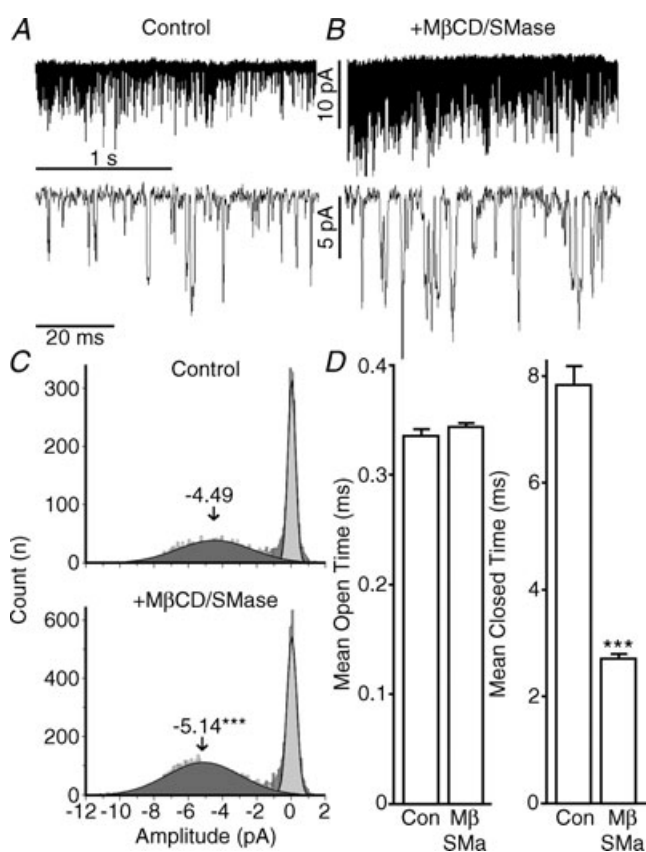


Figure 3. Effects of lipid raft disruption on neuronal $\alpha 7$ nAChR single-channel behaviour

Hippocampal neurons coexpressing wild-type rat $\alpha 7$ nAChR, Ric-3 and GFP were incubated for 15 min with either PBS (control) or a combination of M β CD/SMase and subjected to a 4 s application of 50 μ M ACh. A and B, single-channel traces using the outside-out configuration; M β CD/SMase treatment resulted in larger amplitudes and increased activity. C, all-points histogram of amplitudes of the single-channel current from six control and four M β CD/SMase-treated patches. The mean amplitudes were calculated using a single-exponential Gaussian fit and compared using GraphPad Prism 5. D, mean open and closed times were compared in control and M β CD/SMase-treated patches. Plots show means \pm SEM and were subjected to Student's unpaired *t* test; $***P < 0.0001$.

Effects of lipid raft disruption on the ACh response of native neuronal $\alpha 7$ nAChRs

We examined the effects of lipid raft disruption on native $\alpha 7$ nAChRs in hippocampal neurons that were cultured for a much longer period of time (i.e. 13–14 days), which is a period of high $\alpha 7$ nAChR expression in the hippocampus (Arnaiz-Cot *et al.* 2008). As before, neurons were exposed to a 1 s rapid application of ACh (3 mM) at a holding potential of -70 mV. This resulted in the activation of an inward current response with properties indistinguishable from those of expressed $\alpha 7$ nAChRs (Fig. 1), including a mean amplitude of -1066 ± 127 pA, a desensitization half-time of 37 ± 4 ms, and a biphasic onset of desensitization with a fast ($\tau_{\text{fast}} = 27 \pm 2$ ms; $74 \pm 2\%$) and a slow component ($\tau_{\text{slow}} = 302 \pm 52$ ms; $8 \pm 1\%$; Fig. 4; $n = 14$ cells). In addition, native $\alpha 7$ nAChRs cotreated with M β CD and SMase responded in a similar fashion to the expressed receptors; cotreatment resulted in a significant increase in desensitization half-time to 59 ± 4 ms ($n = 11$ cells; versus 37 ± 4 ms in control conditions; Fig. 4C). While the time constants of desensitization were not significantly different ($\tau_{\text{fast}} = 39 \pm 4$ versus 27 ± 2 ms in control conditions; $\tau_{\text{slow}} = 418 \pm 61$ ms versus 302 ± 52 ms in control conditions), the contribution of the fast and slow components was significantly affected by M β CD/SMase cotreatment; the fast component was $57 \pm 2\%$ (versus $74 \pm 2\%$ in control conditions) and the slow component was $25 \pm 2\%$ (versus $8 \pm 1\%$ in control conditions; Fig. 4D). Finally, current amplitude was not significantly different (-1066 ± 127 versus 1175 ± 182 pA in control conditions; $n = 11$ versus $n = 14$, respectively).

To further dissect the effects of M β CD/SMase cotreatment on $\alpha 7$ nAChR desensitization, we examined the rate of recovery from desensitization. This was done by continuously applying ACh (3 mM for 10 s) to induce near-complete ($>90\%$) desensitization and, after washing out ACh for various amounts of time (1.5, 2.5, 3.5, 5 and 10 s in control conditions; 1.5, 2, 2.5, 3

and 6 s for M β CD/SMase), a brief pulse (2 s) of ACh was applied to assess the extent of recovery from the desensitized state. After M β CD/SMase cotreatment, the $\alpha 7$ nAChRs recovered from desensitization significantly faster; in 2.5 s, the recovery in M β CD/SMase-treated cells was $48 \pm 5\%$ ($n = 7$ cells) versus $29 \pm 4\%$ ($n = 6$ cells) in control conditions. The rate of recovery of control and treated cells was monophasic (Fig. 4E; Gay *et al.* 2008), with time constant values of 3.1 s ($n = 6$ cells) in control conditions and 1.7 s ($n = 7$ cells; $P < 0.0001$) in M β CD/SMase-treated cells, suggesting that lipid raft disruption results in a decreased probability that neuronal $\alpha 7$ nAChRs would become and remain in the desensitized state.

Effects of lipid raft disruption on the $\alpha 3\beta 2$ nAChR

We examined the effects that lipid raft disruption has on the $\alpha 3\beta 2$ nAChR subtype.

Cultured hippocampal neurons coexpressing wild-type rat $\alpha 3$ and $\beta 2$ nAChR subunits (along with GFP) were exposed to a 1 s rapid application of ACh (3 mM) at a holding potential of -70 mV; this resulted in the rapid activation of an inward current that desensitized in the continued presence of agonist (Fig. 5A), with a desensitization half-time of 100 ± 17 ms (Fig. 5D). Similar to the $\alpha 7$ nAChR, the onset of desensitization was biphasic; the fast component ($\tau_{\text{fast}} = 95 \pm 17$ ms) comprised $58 \pm 4\%$, while the slow component ($\tau_{\text{slow}} = 600 \pm 143$ ms) comprised $25 \pm 2\%$ of the total desensitization (Fig. 5E; $n = 7$ cells).

Interestingly, co-application of M β CD and SMase resulted in the opposite effect on the desensitization kinetics of the $\alpha 3\beta 2$ nAChR in comparison to the $\alpha 7$ nAChR; the desensitization half-time was significantly decreased from 100 ± 17 ms in control conditions to 47 ± 16 ms in treated cells ($n = 6$ cells; Fig. 5D). Although both fast and slow time constants were not significantly different, the contribution of the fast and slow components to desensitization was significantly affected; the fast component was $73 \pm 3\%$ (versus $58 \pm 4\%$ in control conditions) and the slow component was $11 \pm 3\%$ (versus $25 \pm 2\%$ in control conditions; Fig. 5E). Furthermore, the current amplitude was significantly decreased from -1040 ± 140 pA in control conditions to -380 ± 68 pA in treated cells ($n = 7$ versus $n = 6$, respectively; Fig. 5C). These results suggest that $\alpha 3\beta 2$ nAChRs are also susceptible to changes in the lipid environment, although the changes in the kinetics of desensitization appear to be opposite to the effects seen with the $\alpha 7$ nAChRs.

Effects of cholesterol and sphingomyelin synthesis inhibitors on $\alpha 7$ nAChR function

So far we have demonstrated that cholesterol depletion through acute treatment with M β CD and SMase affects

$\alpha 7$ nAChR function. These effects were reversed by cotreatment of the cells with the corresponding lipid molecules. Therefore, these treatments show the acute effects that decreasing cholesterol and sphingomyelin levels have on $\alpha 7$ nAChR function. To further investigate the correlation between cholesterol and sphingomyelin levels and $\alpha 7$ nAChR function, we inhibited the *de novo* synthesis of both cholesterol and sphingomyelin. We used myriocin, an inhibitor of serine palmitoyltransferase, the

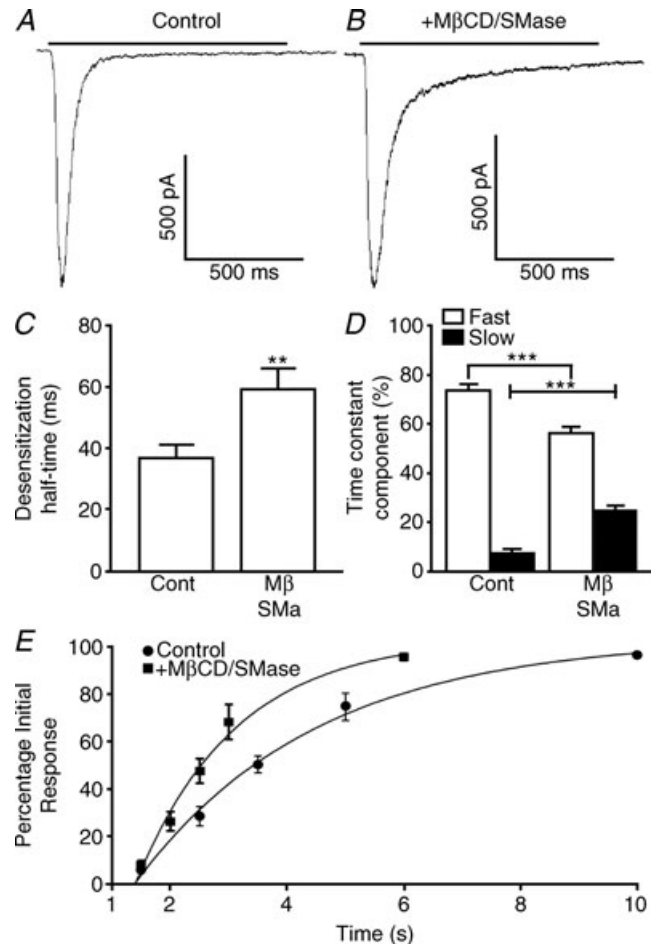


Figure 4. Effects of lipid raft disruption on the response of native neuronal $\alpha 7$ nAChRs

Hippocampal neurons were subjected to a 1 s application of 3 mM ACh at Day in vitro 13–14 (DIV 13–14) to record the response of native channels to a 15 min treatment with either PBS (control) or a combination of 5 mM M β CD and 0.5 U ml $^{-1}$ SMase. A and B, responses to ACh application in control (A) and M β CD/SMase-treated neurons (B). C, M β CD/SMase resulted in a significant increase in the desensitization half-time. D, desensitization time constants (τ) were calculated using Clampfit and fitted with a two-exponential equation. Although no difference was seen in τ values, the ratio of fast and slow desensitization was affected by M β CD/SMase cotreatment. E, recovery from desensitization was studied by consecutive 1 s applications of 3 mM ACh. The time of application was adjusted depending on treatment. ** $p < 0.001$; *** $p < 0.0001$.

key enzyme in the *de novo* synthesis of sphingolipids used to deplete cells of sphingolipids (Szoke *et al.* 2010), and mevastatin, an inhibitor of cholesterol synthesis (Alberts *et al.* 1980); both of these have been shown to disrupt lipid rafts (Simons *et al.* 1998).

Cells were treated with 2 μM mevastatin and 400 nM myriocin in serum-free medium for 24–48 h. Then cells were exposed to the rapid application of ACh to activate the $\alpha 7$ nAChRs (3 mM for 2 s; Fig. 6A and B). Treatment with these synthesis inhibitors showed a partial effect on $\alpha 7$ nAChR function; alterations in desensitization kinetics were observed in nine of

21 cells. The affected cells showed no significant changes in current amplitude (-1767 ± 203 versus -1681 ± 400 pA in control conditions; $n = 9$ versus $n = 10$, respectively) or desensitization half-time (50 ± 5 versus 39 ± 3 ms control conditions), in contrast to changes seen with the M β CD/SMase cotreatment (143 ± 41 versus 27 ± 2 ms in control conditions) and desensitization time constants ($\tau_{\text{fast}} = 18 \pm 2$ versus 21 ± 4 ms in control conditions; $\tau_{\text{slow}} = 180 \pm 56$ versus 285 ± 87 ms in control

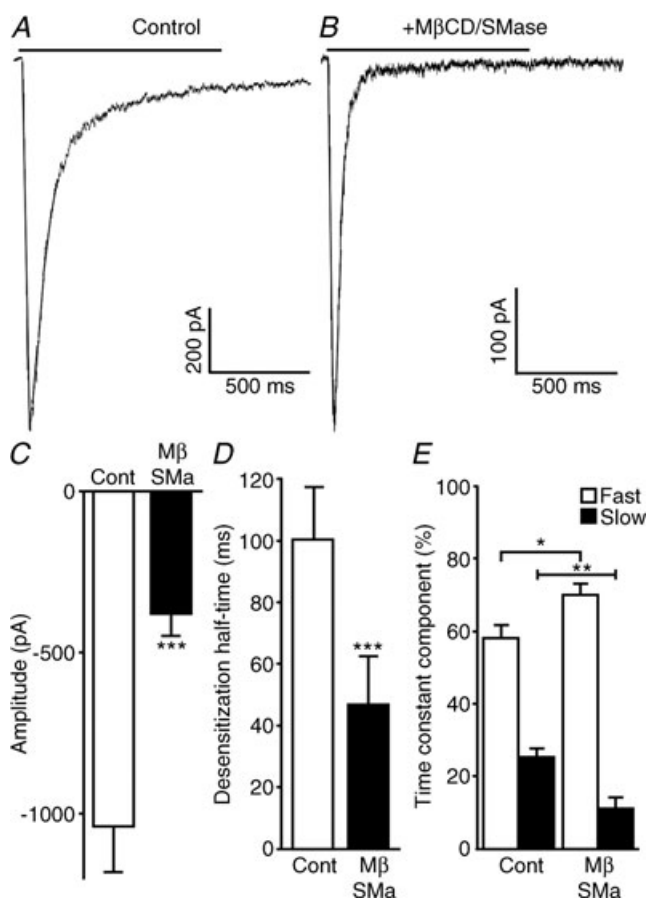


Figure 5. Effects of lipid raft disruption on the responses of $\alpha 3\beta 2$ nAChRs

Hippocampal neurons coexpressing wild-type rat $\alpha 3$ and $\beta 2$ nAChR subunits (along with GFP) were incubated for 15 min with either PBS (control) or a combination of 5 mM M β CD and 0.5 U ml $^{-1}$ SMase and subjected to a 1 s application of 3 mM ACh. A and B, response to ACh application in control (A) and M β CD/SMase-treated neurons (B). C, M β CD/SMase resulted in a significant decrease in current amplitude. D, likewise, desensitization half-time decreased following M β CD/SMase cotreatment. E, desensitization time constants (τ) were calculated using Clampfit and fitted with a two-exponential equation. Although no difference was seen in τ values, the ratio of fast and slow desensitization was affected by M β CD/SMase cotreatment. Plots show means \pm SEM, and were subjected to Student's *t* test; * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

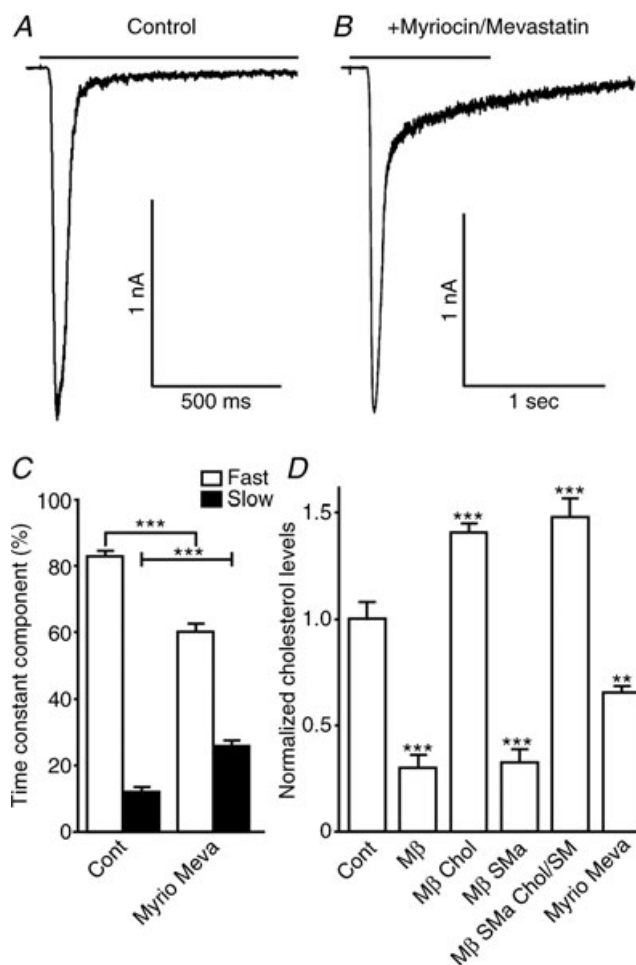


Figure 6. Inhibition of cholesterol and sphingomyelin synthesis affects the desensitization kinetics of $\alpha 7$ nAChRs

Hippocampal neurons were incubated for 24–48 h with 400 μM myriocin and 2 mM mevastatin in serum-free media and tested for their response to a 2 s ACh application. A and B, traces showing the effects of myriocin and mevastatin cotreatment in the ACh response. C, desensitization time constants (τ) were calculated using Clampfit and fitted with a two-exponential equation. Although no difference was seen in τ values, the ratio of fast and slow desensitization was affected by myriocin and mevastatin cotreatment. D, cholesterol levels measured after drug applications were normalized to protein concentration and cholesterol levels in control conditions ($n = 9$). Plots show means \pm SEM; desensitization time constant and cholesterol measurements were analysed using Student's unpaired *t* test and one-way ANOVA, respectively; ** $P < 0.001$; *** $P < 0.0001$.

conditions). However, the ratio of fast to slow components of desensitization was significantly changed to $60 \pm 2\%$ (versus $83 \pm 2\%$ in control conditions) and $26 \pm 2\%$ (versus $12 \pm 1\%$ in control conditions; Fig. 1E), respectively, indicating that inhibition of the synthesis of cholesterol and sphingomyelin had a significant effect on desensitization kinetics.

Cholesterol levels decrease after treatment with cholesterol scavenger and synthesis inhibitors

To compare the effects of acute removal of cholesterol (M β CD) and the enzymatic breakdown of sphingomyelin (SMase) with the chronic inhibition of cholesterol synthesis (mevastatin) and sphingomyelin synthesis (myriocin) on cholesterol levels, we used the Amplex Red Cholesterol Assay Kit. Samples were tested in parallel with a BCA protein kit and first normalized to protein levels, then to cholesterol levels in control conditions. Consistent with the functional data showing partial effects of synthesis inhibitors on $\alpha 7$ nAChR function (Fig. 6A–C), cholesterol levels were decreased approximately 35% by cotreatment with mevastatin and myriocin. In contrast, treatment with M β CD and cotreatment with M β CD and SMase resulted in a decrease of approximately 70%, which is also consistent with the functional data (Fig. 1C–E) because these treatments resulted in larger effects on desensitization kinetics. Treatment with SMase alone showed no effects on cholesterol levels, and there was no difference in cholesterol levels between M β CD and M β CD/SMase cotreatment.

In addition, treatment with M β CD in the presence of cholesterol and M β CD/SMase in the presence of cholesterol and sphingomyelin resulted in enrichment (140 and 147% cholesterol respectively, when compared with control conditions; Borroni *et al.* 2007), which is consistent with the reversal these treatments had on the functional effects of M β CD and M β CD/SMase on $\alpha 7$ nAChR desensitization (Fig. 2B–D).

Discussion

The $\alpha 7$ nAChRs have previously been shown to be localized into lipid rafts (Bruses *et al.* 2001; Oshikawa *et al.* 2003; Fernandes *et al.* 2010); however, the role that this localization has on channel function has not been resolved. Here we have used pharmacological agents to acutely and chronically decrease the levels of cholesterol and sphingomyelin, the major lipids in lipid rafts, and show that the disruption of lipid rafts alters the functional properties of these receptors by slowing the kinetics of desensitization (in part by increasing the rate of recovery from desensitization), and by increasing agonist affinity and single-channel conductance. These data provide new

insights into the mechanism of desensitization of $\alpha 7$ nAChRs by providing evidence that the lipid composition of the plasma membrane can modulate the activity of the $\alpha 7$ nAChRs.

Although the mechanism of desensitization is not completely understood, desensitization is an important characteristic of nAChRs. For example, differences in desensitization rates of nAChR subunits are thought to play a role in nicotine addiction; low doses of nicotine desensitize non- $\alpha 7$ nAChRs on dopaminergic and GABAergic neurons, whereas at the same time it activates $\alpha 7$ receptors, enhancing glutamate-mediated excitatory inputs to the dopaminergic neurons and facilitating dopamine release onto Nucleus accumbens (NAc) neurons (Mansvelder & McGehee, 2002). Moreover, abnormalities in nAChR desensitization are thought to be involved in some nAChR-related diseases, such as autosomal dominant nocturnal frontal lobe epilepsy (which is thought to be caused by mutations in $\alpha 4$ and $\beta 2$ nAChR subunits; Bertrand *et al.* 2002) and congenital myasthenic syndrome (caused by mutations in endplate nAChRs; Milone *et al.* 1997).

Therapeutic drugs are currently being developed to treat Alzheimer's disease and other neurological disorders (Young *et al.* 2008) by potentiating $\alpha 7$ nAChRs through the removal of desensitization (e.g. type II $\alpha 7$ PAMs, such as PNU-120596; (Bertrand & Gopalakrishnan, 2007). However, this can in some cases (but not all) result in excessive calcium influx, resulting in cytotoxicity (Ng *et al.* 2007); therefore, it is critical to have a greater understanding of the mechanisms that govern the desensitization of the $\alpha 7$ nAChRs to aid in the development of $\alpha 7$ nAChR-selective PAMs that do not alter desensitization (e.g. type I PAMs).

Here we have shown that changing the levels of cholesterol and sphingomyelin in the plasma membrane using both M β CD and SMase causes a dramatic change in the desensitization kinetics of the $\alpha 7$ nAChRs. Furthermore, treatment with SMase alone showed no significant difference from control conditions in cholesterol levels and in ACh-evoked $\alpha 7$ desensitization kinetics, suggesting that significant changes in cholesterol levels are necessary for the functional effects reported here. Interestingly, the addition of SMase to M β CD was necessary for the fivefold decrease in desensitization half-time (compared with no significant change with M β CD alone), a decrease in the fast time constant of decay, and an increase in the contribution of the slow component of desensitization when compared with both control conditions and M β CD alone. Taken together, these results suggest that the cotreatment with M β CD and SMase is more effective at disrupting lipid rafts than M β CD alone. This is consistent with a previous study showing that the combination of both agents has more

pronounced effects than M β CD alone on the activity of both KCC2 and NKCC1 (K⁺–Cl[–] and Na⁺–K⁺–2Cl[–] co-transporters, respectively), which are also associated with lipid rafts (Hartmann *et al.* 2009).

Here we show that disruption of lipid rafts through cotreatment with M β CD and SMase affects the functional and pharmacological properties of the α 7 nAChRs, supporting the idea that lipid raft integrity is critical for proper channel function. We have also shown that the disruption of lipid rafts also affects desensitization of the α 3 β 2 subunit-containing nAChRs (however, the effect on kinetics is opposite to the α 7 receptor subtype), suggesting that lipid rafts play an important role in the modulation of more than one type of neuronal nAChRs. These modulatory effects can be mediated by changes in channel localization or by a loss of lipid raft-mediated protein–protein or protein–lipid interactions, which have been shown to result in changes to receptor conformation (Allen *et al.* 2007). In this study, we cannot tell whether these changes are caused by changes in receptor localization or by the loss of a physical interaction. However, cholesterol depletion with M β CD has been shown to increase the mobility (Fernandes *et al.* 2010) and to disperse clusters (Bruses *et al.* 2001) of α 7 nAChRs in neurons, whereas it has been shown to cause the internalization of endplate nAChRs (Borroni *et al.* 2007). Although we found that cholesterol levels were not different between M β CD and M β CD/SMase treatments, current amplitude is decreased by M β CD treatment alone, suggesting that SMase breakdown of sphingomyelin into ceramide and phosphocholine is able to prevent some of the effects of M β CD on α 7 nAChR function. Previous studies have shown that ceramide produced by SMase treatment displaces cholesterol from lipid rafts and forms clusters that prevent the dispersion of raft resident proteins (Yu *et al.* 2005). Furthermore, the generation of ceramide through pretreatment of cells with SMase has been shown to be protective against the effects of M β CD on Ca²⁺-triggered membrane fusion (Rogasevskaja & Coorsen, 2006). Altogether, our data suggest that differences in α 7 response current amplitude between M β CD and M β CD/SMase treatments are due to changes in localization, rather than cholesterol levels.

Both cholesterol and α 7 nAChRs have been shown to play a role in the development of Alzheimer's disease. Early development of Alzheimer's disease is characterized by both cholesterol dysregulation and a degeneration of cholinergic neurons (Barrantes *et al.* 2009). Alzheimer's disease is a neurodegenerative disorder characterized by the generation of β -amyloid (A β) fragments, and the α 7 nAChRs have been implicated in neuroprotection against A β toxicity (Kihara *et al.* 2001). Conflicting reports have recently surfaced on the possible involvement of the α 7 nAChR in Alzheimer's disease; one study using an animal model of Alzheimer's disease found

that knocking out the α 7 nAChR results in more severe memory (hippocampus-dependent) deficits (Hernandez *et al.* 2010), while another study using the same animal model found that knocking out the α 7 nAChR lessens the cognitive deficiencies of Alzheimer's disease (Dziewczapolski *et al.* 2009). This discrepancy is explained by Hernandez *et al.* (2010) as a difference in the age of the animals used; the α 7 nAChR activity appears to be neuroprotective in early Alzheimer's disease, whereas it may be deleterious in later stages of Alzheimer's disease (Dziewczapolski *et al.* 2009; Hernandez *et al.* 2010).

Chronic treatment of cells with a cholesterol synthesis inhibitor, followed by the acute treatment with M β CD, is able to inhibit A β generation in hippocampal neurons (Simons *et al.* 1998). Therefore, cholesterol-lowering drugs appear to be a good therapeutic approach in early Alzheimer's disease because they decrease the generation of A β , while at the same time they improve α 7 nAChR signalling. In this study, we have shown that a decrease in cholesterol levels through synthesis inhibition is able to cause changes in desensitization kinetics without affecting the current amplitude of the α 7 nAChRs, suggesting that treatment with these compounds will result in enhanced cholinergic responses in the hippocampus; this latter effect has been shown to result in improvements in cognition and memory in animal models (Hernandez *et al.* 2010).

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Author contributions

Both authors contributed to either the conception and design or the analysis and interpretation of data. Drafting of the article was done primarily by J.O.C.-S., while both authors participated in revisions. Both authors approved the final version of the manuscript for publication.

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