Palmitoylation of Estrogen Receptors Is Essential for Neuronal Membrane Signaling

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In addition to activating nuclear estrogen receptor signaling, 17β-estradiol can also regulate neuronal function via surface membrane receptors. In various brain regions, these actions are mediated by the direct association of estrogen receptors (ERs) activating metabotropic glutamate receptors (mGluRs). These ER/mGluR signaling partners are organized into discrete functional microdomains via caveolin proteins. A central question that remains concerns the underlying mechanism by which these subpopulations of ERs are targeted to the surface membrane. One candidate mechanism is *S*-palmitoylation, a posttranscriptional modification that affects the subcellular distribution and function of the modified protein, including promoting localization to membranes. Here we test for the role of palmitoylation and the necessity of specific palmitoylacyltransferase proteins in neuronal membrane ER action. In hippocampal neurons, pharmacological inhibition of palmitoylation eliminated 17β-estradiol-mediated phosphorylation of cAMP response element-binding protein, a process dependent on surface membrane ERs. In addition, mutation of the palmitoylation site on estrogen receptor (ER) α blocks ERα-mediated cAMP response element-binding protein phosphorylation. Similar results were obtained after mutation of the palmitoylation site on ERβ. Importantly, mutation of either ERα or ERβ did not affect the ability of the reciprocal ER to signal at the membrane. In contrast, membrane ERα and ERβ signaling were both dependent on the expression of the palmitoylacyltransferase proteins DHHC-7 and DHHC-21. Neither mGluR activity nor caveolin or ER expression was affected by knockdown of DHHC-7 and DHHC-21. These data collectively suggest discrete mechanisms that regulate specific isoform or global membrane ER signaling in neurons separate from mGluR activity or nuclear ER function. **(*Endocrinology* 154: 4293–4304, 2013)**

Neuronal function is rapidly modulated by 17β-estradiol via membrane-initiated events. Membrane estrogen receptor (ER) signaling alters neuronal electrophysiological properties, intracellular signaling cascades, downstream gene expression, and neuroanatomical structure. All of these occur via activation of second-messenger pathways more commonly associated with activation of G protein–coupled receptors (1–10).

Several models have been put forth regarding the mechanism by which 17β-estradiol acts on the surface membrane of neurons to affect cellular excitability (11–14). One prominent mechanism includes 17β-estradiol being able to directly activate metabotropic glutamate receptor (mGluR) signaling through the physical association of membrane-localized ERα and/or ERβ with group I and II mGluRs (15). This interaction of ERs with mGluRs is dependent on distinct functional microdomains generated by caveolins 1 and 3 (16). This coupling of membrane ERs to mGluRs is found across the nervous system, including neurons of the hippocampus (16–19), striatum (20), cor-

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Abbreviations: APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; 2-BR, 2-methyl-6-(phenylethynyl)pyridine hydrochloride; d.i.v., days in vitro; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; ER, estrogen receptor; ERE, estrogen response element; EYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; MAP2, microtubule-associated protein 2; mGluR, metabotropic glutamate receptor; qPCR, quantitative PCR; PPT, 4,4′,4′-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol; siRNA, small interfering RNA.

tex (21, 22), arcuate nucleus (23–25), and dorsal root ganglion (26) and hypothalamic astrocytes (27–29).

Whereas the signaling pathways and sexual differentiation underlying ER/mGluR modulation of neuronal function have been elucidated in detail (16, 17, 19), it is not clear how ERα and ERβ are targeted to the neuronal membrane to initiate this process. One candidate mechanism is *S*-palmitoylation. Palmitoylation is a reversible posttranscriptional modification, typically of cysteine residues, that regulates protein trafficking and function both inside and outside the nervous system (30–32). In nonneuronal cells, palmitoylation of ERα by the palmitoylacyltransferase proteins DHHC-7 and DHHC-21 is essential for receptor trafficking to the surface membrane (33–37). Yet, the functional role of palmitoylation in neuronal ER signaling is unknown. Hence, we tested the role of palmitoylation and these specific palmitoylacyltransferase proteins in membrane-initiated signaling of ERα and ERβ within hippocampal neurons. ER palmitoylation was found to be crucial for membrane ER action. Furthermore, the palmitoylacyltransferase proteins DHHC-7 and DHHC-21 are indeed essential for both membrane ERα and ERβ activation of mGluR signaling. These data establish that palmitoylation plays a central role in 17β-estradiol acting at the neuronal membrane and provide novel tools to disrupt ERα and/or ERβ membrane signaling without compromising mGluR activity or nuclear ER function.

Materials and Methods

Cell culture

Hippocampal neurons were cultured from female Sprague-Dawley 1- to 2-day-old rat pups as described previously (19, 38), using a protocol approved by the animal care and use committee at the University of Minnesota. After decapitation, the hippocampus was isolated in ice-cold modified Hanks' balanced salt solution containing 20% fetal bovine serum (FBS) (Atlanta Biologicals) and 4.2 mM $NaHCO_3$ and 1 mM HEPES (pH 7.35, 300 mOsm). The tissue was then washed and digested for 5 minutes in a trypsin solution (type XI; 10 mg/mL) containing 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 25 mM HEPES, and 1500 U of DNase (pH 7.2, 300 mOsm). After additional washes, tissue was dissociated and pelleted twice by centrifugation to remove contaminants. Cells were then plated onto 10-mm coverslips (treated with Matrigel to promote adherence; BD Biosciences) and incubated for 20 minutes at room temperature. Then 2 mL of MEM (Invitrogen) containing 28 mM glucose, 2.4 mM $NaHCO_3$, 0.0013 mM transferrin (Calbiochem), 2 mM glutamine, and 0.0042 mM insulin with 1% B-27 supplement (Invitrogen), and 10% FBS (pH 7.35, 300 mOsm) were added to each coverslip. To inhibit glial growth, 1 ml of medium was replaced with a solution containing 4 μM cytosine 1-β-D-arabinofuranoside and 5% FBS 48 hours after plating, and 96 hours later, 1 mL of medium was replaced with modified MEM solution containing 5% FBS. Gentamicin (2 μg/mL; Invitrogen) was added to all media solutions to eliminate bacterial growth.

Drugs

The drugs from Tocris were 17β-estradiol (1 nM), tetrodotoxin (1 μM), D-(−)-2-amino-5-phosphonopentanoic acid (25 μM), (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (50 μM), (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) (10 μM), 4,4′,4′-(4-propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol (PPT) (1 nM), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) (10 nM), and 2-methyl-6-(phenylethynyl)pyridine hydrochloride (5 μM). The drug from Sigma-Aldrich was 2-bromohexadecanoic acid (2-BR; also called 2-bromopalmitate) (10 μM).

Immunocytochemistry

Protocols for immunocytochemistry followed those described previously (17, 19). Neurons (8–9 days IV) were incubated in a Tyrode solution containing tetrodotoxin (1 μM) and D-(−)-2-amino-5-phosphonopentanoic acid (25 μM) at room temperature for 1.5 to 3.0 hours. Cell stimulations were performed as follows: vehicle,, 10 minutes; 1 nM 17β-estradiol, 5 minutes; 50 μM DHPG, 5 minutes; 10 μM APDC, 5 minutes; 20 mM K^+, 3 minutes; 17β-estradiol or APDC, 5 minutes; and then 17β-estradiol or APDC and 20 mM K^+, 3 minutes. Pretreatment with 2-BR was 2 hours. Cells were fixed using ice-cold 4% paraformaldehyde (Electron Microscopy Sciences) in PBS containing 4 mM EGTA. Cells were washed in PBS, permeabilized with 0.1% Triton X-100 (VWR Scientific), washed, and blocked at 37°C for 30 minutes in PBS containing 1% BSA and 2% goat serum (Jackson ImmunoResearch). The cells were incubated at 37°C for 1 hour in a block containing a monoclonal antibody directed against serine 133–phosphorylated cAMP response element–binding protein (pCREB, 1:1000; catalog no. 05–667, Upstate Biotechnology), and a polyclonal antibody targeting microtubule-associated protein 2 (MAP2) (1:1000; catalog no. AB5622; Calbiochem). Cells were washed and incubated for 1 hour at 37°C in block solution with Alexa Fluor 488–conjugated anti-rabbit and Alexa Fluor 635–conjugated anti-mouse (1:1000, A11008 and A31574; Invitrogen) secondary antibodies for visualization of MAP2 and pCREB, respectively. Cells were washed and mounted using Citifluor (Ted Pella). Nuclear fluorescence intensities for pCREB were acquired using a Leica DM5500Q confocal system and quantified with Leica LAS AF (version 1.9.0; Leica).

Following established protocols (17), the confocal excitation and detection settings for each experiment were determined using 20 mM K^+–stimulated coverslips. Intercoverslip variability was accounted for by subjecting 2 coverslips to each treatment. Neurons were selected randomly across both coverslips using MAP2 fluorescence, allowing blind acquisition of pCREB intensities. The data acquisition order was random. Images were captured through the approximate midline of each cell. During data analysis, MAP2 staining was used to draw a region of interest outlining the nucleus of each neuron, blinding analysis of pCREB intensity. The region of interest was then transferred to the pCREB image, and average fluorescence intensities within the nucleus were recorded (n = ~25 neurons/group). The background from a region of the image not containing pCREB fluo-

rescence was subtracted from the average pCREB fluorescence. Experiments were replicated at least 3 times to verify the results.

DNA constructs

Rat ERα and ERβ were cloned into pCNDA 3.1 using standard techniques. The estrogen response element (ERE) was cloned into a pGL3 luciferase vector (Promega). The cysteine to alanine point mutations were generated using a QuikChange II site-directed mutagenesis kit (Stratagene). Successful DNA manipulation was verified by direct sequencing. The caveolin 1 expression vector was a gift from Drs R. Massol and T. Kirchhausen (Harvard University, Boston, Massachusetts). Following established protocols, cultured neurons were transfected 7 to 9 days in vitro (d.i.v.) with 1 μg of DNA/coverslip using either the calcium phosphate method (39, 40) or Lipofectamine 2000 (Invitrogen), with 2 μL/coverslip of Lipofectamine. No gentamicin was included in the medium or solutions used for transfection. Cells were exposed to Lipofectamine transfection solution for 4 hours. Once transfected, cells were washed and then were incubated in serum-free DMEM (Invitrogen) with 1% B-27 at 37°C. Experiments were conducted 2 d.i.v. after transfection.

Small interfering RNA (siRNA) transfections

Following previously published methods (16), all siRNA reagents were obtained from Dharmacon/Thermo Scientific unless stated otherwise. Cultured hippocampal neurons were cotransfected 7 d.i.v. with ON-TARGETplus SMARTpool siRNAs for rat DHHC-7, DHHC-10, DHHC-21, or control siRNAs against no known gene target, in addition to the siGLO transfection indicator. Transfection protocols followed the manufacturer's instructions using DharmaFECT, except that cells were incubated with 250 μL of the transfection mixture and incubated at 37°C for 5 hours. After transfection, cells were washed once with DMEM before being placed back in their original medium. Based on siGLO fluorescence, >90% of the cultured cells took up the siRNA. To assay relative changes in mRNA abundance, cells were processed 24 hours after transfection for quantitative PCR (qPCR). For all other experiments requiring commensurate changes in protein, cells were used 48 hours after transfection (16).

Luciferase-based gene reporter assays

Following previously published techniques (16), cultured hippocampal neurons were transfected 8 d.i.v. with a luciferase-based reporter of ERE-dependent transcription and either enhanced yellow fluorescent protein (EYFP), EYFP wild-type ERα, or EYFP-C452A ERα using a calcium phosphate-based method (39, 41). During transfection, 1 μg of each DNA plasmid was added to each culture well (n = 8–10 coverslips/group). Once transfected, cells were incubated in serum-free DMEM (Invitrogen) supplemented with insulin-transferrin-selenium A (ITS; Invitrogen) and containing 1 nM 17β-estradiol, 1% B-27, and 2 μg/mL gentamicin to prevent bacterial growth. The next day, cells were lysed and assayed for luciferase expression using a standard luminometer (Monolight 3010; PharMingen). The experiment was repeated twice to verify the results.

Western blotting

Cultured hippocampal neurons (8–9 d.i.v.) plated on standard 6-well plates were washed with ice-cold PBS, followed by a 10-minute lysis on ice using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) containing HALT phosphatase and protease inhibitor mixture (Thermo Scientific). The lysate was sonicated for 5 minutes and then briefly centrifuged (16). Each sample was analyzed for total protein using the DC Protein Assay (Bio-Rad Laboratories). A 40-μg sample of total protein and a prestained protein standard (Precision Plus Protein All Blue Standards; Bio-Rad Laboratories) were electrophoretically resolved on a 4% to 15% TGX gel (Bio-Rad Laboratories) under reducing conditions for approximately 60 minutes at 100 V. Resolved proteins were then transferred to a nitrocellulose membrane overnight at 25 V for subsequent immunoblotting. Nonspecific binding sites were blocked with Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% milk for 1 hour at room temperature with shaking. Blots were then incubated overnight at 4°C with shaking with the appropriate mixed primary antibodies in TBST with 5% milk for caveolin 1 (1:200, catalog no. 610060; BD Biosciences), Flotillin (1:3000, catalog no. 610820; BD Biosciences), GAPDH (1:1000, catalog no. MAB374; EMD Millipore) and/or ERα (1:200, MC-20, catalog no. sc-542; Santa Cruz Biotechnology). ERβ was not examined because of known specificity problems with the currently available commercial antibodies (42). Membranes were rinsed in TBST and incubated with a mixture of infrared labeled secondary antibodies, goat anti-rabbit IRDye 680 (1:20,000; Li-Cor Biotechnology) and goat anti-mouse IRDye 800 (1:20,000; Li-Cor Biotechnology) in TBST with 5% milk for 1 hour at room temperature. Membranes were rinsed again in TBST before imaging and were scanned using a Li-Cor Odyssey Infrared Imaging System scanner (Li-Cor Biotechnology). Experiments were repeated at least 3 times to verify results.

PCR

qPCR was performed using standard protocols (38, 43). mRNA was extracted and reverse transcribed from cultured hippocampal neurons or adult P70 intact male and female hippocampus using standard kits (RNAeasy Mini or Midi Kit or QuantiTect Reverse Transcription Kit; QIAGEN). qPCR amplification was performed using a LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 II PCR machine (Roche) (44). Threshold values were calculated using the second derivative maximum method and standardized to the ribosome-related gene *rpl13a* (LightCycler 480 Software 1.5; Roche). The thermal cycling program used was a preincubation step at 95°C for 5 minutes, followed by at least 45 cycles consisting of a 10-second denaturing step at 95°C, an annealing step for 10 second at 60°C, an extension step for 10 second at 72°C, and a measurement of fluorescence intensity. At the end of each cycling program, a melting curve was run. PCR for individual cDNA samples was performed in triplicate, and overall experiments were repeated at least twice.

The upper and lower primer sequences used were as follows: rpl13a (GenBank accession number NM_173340), 5′-TGCTGCCGCACAAGACCAAA-3′ and 5′-AACTTTCTGGTAGGCTTCAGCCGC-3′; caveolin 1 (GenBank accession number NM_031556), 5′-GCAGTTGTACCGTGCATCAAGAG-3′ (nucleotides 385–407) and 5′-CGGATATTGCTGAATATCTTGCC-3′; caveolin 3 (GenBank accession number NM_019155.2), 5′-TGGAGGCACGGATCATCAAG-3′ and 5′-ACACGCCATCGAAGCTGTAA-3′; DHHC-7 (GenBank accession number NM_133394.1), 5′-CAATATGCAATGAC-

GAAACTGAG-3′ and 5′-GAAGACAGCTTCATCCCTTCC-3′; DHHC-10 (GenBank accession number NM_001039342.2), 5′-AACAACTTGACTTGGCCTACG-3′ and 5′-TGGCGAAAGAGTAGACAGCA-3′; DHHC-21 (GenBank accession number AY886536.1) 5′-GATGGGAGCGCTTCGGCCTC-3′ and 5′-CCACATGCAGAGCGGGAGCTG-3′; ERα (GenBank accession number NM_012689.1), 5′-TTTCTTTAAGAGAAGCATTCAAGGA-3′ and 5′-TTATCGATGGTGCATTGGTTT-3′; and ERβ (GenBank accession number NM_012754.1), 5′-ATGTACCCCTTGGCTTCTGC-3′ and 5′-ACTGCTGCTGGGAGGAGATA-3′.

Statistics

Experimental results were analyzed with ANOVAs and Tukey multiple comparison post hoc tests or the Student *t* test, using Prism 4.03 (GraphPad Software). Probability values ≤ .05 were considered a priori significant. Data are presented as means ± SEM. Of note, in each figure, statistically different groups are denoted by different lower case letters within each bar graph. In addition, shaded bars indicate groups in which the experimental manipulation produced an outcome statistically different outcome from the corresponding control.

Results

Palmitoylation is necessary for membrane ER regulation of CREB phosphorylation

Administered alone, 17β-estradiol rapidly phosphorylates the transcription factor CREB in female hippocampal pyramidal neurons. In contrast, a 2-minute preexposure to 17β-estradiol attenuates L-type calcium channel-dependent CREB phosphorylation, triggered by a 3-minute 20 mM K^+ depolarization (17, 19). These rapid actions of 17β-estradiol are mediated via membrane-associated ERs coupled to mGluRs and its associated downstream signaling pathways, which are organized into 2 discrete functional signaling pathways via caveolins 1 and 3 (16). One pathway couples membrane ERα to mGluR1a via caveolin 1; the second pathway couples ERα and ERβ to mGluR2 via caveolin 3.

We first tested whether global blockade of palmitoylation affected 17β-estradiol regulation of CREB phosphorylation. In hippocampal neurons, pharmacological blockade of palmitoylation by preincubating cultures for 2 hours with the broad-spectrum palmitoylation inhibitor 2-BR (45) eliminated 17β-estradiol–induced CREB phosphorylation [$F_{(3, 120)} = 15.98$; $P < .05$] (Figure 1, A and B). Pretreatment with 2-BR also eliminated 17β-estradiol-induced attenuation of L-type calcium channel-dependent CREB phosphorylation, observed via cell depolarization using 20 mM K^+ [$F_{(4, 178)} = 44.04$; $P < .05$] (Figure 1C). Interestingly, treatment with 2-BR did not alter L-type calcium channel–dependent CREB phosphorylation in the absence of 17β-estradiol. These results indicate that palmitoylation is necessary for membrane ER signaling in neurons. However, these experiments do not determine which protein(s) need to be palmitoylated for membrane ER signaling to occur.

Mutation of the palmitoylation cysteine residue on ERα and ERβ specifically eliminates membrane ER signaling

Palmitoylation typically occurs on cysteine residues. In rat, the palmitoylation site on ERα is at cysteine 452 and is embedded within a highly conserved 9-amino acid motif (ERα 446–461: QGEEFVCLKSIILLNS) (35, 37). This motif is located within the "E" region of ERα (46). Work in nonneural cells indicates that mutation of the consensus palmitoylation cysteine residue in ERα disrupts its asso-

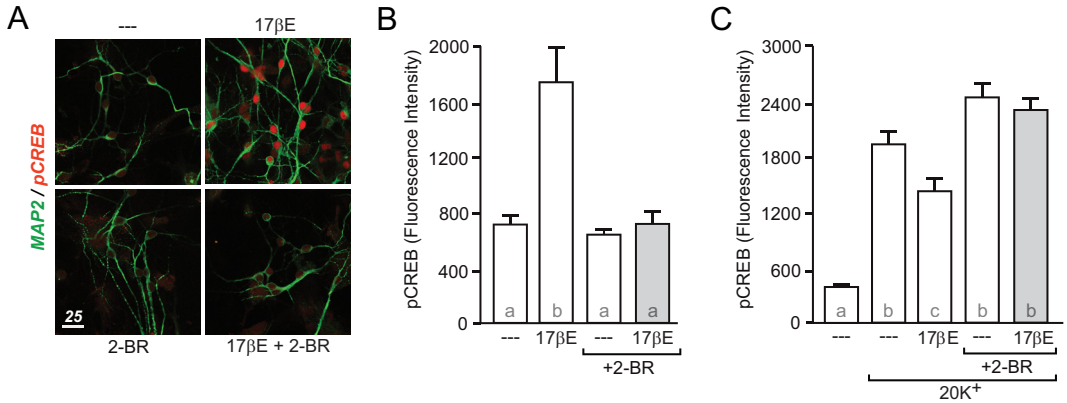

Figure 1. Inhibition of palmitoylation blocks 17β-estradiol-induced CREB phosphorylation. A, Confocal images of cultured female hippocampal neurons immunolabeled with MAP2 (green) and CREB phosphorylated at serine 133 (red). Administration of 2-BR blocked 17β-estradiol-induced CREB phosphorylation. Treatments: top left, vehicle; top right, 17β-estradiol (17βE); bottom left, 2-BR; bottom right, 17β-estradiol and 2-BR. Scale bar corresponds to 25 μm. B, Quantification of immunofluorescence demonstrating that 2-BR blocks 17β-estradiol-induced CREB phosphorylation. C, 2-BR also blocked 17β-estradiol-mediated attenuation of L-type calcium channel-dependent CREB phosphorylation, triggered by treatment with 20 mM K^+ ($20K^+$). Different lower case letters within each bar graph indicate statistically different groups.

ciation with caveolin 1 (35, 37). Because ERα interactions with caveolin 1 are required for membrane signaling, these data suggest that ERα palmitoylation is necessary for membrane ER signaling.

The next set of experiments specifically tested this hypothesis, ie, that mutation of the ERα palmitoylation site eliminated membrane ER signaling. Cultured hippocampal neurons were transfected with DNA encoding either EYFP (Figure 2A) or EYFP-tagged ERα C452A (Figure 2B). EYFP -transfected neurons showed typical 17β-estradiol modulation of CREB phosphorylation [$F_{(7, 232)}$ = 1406.00; $P < .05$] (Figure 2A). Exposure to 17β-estradiol or the ERα agonist PPT (but not the ERβ agonist DPN) increased CREB phosphorylation compared with that for the vehicle (first 4 bars). In addition, 17β-estradiol as well as both ER receptor agonists attenuated 20 mM K^+–induced CREB phosphorylation (last 4 bars).

These results are in contrast to those for neurons transfected with EYFP-ERα C452A [$F_{(7, 232)}$ = 7417.00; $P <$ .05], for which 17β-estradiol–induced CREB phosphorylation was eliminated (Figure 2B, first 4 bars). Of particular importance, 17β-estradiol attenuation of L-type calcium channel calcium channel-dependent CREB phosphorylation was still observed in these neurons. This was due to membrane ERβ signaling alone, because the ERα agonist PPT had no effect on 20 mM K^+–induced CREB phosphorylation (last 4 bars). These data indicate that overexpression of EYFP-ERα C452A eliminated membrane ERα signaling but left surface membrane ERβ signaling intact.

If overexpression of ERα C452A could specifically eliminate membrane ERα action, we speculated that the analogous manipulation in ERβ would have reciprocal effects. In rat, ERβ is palmitoylated at cysteine 354 and is also embedded within the highly conserved 9-amino acid motif (ERβ 348–363: QHKEYLCVKAMILLNS). Expression of EYFP-tagged ERβ C354A in hippocampal neurons specifically abolished ERβ-induced attenuation of 20 mM K^+–induced CREB phosphorylation [$F_{(7, 232)}$ = 1766.00; $P < .05$] (Figure 2C, last 4 bars). Notably, ERα-mediated attenuation of 20 mM K^+–induced CREB phosphorylation, as well as ERα-dependent CREB phosphor-

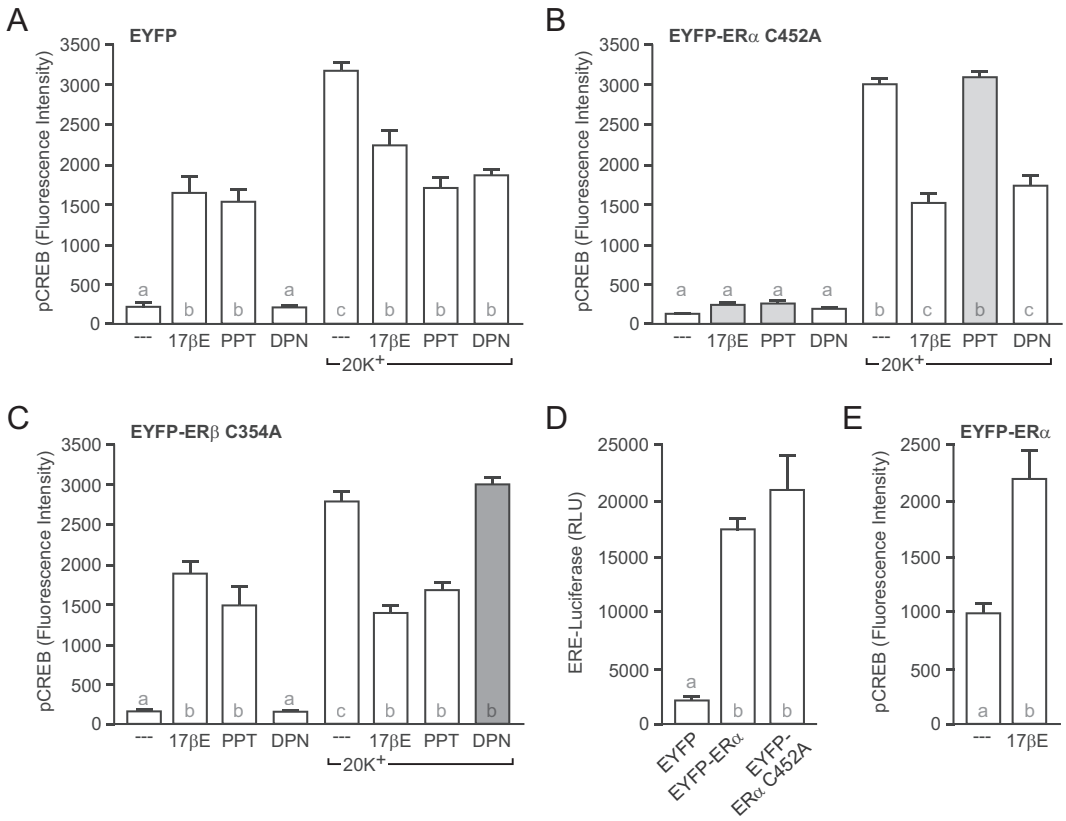

Figure 2. The ER palmitoylation site regulates membrane estrogen receptor signaling. A, Hippocampal neurons transfected with EYFP exhibit characteristic 17β-estradiol modulation of CREB phosphorylation. 17β-Estradiol-mediated CREB phosphorylation is dependent on ERα, whereas attenuation of L-type calcium channel-dependent CREB phosphorylation is mediated by either ERα or ERβ. Receptor specificity was determined using the ERα agonist PPT and the ERβ agonist DPN. B, Expression of EYFP-ERα C452A blocked membrane ERα but not ERβ signaling. C, Expression of EYFP-ERβ C354A eliminated membrane ERβ but not ERα signaling. D, EYFP-ERα and EYFP-ERα C452A were equally effective in stimulating nuclear ER function, as measured by ERE-dependent luciferase expression. RLU, relative light units. E, Expression of EYFP-ERα does not compromise 17β-estradiol-induced membrane signaling. Different lower case letters within each bar graph indicate statistically different groups.

ylation (first 4 bars), was not affected by this manipulation.

In nonneuronal cells, mutation of the palmitoylated cysteine on ERα does not affect ERE-mediated gene expression (37). Similar results were observed in cultured hippocampal neurons. Transfection of either EYFP-tagged ERα or EYFP-ERα C452A resulted in a significant increase in ERE-mediated luciferase expression ($F_{(2, 12)}$ = 24.15; $P < .05$) (Figure 2D).

Because overexpression of EYFP-ERα was found to directly affect cell function (ie, increase ERE-mediated gene expression), it raised an alternative possibility regarding the effects of EYFP-ERα C452A observed in Figure 2B, namely, that the loss of membrane ERα signaling with EYFP-ERα C452A was simply due to the overexpression of the receptor, regardless of whether it was capable of being palmitoylated. The most straightforward approach to test this hypothesis was to overexpress EYFP-ERα and monitor a membrane ERα-mediated event. Neurons transfected with EYFP-ERα were found to still exhibit 17β-estradiol–induced CREB phosphorylation [$t_{(41)}$ = 5.904] (Figure 2E), indicating that overexpression of the receptor does not negatively affect membrane signaling, eliminating this alternative hypothesis.

Collectively, these data demonstrate that nonpalmitoylatable mutants of ERα and ERβ act as dominant negatives to specifically eliminate membrane ER signaling of their endogenous counterparts in neurons.

DHHC-7 and DHHC-21 are requisite palmitoyl acyltransferase proteins for both ERα and ERβ membrane signaling

In mammalian cells, palmitoylation is potentially performed by at least 23 different enzymes, all of which contain an Asp-His-His-Cys (DHHC) amino acid sequence (47, 48). In hippocampal neurons, mRNA for all 23 DHHC proteins was detected using qPCR (data not shown). In nonneural cells, DHHC-7 and DHHC-21 are the necessary palmitoylacyltransferases for ERα (36). It is not known whether these palmitoylacyltransferases affect membrane ERβ signaling. The next experiments tested whether these proteins were essential for membrane ER signaling in neurons. For controls, we used siRNA against no known target and the palmitoylacyltransferase DHHC-10. DHHC-10 has no known connection to ER palmitoylation in nonneuronal cells (36, 47, 49).

Neurons were first transfected with siRNA against no known target or mock transfected (Figure 3A). Neurons transfected with siRNA against no known target did not show differences in mRNA expression of DHHC-7 [$t_{(4)}$ = 1.712], DHHC-21 [$t_{(4)}$ = 0.0076], or DHHC-10 [$t_{(4)}$ = 0.1764] compared with mock-transfected neurons. In addition, neurons transfected with siRNA against no known target showed normal 17β-estradiol and ER agonist modulation of CREB phosphorylation [$F_{(7, 248)} = 36.95$, $P < .05$] (Figure 3B). In contrast, transfection with siRNA against DHHC-7 decreased DHHC-7 expression [$t_{(4)}$ = 6.318. $P < .05$] (Figure 3C), without affecting the expression of DHHC-21 [$t_{(4)}$ = 0.2900] or DHHC-10 [$t_{(4)}$ = 0.2284]. Knockdown of DHHC-7 eliminated both membrane ERα and ERβ signaling to CREB [$F_{(7, 220)} = 134.7$, $P < .05$] (Figure 3D).

Similar results were observed targeting DHHC-21. Transfection with siRNA against DHHC-21 resulted in decreased DHHC-21 expression [$t_{(4)} = 2.816$, $P < .05$] (Figure 3E), but not DHHC-7 [$t_{(4)}$ = 0.2684] or DHHC-10 [$t_{(7)}$ = 0.4079]. Transfection with siRNA against DHHC-21 also abolished 17β-estradiol modulation of CREB phosphorylation [$F_{(7, 262)} = 273.6$, $P < .05$] (Figure 3F). Finally, transfection with siRNA against DHHC-10 resulted in decreased expression of DHHC-10 expression [$t_{(4)} = 5.411$, $P < .05$] (Figure 3G) but not of DHHC-7 [$t_{(4)}$ = 2.255] or DHHC-21 [$t_{(4)}$ = 1.535]. Transfection with siRNA against DHHC-10 did not affect 17β-estradiol modulation of CREB phosphorylation [$F_{(7, 256)} = 36.95$, $P < .05$] (Figure 3H).

mGluR signaling and expression of caveolins and ERs are unaffected by DHHC-7 and DHHC-21 knockdown

Our data suggest that DHHC-7 and DHHC-21 are essential for the palmitoylation of ERα and ERβ and necessary for 17β-estradiol–induced surface membrane signaling. As controls, we verified that knockdown of DHHC-7 or DHHC-21 did not affect mGluR signaling or alter the global expression of either caveolin proteins or ERs.

To test whether DHHC-7 or DHHC-21 knockdown affected mGluR signaling, neurons were transfected with siRNA against no known target (Figure 4A), DHHC-7 (Figure 4B), or DHHC-21 (Figure 4C). Two days later we assessed neuronal sensitivity to the group I mGluR agonist DHPG and the group II mGluR agonist APDC. Under each siRNA transfection condition, DHPG increased CREB phosphorylation and APDC attenuated 20 mM K^+–induced CREB phosphorylation [no target: $F_{(4, 159)} = 48.28$, $P < .05$; DHHC-7: $F_{(4, 148)} = 48.70$, $P < .05$; DHHC-21: $F_{(4, 141)} = 66.87$, $P < .05$]. Hence, knockdown of DHHC-7 or DHHC-21 did not affect group I or II mGluR signaling to CREB.

qPCR was used to determine that siRNA targeting DHHC-7 or DHHC-21 did not alter expression of caveolin 1 (Figure 4D) or caveolin 3 (Figure 4E). In comparison to no target siRNA controls, caveolin 1 was unaltered by

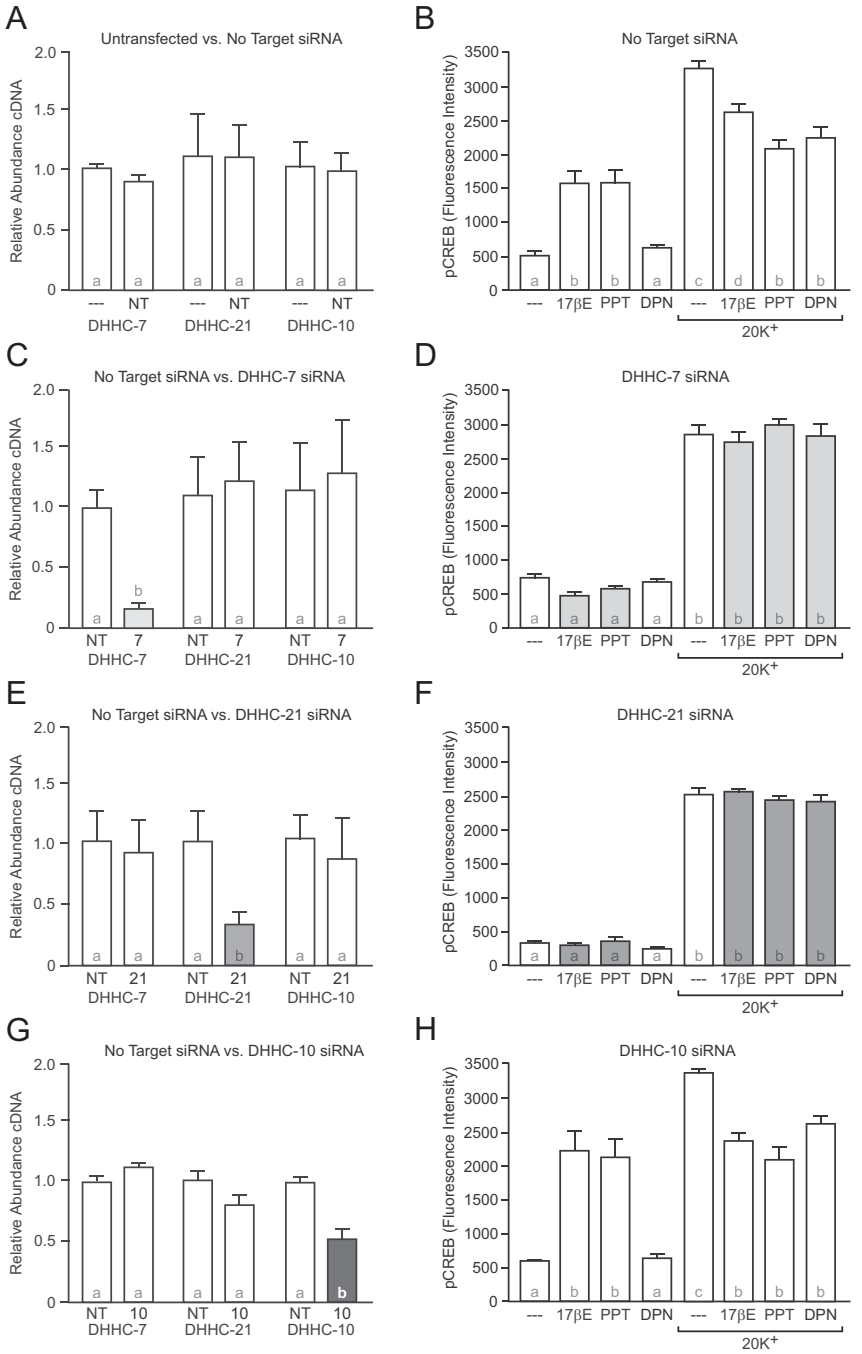

Figure 3. DHHC-7 and DHHC-21 are both necessary for ERα- and ERβ-mediated 17β-estradiol modulation of CREB phosphorylation. A, Transfection of hippocampal neurons with siRNA against no known target did not affect expression of the palmitoylacyltransferases DHHC-7, DHHC-21, or DHHC-10) compared with that for nontransfected neurons. B, In addition, no target (NT) siRNA had no effect on 17β-estradiol regulation of CREB phosphorylation. C, Transfection with siRNA against DHHC-7 knocked down DHHC-7 mRNA but not DHHC-21 or DHHC-10. D, siRNA against DHHC-7 completely eliminated 17β-estradiol regulation of CREB phosphorylation. E, Transfection with siRNA against DHHC-21 reduced DHHC-21 mRNA but not DHHC-7 or DHHC-10. F, siRNA against DHHC-21 also abolished the effects of 17β-estradiol on CREB phosphorylation. G, Transfection of siRNA against DHHC-10 decreased DHHC-10 mRNA, without affecting DHHC-7 or DHHC-21 expression. H, DHHC-10 knockdown did not affect 17β-estradiol modulation of CREB phosphorylation. Different lower case letters within each bar graph indicate statistically different groups.

siRNA against DHHC-7 [$t_{(4)}$ = 0.1763] or DHHC-21 [$t_{(4)}$ = 0.4932]. Similar results were found in examination of the expression of caveolin 3 after transfection of siRNA against DHHC-7 [$t_{(4)}$ = 0.8795] or DHHC-21 [$t_{(4)}$ = 0.5010]. Furthermore, knockdown of either DHHC-7 or DHHC-21 did not affect caveolin 1 protein expression [$F_{(2, 6)}$ = 0.6209] (Figure 4F). Western blot experiments were limited to caveolin 1 based on the availability of quality antibodies. qPCR was then again used to determine that DHHC manipulation did not affect cDNA expression of either ERα (Figure 4G) or ERβ (Figure 4H). In comparison to no target siRNA controls, ERα and ERβ mRNA expression were unaffected by siRNA against either DHHC-7 (ERα: $t_{(4)}$ = 0.2277; ERβ: $t_{(4)}$ = 0.2664) or DHHC-21 (ERα: $t_{(12)}$ = 0.5010; ERβ, $t_{(4)}$ = 0.0931). Finally, siRNA targeting DHHC-7 or DHHC-21 did not affect ERα protein expression ($F_{(2, 6)}$ = 0.905) (Figure 4I). Western blot experiments were limited to ERα because of specificity issues related to currently available ERβ antibodies (42). Of note, consistent with previous findings from primary cultures taken from the nervous system (23, 28, 50, 51), the hippocampal cultures appeared to primarily express the 52-kDa isoform of ERα (52).

Discussion

Estrogens work on the nervous system through the 2 soluble intracellular receptors, ERα and ERβ. Part of a family of steroid hormone receptors originally characterized as ligand-activated transcription factors (53, 54), ERα and ERβ can also affect cellular function through receptors located at the surface membrane (55). These membrane actions of

ERs, either in isolation or in combination with activation of the nuclear receptors, regulate a diverse array of behaviors. These include sexual receptivity, learning and memory, drug intake, cognition, motor control, and nociception (24, 56–62). Limiting our progress in identifying roles for membrane receptors in normal neuronal function is a lack of understanding regarding how intracellular steroid hormone receptors are modified to act as membrane signaling proteins. This work demonstrates that palmitoylation is an essential posttranslational modification by which ERα and ERβ are adapted to function as membrane signaling proteins in neurons.

ERα and ERβ each contain a unique consensus palmitoylation regulatory site (63, 64). Previous work in cell lines demonstrated that ERα incorporates radiolabeled palmitate (37). Substitution of the palmitoyl cysteine res-

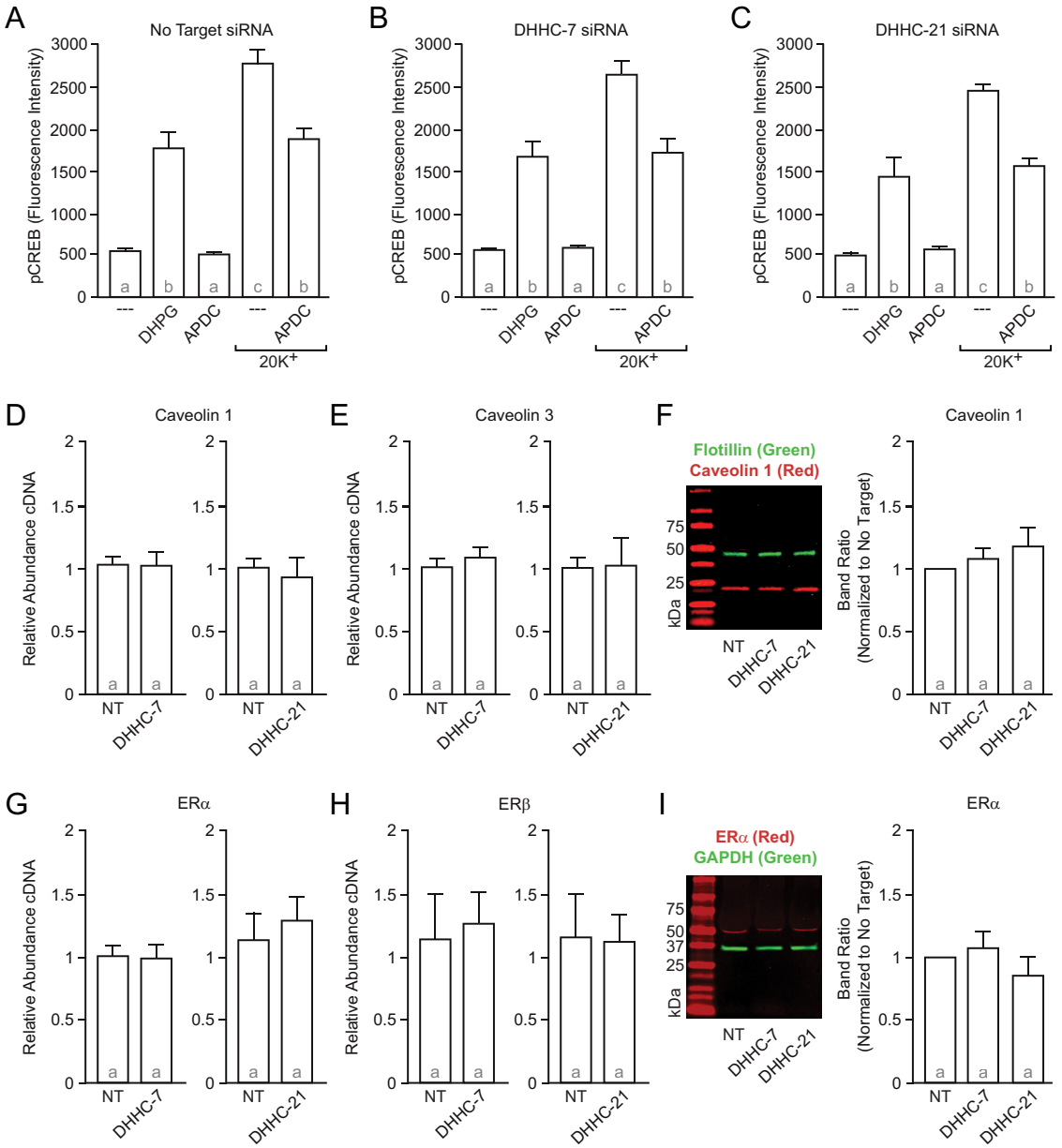

Figure 4. siRNA knockdown of either DHHC-7 or DHHC-21does not affect mGluR signaling, caveolin expression, or ER expression. A–C, mGluR1a-mediated CREB phosphorylation and mGluR2-mediated attenuation of L-type calcium channel–dependent CREB phosphorylation in hippocampal neurons are unaffected by transfection of siRNAs against no known target (A), DHHC-7 (B), or DHHC-21 (C). mGluR1a was activated using the group I mGluR agonist DHPG; mGluR2 was activated using the group II agonist APDC. D and E, siRNA against DHHC-7 and DHHC-21 had no effect on mRNA expression of either caveolin 1 (D) or caveolin 3 (E). F, Western blot and band analysis indicating that knockdown of DHHC-7 or DHHC-21 did not affect caveolin 1 protein. Flotillin was used as the loading control. G and H, siRNA against DHHC-7 and DHHC-21 had no effect on mRNA expression of either ERα (G) or ERβ (H). I, Western blot and band analysis indicating that knockdown of DHHC-7 or DHHC-21 did not affect ERα protein. GAPDH was used as the loading control. Different lower case letters within each bar graph indicate statistically different groups. NT, no target.

idue with alanine blocks both radiolabeling of ERα and membrane-initiated ERα responsiveness (34, 35, 37, 64; cf. Ref. 65). The data presented here extend these findings. We report that palmitoylation of ERα and ERβ is essential for membrane ER function. Elimination of the palmitoylated cysteine residue on both ERs eliminates membrane signaling to CREB. Expression of nonpalmitoylatable ERα eliminates membrane ERα signaling without compromising membrane signaling of ERβ. The nonpalmitoylatable ERβ mutant acts as a dominant negative to membrane ERβ signaling in a similar manner. Mechanistically, it appears that nonpalmitoylated ERs are unable to functionally associate with caveolin proteins and incapable of activating mGluRs. The expression of the palmitoyl acyltransferases DHHC-7 and DHHC-21 is required for membrane ER signaling to occur. Importantly, disruption of ER palmitoylation eliminates membrane signaling without overtly affecting nuclear ER function.

Palmitoylation provides an explanation regarding how nuclear receptors can be targeted to the surface membrane. Although often understudied within neurons, palmitoylation is a common posttranslational protein modification (30). Palmitoylacyltransferase proteins, typically located in the endoplasmic reticulum, Golgi, and plasma membrane, form a labile thioester bond between a saturated 16-carbon palmitic acid and a cysteine residue that is part of a conserved 9-amino acid motif (66–68). The addition of the palmitic acid creates a hydrophobic site on the protein, facilitating association with lipid membranes and other proteins, including caveolins (35). In addition to membrane signaling of ERs, palmitoylation is essential for the trafficking and function of postsynaptic density protein 95 (PSD-95) (47) as well as several ion channels, G protein–coupled receptors, second messenger systems, and scaffolding proteins (30). Caveolin proteins themselves are also palmitoylated (69).

Interestingly, mGluRs do not appear to be palmitoylated (70, 71). The nonessential nature of palmitoylation in mGluR signaling was necessary for our experimental design. Specifically, it allowed us to resolve membrane ER signaling through the monitoring of mGluR regulation of CREB phosphorylation. Thus, the experimental manipulations can be directly attributed to producing a loss of surface membrane ERs and/or a disruption of caveolin-dependent ER/mGluR interactions.

Disruption of either DHHC-7 or DHHC-21 expression resulted in a loss of membrane ER signaling. These data are not consistent with a model in which either DHHC-7 or DHHC-21 could alone palmitoylate and/or maintain ER palmitoylation. These data, instead, indicate 3 possible models. The first model is simultaneous, in that the 2 DHHC proteins simultaneously cooperate to palmitoylate ER as part of a multiprotein functional unit. This process has been identified in neurons, although not well characterized (47). The second model is serial, in that both palmitoyl acyltransferases work in sequence to promote membrane ER function. The third model is parallel, in that the 2 DHHC proteins act during the same temporal window to regulate separate but equally necessary processes. We will now further discuss the serial and parallel models.

The serial model posits that whereas one palmitoyl acyltransferase is responsible for the initial palmitoylation of the ER, the second is required for its maintenance. Because *S*-palmitoylation is a reversible process, proteins are often palmitoylated/depalmitoylated multiple times (47). Furthermore, membrane ERs appear to move in and out of the surface membrane as part of its signaling dynamic (51). In the nervous system, ERα is lost from hippocampal synaptosomes after palmostatin B treatment via depalmitoylation (72). In hippocampal neurons, DHHC-7 expression appears localized to the Golgi (73), suggesting that DHHC-7 is involved in ER trafficking to the membrane. Whereas the spatial localization of DHHC-21 has yet to be determined in neurons, DHHC-21 may repalmitoylate ERs, following a process of depalmitoylation that occurs during normal membrane ER signaling. Notably, DHHC-7 regulates the function of a wide array of cellular proteins (47, 74–78), possibly regulating the initial targeting of membrane proteins.

The third model is parallel. It posits that whereas one enzyme directly palmitoylates the ERs, the second DHHC enzyme palmitoylates an accessory protein also required for membrane ER signaling. Caveolin proteins are prime candidates for this accessory protein. Caveolin proteins can be palmitoylated on at least 3 different sites, albeit palmitoylation of caveolin is not necessary for its localization to the cellular membrane (69). Hence, to test whether caveolin palmitoylation is necessary for its interaction with ERs, the palmitoylation sites on these proteins could be mutated and then protein-protein interactions with ERs monitored. We note that the parallel and serial models are not mutually exclusive. Another relevant caveat is that our experiments tested the necessity of only DHHC-7, DHHC-10, and DHHC-21. We note that DHHC-7 and DHHC-21 are the only 2 DHHC proteins that are necessary palmitoylacyltransferases for ERα in nonneural cells (36). However, formally it remains possible that other DHHC proteins may play a role in ER targeting in neurons.

siRNAs targeting either DHHC-7 or DHHC-21 eliminated both ERα and ERβ membrane signaling. This is in contrast to the action of ERα or ERβ mutants lacking the palmitoylation site. These mutants acted as dominant neg-

atives, specifically blocking the membrane signaling of the respective ER subtype.

These tools can be used in future experiments not only to isolate membrane or nuclear ER function but also to delineate membrane ERα vs membrane ERβ effects.

To conclude, the data presented here indicate an essential role for ER palmitoylation in mediation of neuronal membrane-initiated 17β-estradiol signaling in neurons. We further show that the palmitoyl acyltransferases DHHC-7 and DHHC-21 are necessary for membrane ER but not mGluR signaling. Collectively, the data indicate that membrane ERs signaling is independent of mGluR signaling or nuclear ER signaling. Given the widespread association of membrane ER with mGluR, these data may prove broadly useful to understanding estrogen action throughout the nervous system.

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