Cellular/Molecular

Estradiol Rapidly Regulates Membrane Estrogen Receptor α Levels in Hypothalamic Neurons

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Estrogen receptors (ERs) and estrogen-binding proteins have been localized intracellularly and on the cell surface. The membrane-associated proteins initiate signaling that activates a myriad of cellular responses including the modulation of ion channels and ultimately transcription. Although many of the downstream actions of membrane ERs, including ERα and ERβ, have been characterized, the mechanisms regulating membrane ER levels have remained elusive in the nervous system. In the present study, we used surface biotinylation to identify and study the estradiol regulation of membrane ERα in mixed-sex, cultured hypothalamic neurons from rat. Following surface biotinylation, Western blot analysis revealed full-length 66 kDa ERα and several ERα splice variants, most notably a biotinylated 52 kDa ERα-immunoreactive protein. Treatment of the neurons with estradiol caused a rapid and transient increase of the biotinylated 52 kDa and 66 kDa ERα proteins in the plasma membrane. Exposure of the neurons to estradiol also significantly increased internalization of 52 kDa and 66 kDa ERα membrane proteins, a measure of receptor activation. In the hypothalamus, membrane ERα signaling depends on transactivation of metabotropic glutamate receptor-1a (mGluR1a). Estradiol treatment increased the internalization of mGluR1a in parallel with ERα, a finding consistent with the hypothesis of an ERα-mGluR1a signaling unit. These results demonstrate that estradiol regulates the amount of ERα in the membrane, suggesting estradiol can regulate its own membrane signaling.

Introduction

Estrogens are steroid hormones involved in sexual reproduction, neuroprotection, and learning and memory in the brain. Estradiol, the major estrogen, elicits its actions by binding and activating both intracellular estrogen receptors (ERs) and receptors associated with the plasma membrane. While the mechanisms of intracellular ER regulation and function have been largely elucidated, how levels of membrane-associated ER are regulated has remained more elusive (Levin, 2008).

Intracellular ERs, ERα and ERβ, are ligand-induced transcription factors that modulate-specific gene expression, while membrane-associated ERs rapidly alter cellular physiology by activating G-protein-coupled receptor (GPCR)-associated pathways. Some of these membrane actions are initiated through transactivation of metabotropic glutamate receptors (mGluRs) (Boulware et al., 2005, 2007). Membrane-initiated estradiol signaling is an important component of estradiol action in the brain, as evidenced by its role in sexual receptivity, a classical estrogen-dependent behavior (Dewing et al., 2007), and by the synthesis of progesterone (Chaban et al., 2003; Micevych et al., 2007; Kuo et al., 2009b). Both membrane and estradiol-regulated nuclear signaling in the hypothalamus are dependent on ERα (Micevych et al., 2003; Wintermantel et al., 2006; Dewing et al., 2007; Kuo et al., 2009b).

While there is little doubt that membrane-initiated signaling is an important component of estradiol effects on brain function, questions persist about whether (1) intracellular ERs are intrinsic membrane proteins or receptors associated with the cell membrane; (2) full-length ER or alternatively spliced proteins (Friend et al., 1995; Poola et al., 2002; Li et al., 2003; Moriarty et al., 2006) are membrane ERs; and (3) estradiol regulates levels of its cognate receptor in the plasma membrane.

In general, membrane receptors are regulated in a number of complex ways, only one of which is through transcriptional regulation. More often, membrane receptor levels are regulated by trafficking, the insertion into and removal (internalization) from the cell surface (Sinchak and Micevych, 2003). Intracellular ERα levels are transcriptionally regulated by estradiol and the insertion of ERα into the plasma membrane appears to be estradiol-regulated (Gorosito et al., 2008), but whether ERα is internalized by estradiol has not been elucidated.

An effective way to study membrane receptor trafficking is with surface biotinylation. Membrane proteins are labeled when a portion of the molecule that is exposed to the extracellular space is linked to biotin. Western blots are then used to characterize the biotinylated protein. Cultured hypothalamic neurons were surface biotinylated to identify membrane ERα and to determine whether the population of membrane ERα was stable, or whether estradiol regulated ERα levels in the plasma membrane. These studies demonstrate that in response to estradiol, full-length 66 kDa ERα and an alternatively spliced 52 kDa ERα variant are rapidly and transiently trafficked at the cell surface. Consistent

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with the idea of an ERα-mGluR1a signaling unit, mGluR1a was internalized in tandem with ERα. These findings suggest that estradiol regulates plasma membrane levels of its cognate receptor to modulate cellular response during long-term exposure to the steroid.

Materials and Methods

Animals. Animals were treated in accordance with the principles and procedures of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. Timed, pregnant Long–Evans female rats were purchased from Charles River. Wild-type C57BL/6 mice and ERα knock-out (ERαKO) mice were purchased from The Jackson Laboratory. Upon their arrival, animals were housed in a climate-controlled environment on a 12 h light/dark cycle and provided food and water *ad libitum*. To establish primary cultures or collect tissue, animals were removed from their home cage and anesthetized with isoflurane.

Primary hypothalamic cultures. Hypothalamic neurons were extracted from prenatal pups on embryonic day 18 (E18). Under sterile conditions, brains were removed and placed into chilled calcium- and magnesium-free Hanks' balanced salt solution (HBSS-CMF). Using a stereoscope, the hypothalamus was dissected. Briefly, coronal sections were made rostral to the mammillary bodies and caudal to the optic chiasm. Each hypothalamus was isolated by removing extrahypothalamic tissue parasagittally along both lateral sulci and below the thalamus. Hypothalamic tissue was placed into chilled HBSS-CMF and enzymatically dissociated with 0.25% trypsin and 0.1% DNase for 10 min at 37°C. Enzymatic dissociation was halted using 10% charcoal-stripped fetal bovine serum in HBSS-CMF, and the samples were subsequently centrifuged at 800 × *g* for 5 min at 4°C, resuspended, and washed with chilled Neurobasal-A medium (NB). The dispersed tissue was mechanically dissociated with fire-polished Pasteur pipettes and cells passed through a 40 μm cell strainer and plated onto poly-D-lysine (0.1 mg/ml)-coated 6-well culture dishes at a density of ~1×10^6 cells/ml. Cultures were maintained in serum-free supplemented NB medium (B27, 2 mM glutamine, 1% penicillin/streptomycin; Invitrogen) and kept in a constant environment of 37°C and 5% CO_2. A complete medium exchange was performed 1 h after plating and then a 50% exchange was done 24 h later and every fourth day while in culture. The day before experimentation, cells were switched to supplemented NB (insulin 10 mg/ml, transferring 5.5 mg/ml, selenium 5 μg/ml, putrescine 100 μg/ml, 1% penicillin/streptomycin; Sigma). On the day of the experiment, culture medium was completely replaced with fresh NB medium 2–3 h before experimental treatment. Estradiol (17β-estradiol; Sigma) was solubilized in DMSO (vehicle) and prepared fresh in NB medium for each experiment.

Tissue. Hypothalamic tissue was taken from Long–Evans postnatal day 9 (PND9) rat pups or adult wild-type and ERαKO mice. Brains were removed from the cranium and hypothalamic tissue was isolated following the description above and placed into chilled HBSS-CMF. Uterine and ovarian tissue was extracted from timed, pregnant female Long–Evans rats (E18) or taken from adult female mice. Uterine and ovarian tissue was identified, collected, and placed into chilled HBSS-CMF. Tissue was frozen and stored at −80°C until used. Tissue was homogenized and lysed with a Kontes Dounce tissue grinder and radioimmunoprecipitation assay (RIPA) buffer (Triton X-100 1.25%, SDS 0.1%, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.6) supplemented with a mixture of protease and phosphatase inhibitors (HALT, Pierce). Cell lysate was collected and placed on ice for 20 min, vortexed, and triturated 10 times with a pipette tip and then with a 26 gauge needle. The lysate was clarified by centrifugation at 14,000 rpm for 5 min at 4°C. The supernatant was collected and an aliquot was saved for BCA protein determination. Samples were prepared for Western blot analysis in Laemmli buffer plus 5% β-mercaptoethanol.

Cell surface biotinylation. On day 10–14 *in vitro*, hypothalamic neurons were treated with 1 nM estradiol or vehicle in a constant environment of 37°C and 5% CO_2. At the end of estradiol treatment, cell culture dishes were placed on ice to prevent exocytosis and endocytosis. Estradiol-containing NB medium was immediately aspirated and replaced with ice-cold PBS, pH 7.4, supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$ (PBS-CM). To label and isolate cell surface ERα and mGluR1a, the water-soluble, cleavable, and membrane-impermeable biotin analog sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce) was diluted in PBS-CM (0.5 mg/ml, pH 7.6) immediately before use and incubated with cells on ice for 20 min with occasional rocking. At the end of incubation, excess free sulfo-NHS-SS-biotin was quenched with three incubations with 50 mM NH_4Cl and 50 mM glycine in PBS-CM. Quenching solution was aspirated and cells washed three times with ice-cold PBS-CM.

Internalization. On day 10–14 *in vitro*, hypothalamic neurons were placed on ice and NB medium was aspirated and replaced with ice-cold PBS-CM. To label cell surface ERα and mGluR1a, sulfo-NHS-SS-biotin was diluted in PBS-CM (0.5 mg/ml, pH 7.6) immediately before use and incubated with cells on ice for 20 min with occasional rocking. At the end of incubation, excess free sulfo-NHS-SS-biotin biotin was quenched with three incubations with 50 mM NH_4Cl and 50 mM glycine in PBS-CM. The quenching solution was aspirated, cells washed three times with ice-cold PBS-CM, and then treated with 1 nM estradiol or vehicle for the indicated times in a constant environment (37°C and 5% CO_2). At the end of treatment, cells were placed on ice and the NB medium with estradiol was immediately aspirated and replaced with ice-cold PBS-CM. Cells were then incubated three times with membrane-impermeant reducing agent 2-mercaptoethanesulfonic acid (MESNA; Sigma), 100 mM in Tris buffer solution with 1 mM $CaCl_2$ (TBS-C) (pH 7.6) for 10 min to reduce the cell surface sulfo-NHS-SS-biotin. MESNA was oxidized with 50 mM iodoacetamide in PBS-CM (5 min) and then the cells were washed three times with PBS-CM.

Cell lysis and isolation of biotinylated proteins. Cultured neurons were lysed with RIPA buffer supplemented with a mixture of protease and phosphatase inhibitors (HALT, Pierce). Cell lysate was collected and placed on ice for 20 min, vortexed and triturated 10 times with a pipette tip followed by a 26 gauge needle. The lysate was clarified by centrifugation at 14,000 rpm for 5 min at 4°C. The supernatant, containing biotinylated and soluble cytoplasmic proteins, was collected. An aliquot of supernatant was saved for BCA protein determination and examination of cytoplasmic proteins by Western blot analysis. Biotinylated proteins were isolated by binding to NeutrAvidin beads (Pierce). All procedures were performed in Snap Cap spin columns (Pierce) for the remainder of the experiment. NeutrAvidin beads were prepared by washing two times with wash buffer (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.6, and supplemented with protease and phosphatase inhibitors). Supernatant containing biotinylated proteins was added to washed beads (1:1) and incubated overnight at 4°C with end-over-end rotation. The following day, beads were washed three times with wash buffer and once with Tris-HCl, pH 7.5, supplemented with protease and phosphatase inhibitors. Proteins labeled with the sulfo-NHS-SS-biotin were eluted from the beads with Laemmli buffer containing 50 mM DTT (1:1) for 5 min at 95–100°C. The eluted proteins were separated from the NeutrAvidin beads by centrifugation and analyzed by Western blot.

Western blot analysis. For electrophoresis, samples were loaded onto 10% SDS polyacrylamide gels along with molecular weight protein standards (Cell Signaling Technology). Biotinylated protein fraction concentrations were equilibrated with Laemmli buffer containing 50 mM DTT according to BCA protein determination data gathered from cytoplasmic protein samples. For analysis of cytoplasmic proteins from rat hypothalamic, uterine, and ovarian tissue, 15 μg of protein were loaded onto gels. The mouse hypothalamic and ovarian tissue used 50 μg of protein loaded onto gels. For analysis of biotinylated proteins, a volume equivalent to 30 μg of cytoplasmic protein was loaded onto gels. Electrophoresis of proteins was performed with a MiniProtean Transblot (Bio-Rad) for 1 h at 0.03 constant-amps/gel. Gels were electroblotted onto Immobilon-p PVDF (Millipore) membrane using a chilled Hoefer transblotter (TE-42) for 1.5 h at 0.8 constant-amps. After transfer, gels were stained with Coomassie blue dye. Membranes were washed once with TBS plus 0.1% Tween 20 (TBS-T) (pH 7.5) and then nonspecific immunological binding sites were blocked with 5% nonfat milk in TBS-T for 30 min on an

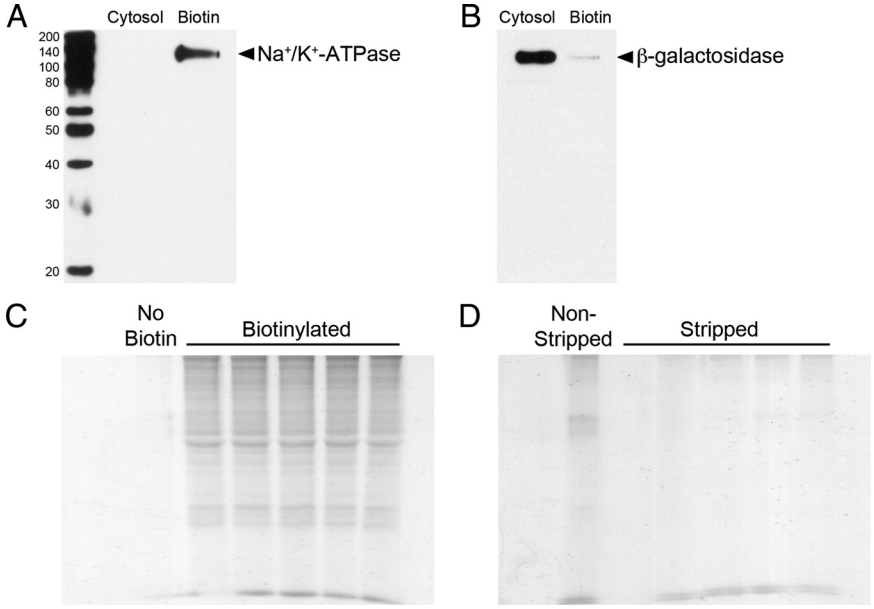

Figure 1. Cell surface biotinylation of hypothalamic neuronal cultures. ***A***, ***B***, Western blots were probed with Na^+/K^+-ATPase (***A***) and β-galactosidase (***B***) antibodies as controls to determine the purity of cytoplasmic (Cytosol) and biotinylated (Biotin) protein fractions, respectively. SDS polyacrylamide gels were stained with Coomassie blue after the protein transfer to examine biotinylation experimental controls and total protein loading of biotinylated samples. ***C***, Gels were loaded with nonbiotinylated (No Biotin) and biotinylated samples from cell surface biotinylation experiments. ***D***, Gels were loaded with non-MESNA-treated (Non-Stripped) and MESNA-treated (Stripped) protein samples from cell surface biotin internalization experiments.

orbital shaker. Primary antibodies were diluted in 5% nonfat milk in TBS-T and incubated overnight at 4°C on a side-to-side rocker. Membranes were washed three times with TBS-T and then with the secondary antibody also diluted in 5% nonfat milk in TBS-T for 1 h on an orbital shaker. Membranes were then washed three times with TBS-T and once with TBS, pH 7.5. Immunoblots were visualized with autoradiography using enhanced chemiluminescence (ECL) (SuperSignal West Pico, Pierce).

Antibodies. ERα antibodies, MC-20 (1:1000) and H-184 (1:1000; both from Santa Cruz Biotechnology; MC-20 lot # G2408 and B1709), directed against the NH_2- and COOH-terminals, respectively, were used. Additionally, anti-mGluR1a (1:1000; Millipore), anti-β-actin (1:10,000; Sigma), anti-Na^+/K^+-ATPase (1:5000; Millipore), and anti-β-galactosidase serum (1:5000; Millipore) were used. Light chain-specific secondary antibodies were peroxidase-labeled mouse anti-rabbit and goat anti-mouse IgG (Jackson ImmunoResearch Laboratories).

Densitometric analyses. To quantify protein levels, autoradiographic films were digitally scanned and analyzed. Band densities were analyzed using ImageJ software (version 1.41). Total band intensity values were calculated by subtracting the background for each film to account for any variation in background intensity across films. Data were then expressed as relative ratios, calculated as biotinylated values divided by cytoplasmic β-actin values obtained by Western blot analysis of the two protein fractions, and multiplied by 100 to obtain the percentage of protein ratio.

Statistical analysis. One-way ANOVA followed by a Tukey's *post hoc* test was used to determine statistical significance between experimental treatments. Data were analyzed using GraphPad Prism 4 software, and significance level was set at $p \leq 0.05$ for all experiments.

Results

Cell surface biotinylation

We surface biotinylated hypothalamic neurons with membrane-impermeable sulfo-NHS-SS-biotin using two different experimental protocols to investigate receptor trafficking (insertion and internalization) at the cell surface. Before these treatments, control experiments were conducted to ensure biotinylation efficiency of surface membrane proteins. To determine that only cell surface proteins were labeled with the membrane-impermeable biotin, and that labeled protein fractions contained no cytoplasmic proteins, Western blot analysis of biotinylated (Fig. 1*A*) and cytoplasmic (Fig. 1*B*) proteins was used to test for the presence of membrane and cytoplasmic markers. The biotinylated fraction (Fig. 1*A*) contained the membrane localized Na^+/K^+-ATPase, but this protein was not detected in the cytoplasmic fraction (Fig. 1*B*). The cytoplasmic marker β-galactosidase was not found in the biotinylated fraction compared with the cytoplasmic fraction (Fig. 1*B*). The biotinylated fraction did contain a light β-galactosidase-immunoreactive band; however, this is likely due to a low level of membrane localized β-galactosidase (Aureli et al., 2009). These findings demonstrate that biotinylated fractions contain proteins that were localized to the plasma membrane and were not contaminated with cytoplasmic proteins.

To check for unspecific protein binding during surface biotinylation experiments, cells that were not labeled with biotin were processed along with biotinylated samples. Coomassie Blue stained SDS polyacrylamide gels revealed that nonbiotinylated samples did not contain detectable levels of bound unspecific protein (Fig. 1*C*). Stained gels also revealed that biotinylated samples were equally loaded onto SDS polyacrylamide gels (Fig. 1*C*).

For internalization experiments, we tested whether surface biotinylated proteins would be internalized by estradiol stimulation. Neurons were surface biotinylated, stimulated and then treated with MESNA, a membrane-impermeable reducing agent, to strip away the sulfo-NHS-SS-biotin label from proteins on the cell surface. The presence of any remaining biotinylated proteins not reduced by MESNA indicated they were no longer on the cell surface and it was assumed they were internalized. To test the efficiency of biotin stripping, two controls were done: first, non-stripped controls detected the total amount of biotinylated protein on the plasma membrane (Fig. 1*D*); second, vehicle stimulated neurons did not contain biotinylated proteins, suggesting that MENSA effectively removed (first lane, Stripped) the biotin label from cell surface proteins (Fig. 1*D*).

To examine the specificity of the ERα antibody MC-20, a series of controls were conducted in rat and mouse hypothalamic, uterine, and ovarian tissue. MC-20 is a polyclonal antibody directed toward amino acids on the COOH-terminal (a.a. 579–599) of mouse nuclear ERα protein (Clarke et al., 2000). Western blot analysis of the cytoplasmic proteins showed full-length 66 kDa ERα as well as several ERα-immunoreactive proteins (Fig. 2). In hypothalamic tissue, in addition to the 66 kDa ERα band other ERα-immunoreactive bands (~52, 73, 78, 100, 110, and 125 kDa) were revealed before the detection of full-length ERα (Fig. 2*A*). In the uterus, while full-length ERα was not seen using the MC-20 antibody, the ovary did contain 66 kDa ERα as well as other ERα-immunoreactive proteins seen in hypothalamic tissue (Fig. 2*A*).

Surface biotinylation revealed several ERα-immunoreactive proteins on the plasma membrane of hypothalamic neurons (Fig. 2*B*). The primary immunoreactive protein identified with the

MC-20 antibody migrated at ~52 kDa (Fig. 2*B1*). When Western blot autoradiographic films were exposed to ECL-treated immunoblots for ~2 h, full-length 66 kDa ERα (Fig. 2*B2*) was revealed along with other ERα-immunoreactive proteins (~46, 78, and 125 kDa). Biotin labeling of these proteins indicated they were present in the plasma membrane. Biotinylation of protein occurs when biotin covalently attaches to primary amines (e.g., lysine) present within the protein sequence, so membrane ERα labeled by membrane-impermeable biotin suggests that a portion of the receptor is exposed to the extracellular space. Although no transmembrane spanning domain has been described for ERs, the ligand-binding domain (E/F domain) has been predicted to insert into the plasma membrane due to hydrophobic amino acids (Kumar et al., 1986).

Western blot analysis of hypothalamic tissue from ERαKO mice suggests the 52 kDa protein is derived from the same gene (*Esr1*) as full-length ERα (Fig. 2*C*). In wild-type mice, the 52 kDa and 66 kDa ERα proteins are present in hypothalamic tissue, while in homozygous ERαKO (*Esr1*$^{-/-}$) mice both proteins are absent. It is interesting to note that a small amount of ERα-immunoreactivity was detected in homozygous ERαKO mice, possibly representing other unidentified ERα variants (Shao et al., 2007). Preabsorption of the MC-20 antibody with a peptide corresponding to the epitope against which the antibody was generated, a.a. 579–599 of ERα, blocked staining of all the ER-immunoreactive bands (Fig. 2*D*). The presence of the 66 kDa ERα in hypothalamic, uterine, and ovarian tissue was verified using the NH_2-terminally directed antibody H-184 (epitope: ERα a.a. 2–185; Fig. 2*E*). In contrast to MC-20 staining, H-184 also labeled various ERα-immunoreactive proteins but it did not detect the 52 kDa ERα-immunoreactive protein (Fig. 2*E*).

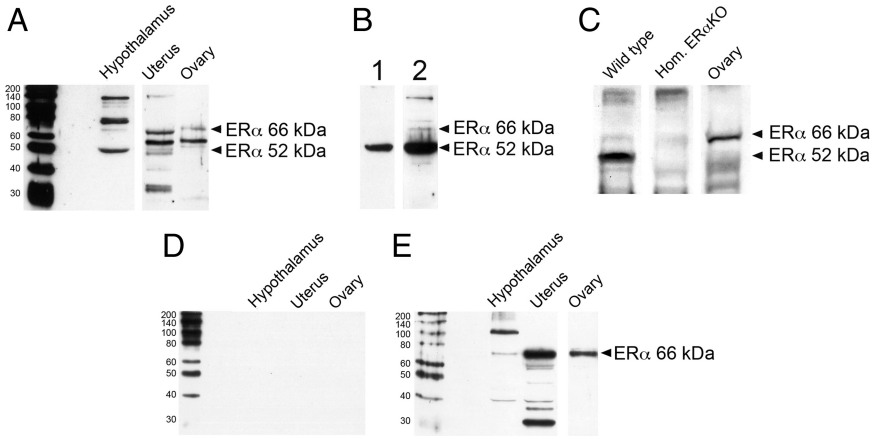

Figure 2. Western blot analysis and identification of ERα protein in tissue. Tissue was taken from PND9 rat pups and pregnant dames and prepared for Western blot analysis. ***A***, Western blot analysis of the cytoplasmic proteins from hypothalamic, uterine, and ovarian tissue using antibody MC-20 revealed a 66 kDa ERα and several other ERα-immunoreactive bands. ***B***, Western blot analysis of surface biotinylated hypothalamic neuronal cultures. A primary immunoreactive band at ~52 kDa appeared on autoradiographic films exposed for short periods (B1; 2 min), longer exposed films (B2; > 2 h) detected full-length 66 kDa ERα on the plasma membrane of hypothalamic neurons. ***C***, Western blot analysis of mouse wild-type and homozygous ERαKO hypothalamic tissue. In female wild-type animals, both 52 kDa and 66 kDa ERα-immunoreactive bands were detected, but both were missing in ERαKO. ERα-immunoreactivity was observed in ERαKO. Wild-type female ovary was used as a control. ***D***, Preabsorption of antibody MC-20 with the blocking peptide prevented ERα-immunodetection. ***E***, Analysis of hypothalamic, uterine, and ovarian tissue with antibody H-184 also detected a 66 kDa ERα as well as other ERα-immunoreactive bands.

Estradiol stimulation increased the level of membrane ERα on the cell surface of hypothalamic neurons

Western blot analysis of biotinylated proteins using MC-20 detected the expression of the major ERα-immunoreactive band at 52 kDa and full-length 66 kDa ERα. We then tested how stimulation of these ERα proteins changed their membrane levels. Estradiol (1 nM) stimulation rapidly increased the insertion of the 52 kDa (ANOVA $F_{(4,30)} = 3.025$, $p = 0.033$) and 66 kDa (ANOVA $F_{(4,15)} = 3.27$, $p = 0.041$) ERα proteins in the plasma membrane of hypothalamic neurons (Fig. 3). Both ERα protein levels reached a significant level in the membrane after 30 min of estradiol treatment. The increase was transient and by 60 min of estradiol treatment, ERα protein levels were elevated but not significantly different from control levels (Fig. 3). Across the time points of the experiment, levels of both ERα proteins remained stable in the cytoplasmic protein fractions (data not shown), suggesting that changes in membrane levels were not directly due to transcriptional downregulation of ERα.

Estradiol induces internalization of membrane ERα

In the second set of experiments, hypothalamic neurons were surface biotinylated and then treated with estradiol to determine whether membrane ERα was internalized. Estradiol (1 nM) rapidly increased the accumulation of biotinylated 52 kDa (ANOVA $F_{(4,25)} = 8.581$, $p = 0.0002$) and 66 kDa (ANOVA $F_{(4,15)} = 4.287$, $p = 0.0164$) ERα proteins in the cytoplasm, indicating that treatment with estradiol facilitated the removal of both ERα variants from the cell surface (Fig. 4). Similar to the experiments that showed ERα insertion into the membrane, the major ERα-immunoreactive band was at 52 kDa and full-length ERα at 66 kDa (Fig. 4). However, 52 kDa ERα protein became internalized after 5 min of exposure to estradiol. In each case, levels of 52 kDa and 66 kDa ERα was significantly increased after 30 min of estradiol treatment but the time course of internalization extended longer than the insertion of the receptor. At 60–120 min, internalization of 52 kDa ERα was still significantly greater than the basal level. This suggest that the two processes, insertion and internalization, are not coupled and may be regulated under different mechanisms.

Estradiol stimulation of membrane ERα-induced mGluR1a trafficking

Membrane-initiated estradiol signaling in the hypothalamus and hippocampus has been shown to be dependent on an ERα transactivation of mGluR1a (Boulware et al., 2005; Dewing et al., 2007, 2008). To determine whether estradiol treatment changed the level of mGluR1a protein in the plasma membrane, cell surface biotinylation was used. We could not demonstrate a significant estradiol-induced increase in membrane mGluR1a levels ($p = 0.1829$). As with ERα, peak insertion (~65%) occurred 30 min after hormone treatment (Fig. 5*A*). Interestingly, estradiol increased the level of biotinylated mGluR1a protein (internalization) in the cytoplasmic fraction (Fig. 5*B*), with the greatest level of internalization observed after 30–60 min of steroid treatment (ANOVA $F_{(4,20)} = 5.72$, $p = 0.0031$). This suggests that activating ERα also induced mGluR1a internalization, an observation consistent with the ERα transactivation of mGluR1a.

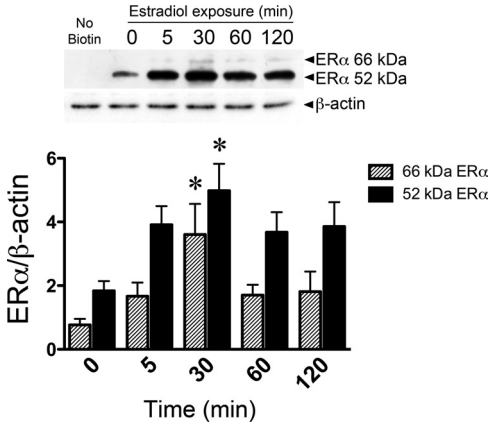

Figure 3. Estradiol stimulation of membrane ERα increased its insertion into the plasma membrane. Hypothalamic neuronal cultures were treated with 1 nM estradiol over time and surface biotinylated. Western blot analysis of surface biotinylated hypothalamic neurons using antibody MC-20 revealed the presence of 52 kDa and 66 kDa ERα (top). Control cultures were incubated without biotin (No Biotin) and processed similar to biotinylated samples. Equal loading of biotinylated samples was determined by probing cytoplasmic fraction blots with an β-actin antibody (bottom). The bar graph depicts that estradiol stimulation rapidly increased the amount of biotinylated ERα on the plasma membrane. A maximum of both 52 kDa and 66 kDa ERα proteins was reached after 30 min, after which concentrations returned toward basal levels (60–120 min). Bars (mean ± SEM) represent the ratio of either 52 kDa or 66 kDa ERα-immunoreactive band optical density value divided by the corresponding β-actin value and normalized (Tukey's *post hoc*, $*p < 0.05$; $n = 7$; time = 0 vs 30 min).

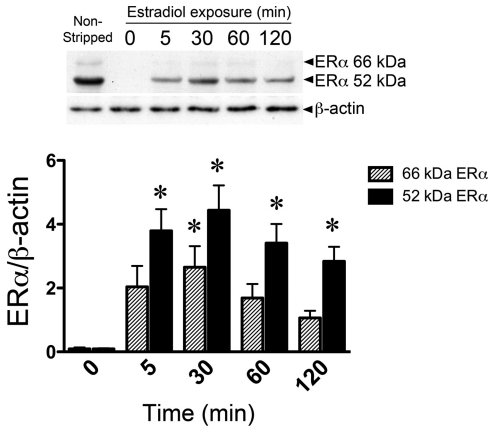

Figure 4. Stimulation of membrane ERα induced internalization of the receptor in hypothalamic neurons. Hypothalamic neuronal cultures were surface biotinylated and treated with 1 nM estradiol over time. Western blot analysis using antibody MC-20 revealed the presence of 52 kDa and 66 kDa ERα (top). Cultures were incubated with membrane-impermeable MESNA to reduce and strip biotin away from cell surface proteins; control cultures were not stripped (Non-Stripped). Equal loading of biotinylated samples was determined by probing cytoplasmic fraction blots with a β-actin antibody (bottom). The bar graph shows that estradiol stimulation of 52 kDa and 66 kDa ERα resulted in the rapid (5 min) accumulation of the receptors in the cytoplasm, with a maximum at 30 min and remaining significant 60–120 min after steroid exposure. The bars (mean ± SEM) represent the ratio of the 52 kDa and 66 kDa ERα-immunoreactive band optical density value divided by the corresponding β-actin value and normalized (Tukey's *post hoc*, $*p < 0.05$; $n = 6$; time = 0 vs 5, 30, 60, 120 min).

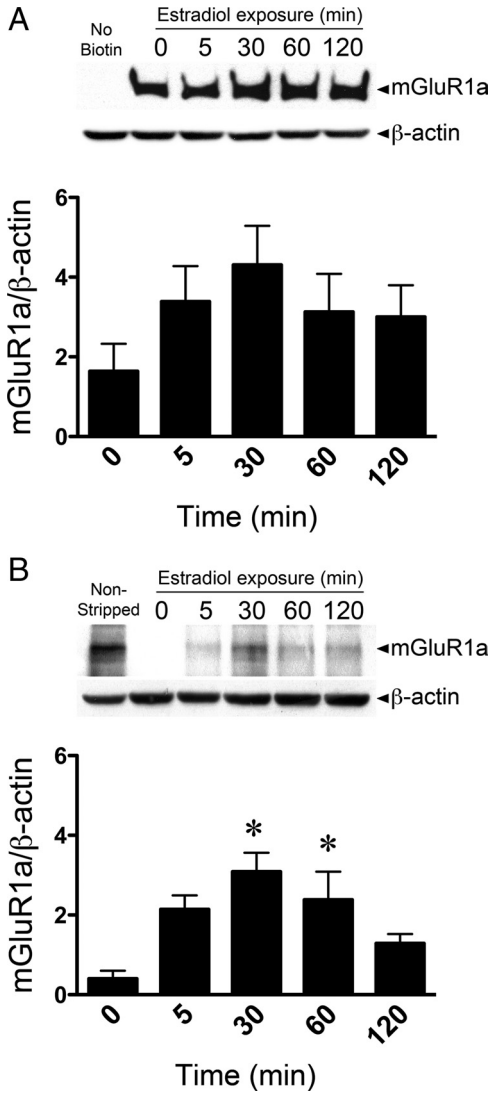

Figure 5. Activation of membrane ERα initiated the trafficking of mGluR1a on hypothalamic neuron plasma membranes. ***A***, Hypothalamic neuronal cultures were treated with 1 nM estradiol for the times indicated (0–120 min) and surfaced biotinylated. Western blot analysis (***A***, top) using an mGluR1a antibody revealed the presence of a 140 kDa immunoreactive band on plasma membranes. Control cultures were incubated without biotin (No Biotin) and processed similar to biotinylated samples. ***B***, Hypothalamic neuronal cultures were biotinylated and treated with 1 nM estradiol for the times indicated (0–120 min). Cultures were incubated with membrane-impermeable MESNA to reduce and strip biotin away from cell surface proteins; control cultures were not stripped (Non-Stripped). Western blot analysis (***B***, top) using an mGluR1a antibody revealed the rapid accumulation of the 140 kDa protein in the cytoplasm with a maximum at 30–60 min. Levels returned toward basal between 60 and 120 min. Equal loading of biotinylated samples was determined by probing cytoplasmic fraction blots with a β-actin antibody (***A*** and ***B***, bottom). Bars (mean ± SEM) represent the ratio of mGluR1a-immunoreactive band optical density value divided by the corresponding β-actin value and normalized (Tukey's *post hoc*, $*p < 0.05$; $n = 5$; time = 0 vs 30, 60 min).

Discussion

Estradiol is an extracellular messenger that activates membrane ERs to initiate cell signaling and activates intracellular ERs to directly modulate gene transcription. Because a number of molecules appear to bind estradiol, the identity of the ER(s) involved in membrane-initiated signaling remains unsettled. A preponderance of evidence, however, suggests that membrane ERs are products of the same genes as intracellular ERα and ERβ (Razandi et al., 1999). These ER proteins transduce membrane-initiated estradiol signaling events which affect cellular physiology and ultimately gene transcription (Micevych and Dominguez, 2009). Other endogenously expressed estradiol-binding proteins such as STX-binding protein or ER-X may also be involved, but whether they are products of ERα or ERβ genes is unknown (Razandi et al., 1999; Toran-Allerand et al., 2002; Revankar et al., 2005; Dominguez-Salazar et al., 2006; Qiu et al., 2008). In terms of female rodent sexual reproduction, ERα in the hypothalamus is crit-

ical (Moffatt et al., 1998; Kudwa and Rissman, 2003; Micevych et al., 2003; Wintermantel et al., 2006).

The present results demonstrate that ERα is biotinylated on the surface of hypothalamic neurons. This supports various studies with membrane-impermeable estradiol constructs that indicate the presence of a cell surface ER (Mermelstein et al., 1996; Chaban et al., 2003; Qiu et al., 2003; Dewing et al., 2007). Moreover, the major immunoreactive membrane ERα protein is not the full-length 66 kDa ERα but rather the 52 kDa protein. However, this may not be due to the difference between the levels of the 52 or 66 kDa ERα but rather due to the affinity of MC-20 for these proteins. Similarly, a number of ERα-immunoreactive proteins have also been identified in mammary tissue (Bollig and Miksicek, 2000; Herynk and Fuqua, 2004).

Membrane ERα and ERβ interact with other membrane receptors to initiate signaling, including various growth factor and neurotransmitter receptors (Carrer and Cambiasso, 2002; Quesada and Etgen, 2002; Evinger and Levin, 2005; Micevych and Dominguez, 2009). Our laboratory has demonstrated that estradiol induction of female sexual receptivity is dependent on transactivation of mGluR1a by activated ERα (Dewing et al., 2007, 2008; Kuo et al., 2009a,b). In our present study, membrane ERα and mGluR1a on hypothalamic neurons were rapid and transiently internalized within minutes of estradiol stimulation. The regulation of membrane ERα and mGluR1a protein levels by trafficking could serve as a method through which hypothalamic neurons regulate their response to estradiol, like other cell surface receptors.

As with other membrane receptors, ER levels are modulated by a series of complex processes that involve the control of expression and posttranscriptional modifications (Francesconi et al., 2009). Perhaps the most dramatic is the trafficking of receptors. Desensitization, a well documented process (Sinchak and Micevych, 2003), involves internalization of membrane receptors following agonist stimulation. Receptors are internalized during desensitization when endocytotic vesicles form and fuse with endosomes, where the ligand and receptor are uncoupled and sorted to be recycled or degraded.

A hallmark of insertion and internalization is the formation of pits on the plasma membrane. Estradiol treatment has been reported to induce pit formation on the membranes of hypothalamic neurons (Garcia-Segura et al., 1987; Olmos et al., 1987). Moreover, membrane ERα is associated with caveolae, lipid rich microdomains that cluster intracellular signaling proteins (Luoma et al., 2008), and clathrin-coated vesicles (Moats and Ramirez, 2000; Sreeja and Thampan, 2004). Such pits may represent the fusion of exocytotic vesicles delivering ERα to the membrane and endocytotic pits responsible for receptor internalization (Garcia-Segura et al., 1987; Olmos et al., 1987; Párducz et al., 1996; Moats and Ramirez, 1998).

Our present and previous results (Bondar et al., 2009) indicate an estradiol-mediated mechanism of insertion into the membrane. Localization of membrane ERα requires palmitoylation and the association with caveolin proteins. Both are required for association with mGluRs and insertion into the plasma membrane (Razandi et al., 2002; Acconcia et al., 2005; Boulware et al., 2007; Luoma et al., 2008). One possibility is that estradiol may regulate the level of palmitoylation of ERα and its association with caveolin. This idea is supported by the observation that estradiol treatment induced the rapid incorporation of ^{3}H-palmitic acid into ERα protein (Acconcia et al., 2005). After 2 h, estradiol decreased the interaction between ERα and caveolin-1, a finding congruent with a decrease in membrane ERα localization (Acconcia et al., 2005; Pedram et al., 2007). The time course of ERα trafficking in the present experiments is very similar to the time course reported in previous studies, implying the events are linked.

Estradiol regulated the internalization of membrane ERα in hypothalamic neurons. Membrane receptor internalization following agonist stimulation is regulated by β-arrestins and G-protein-coupled receptor kinases (GRKs). GRK phosphorylation desensitizes receptors and catalyzes their interaction with β-arrestins, which directs receptors toward endosomes (Marchese et al., 2008). Consistent with this idea, estradiol has been reported to rapidly activate GRK2 and recruit β-arrestin-1 to ERα in cortical neurons (Dominguez et al., 2009). Internalization of activated membrane ERα has been suggested to be mediated by a β-arrestin mechanism.

An interesting parallel between membrane and intracellular ERs is the estradiol modulation of cognate receptors in different cellular compartments. Estradiol downregulates intracellular ER protein and mRNA levels (Lauber et al., 1990; Zhou et al., 1993; Alarid et al., 1999). In the present experiments, peak insertion into the membrane and internalization took place within an hour of estradiol treatment. This occurred without an observable change in ERα levels in the cytoplasmic fraction. This suggests that our observations at the membrane are related to trafficking of ERα and degradation rather than decreased synthesis of the protein. We expect, however, that long-term estradiol treatment may result in a decrease in transcription and decrease in total cellular ER levels as has been reported (Pinzone et al., 2004).

Studies in other cells have observed the movement of ERs to or from the plasma membrane. Similar to our experiments, a number of ERα variants were identified (Karthikeyan and Thampan, 1996; Stirone et al., 2003; Gorosito et al., 2008; Liu et al., 2008; Dominguez et al., 2009). Of particular interest were the studies of Gorosito et al. (2008), which used cell surface biotinylation of neurons to identify an ERα-immunoreactive protein with an apparent molecular weight (MW) in the low 50 kDa range. Similar to our results here and in astrocytes, vascular epithelial and endothelial cells have identified ERα variants as the major membrane ER protein, where as the levels of the 66 kDa protein are low and require a lengthy autoradiographic film exposure to detect full-length ERα (Li et al., 2003; Stirone et al., 2003; Bondar et al., 2009). The present study demonstrated that hypothalamic neurons, like other cell types, have a number of ERα splice variants (Bollig and Miksicek, 2000; Li et al., 2003; Gorosito et al., 2008; Ishunina and Swaab, 2008; Dominguez et al., 2009).

Numerous ERα mRNA splice products have been identified in brain and other tissues (Friend et al., 1995; Bollig and Miksicek, 2000; Poola et al., 2002; Moriarty et al., 2006; Al-Bader et al., 2008). During alternative RNA splicing, exons are deleted or duplicated, resulting in a family of ERα proteins (Herynk and Fuqua, 2004; Perlman et al., 2005). Such proteins have been identified using specific NH_2- and COOH-terminal directed ERα antibodies, but mass spectrometry studies (MALDI-TOF) have not detected these ERα variants in the membrane (Razandi et al., 2003; Heberden et al., 2006; G. Bondar and R. Dominguez, unpublished results). In the hypothalamus, splice variants have been suggested to participate in membrane-initiated estradiol signaling (Shughrue et al., 2002; Dominguez-Salazar et al., 2006). It is unclear what role splice variants play; however, studies report that the most common splice variant, ERαΔ7, which is missing the ligand-binding domain, is a dominant-negative protein (Couse et al., 1995; Bollig and Miksicek, 2000; Kos et al., 2002; Shughrue et al., 2002; Nethrapalli et al., 2005; Dominguez-

Salazar et al., 2006). At present, it is difficult to ascertain which of the ERα-immunoreactive proteins identified in our experiments are the products of alternative splicing. The predominant membrane ERα-immunoreactive protein in our study migrates at ~52 kDa, an MW similar to that of the ERαΔ7 (52 kDa) and the ERαΔ4 (54 kDa), which have been identified in the brain (Fuqua et al., 1992; Bollig and Miksicek, 2000; Deecher et al., 2003; Perlman et al., 2005). Both ERαΔ7 and ERαΔ4 express exon 1 and 2, however in our study H-184 did not detect proteins of either MW in hypothalamic tissue. Similar results have been reported in uterine, ovary, and cell lines (Deecher et al., 2003; Heberden et al., 2006; Shao et al., 2007). Currently, the identity of the 52 kDa ERα remains unknown. In ERαKO astrocytes, both the 52 and the 66 kDa ERα were not expressed, suggesting they are derived from the ERα gene (Bondar et al., 2009). Regardless of the identity of the 52 kDa ERα-immunoreactive protein, membrane-initiated estradiol signaling dependent on mGluR1a requires full-length 66 kDa ERα (Boulware et al., 2005; Dewing et al., 2007, 2008). Thus, the function of the 52 kDa ERα in the membrane is an open question.

Our findings that ERα is present on the plasma membrane and that estradiol stimulation rapidly and transiently induced receptor trafficking suggest that membrane ERα is rapidly regulated by its ligand, which may explain how membrane-initiated estradiol actions are self-limiting. Further studies are needed to elucidate the mechanism underlying these actions.

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