Cellular/Molecular

Estradiol-Induced Estrogen Receptor-α Trafficking

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Estradiol has rapid actions in the CNS that are mediated by membrane estrogen receptors (ERs) and activate cell signaling pathways through interaction with metabotropic glutamate receptors (mGluRs). Membrane-initiated estradiol signaling increases the free cytoplasmic calcium concentration ($[Ca^{2+}]_i$) that stimulates the synthesis of neuroprogesterone in astrocytes. We used surface biotinylation to demonstrate that ERα has an extracellular portion. In addition to the full-length ERα [apparent molecular weight (MW), 66 kDa], surface biotinylation labeled an ERα-immunoreactive protein (MW, ~52 kDa) identified by both COOH- and NH_2-directed antibodies. Estradiol treatment regulated membrane levels of both proteins in parallel: within 5 min, estradiol significantly increased membrane levels of the 66 and 52 kDa ERα. Internalization, a measure of membrane receptor activation, was also increased by estradiol with a similar time course. Continuous treatment with estradiol for 24–48 h reduced ERα levels, suggesting receptor downregulation. Estradiol also increased mGluR1a trafficking and internalization, consistent with the proposed ERα–mGluR1a interaction. Blocking ER with ICI 182,780 or mGluR1a with LY 367385 prevented ERα trafficking to and from the membrane. Estradiol-induced $[Ca^{2+}]_i$ flux was also significantly increased at the time of peak ERα activation/internalization. These results demonstrate that ERα is present in the membrane and has an extracellular portion. Furthermore, membrane levels and internalization of ERα are regulated by estradiol and mGluR1a ligands. The pattern of trafficking into and out of the membrane suggests that the changing concentration of estradiol during the estrous cycle regulates ERα to augment and then terminate membrane-initiated signaling.

Introduction

Estradiol mediates numerous actions in the nervous system ranging from regulation of reproduction to learning and memory, and neuroprotection. The effects of estradiol on the brain involve both classical nuclear- and membrane-initiated actions. Nuclear-initiated actions are mediated through intracellular estrogen receptor-α (ERα) and ERβ that behave as ligand-activated transcription factors. Evidence suggests that the same molecules activate ERα- and ERβ-mediated nuclear- and membrane-initiated signaling. Both receptors have been demonstrated with a variety of methods, including immunoreactivity, Western blotting, and pharmacology, to mediate membrane-initiated steroid signaling (MISS) in neurons and glia (Lagrange et al., 1995, 1996; Ivanova et al., 2001; Kelly and Levin, 2001; Wade et al., 2001; Chaban et al., 2004; Acconcia et al., 2005; Pawlak et al., 2005; Mhyre and Dorsa, 2006; Pedram et al., 2006; Hirahara et al., 2009; for review, see Micevych and Dominguez, 2009; Sakuma et al., 2009). MISS activates G-protein-dependent cell signaling cascades (Hammes and Levin, 2007), including activation of the mitogen-activated protein kinase pathway, activation of protein kinase C (PKC), increase in $[Ca^{2+}]_i$, and phosphorylation of cAMP-responsive element binding protein (CREB) (Dewing et al., 2007, 2008; for review, see Kelly and Ronnekleiv, 2008). MISS acting through pCREB increases the expression of reproductive neuropeptides (Watters and Dorsa, 1998).

The estradiol-activated MISS depends on the transactivation of metabotropic glutamate receptors (mGluRs) by membrane ERα (Boulware et al., 2005, 2007; Dewing et al., 2007, 2008; Micevych and Mermelstein, 2008), which also activates PKC, increases $[Ca^{2+}]_i$, and phosphorylates CREB (Boulware et al., 2005, 2007; Dewing et al., 2007, 2008; Zhao and Brinton, 2007; for review, see Micevych and Mermelstein, 2008). In astrocytes, coimmunoprecipitation studies demonstrate a direct interaction of 66 kDa ERα with the mGluR1a, and blockade of the mGluR1a prevents the estradiol-induced $[Ca^{2+}]_i$ flux needed to stimulate the synthesis of neuroprogesterone (Micevych et al., 2007; Kuo et al., 2009). In addition to the ERα–mGluR1a interaction, when ERα interacts with the mGluR2/3, it inhibits adenylyl cyclase-dependent calcium influx through L-type voltage-gated calcium channels. These variable interactions with mGluRs account for both the facilitatory and inhibitory effects of estradiol (Chaban et al., 2003; Li et al., 2008; Mermelstein and Micevych, 2008).

We examined the regulation of membrane ERα by estradiol using surface biotinylation in astrocytes (Busch et al., 1989; Brandli et al., 1990; Lin et al., 2000; Sabarth et al., 2002; Gorosito et al., 2008). Membrane proteins including ERα were biotinylated by a membrane-impermeable reagent that prevented labeling of intracellular proteins. Exposure to estradiol for 5 min transiently increased 66 and 52 KDa ERα membrane levels and their internalization. This receptor trafficking was dependent on mGluR1a. The estradiol-induced $[Ca^{2+}]_i$ flux coincided with the maximal internalization/activation of ERα levels. Continuous

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exposure to estradiol eventually reduced both membrane ERα and its internalization to basal levels, suggesting downregulation of the receptor. Together, these experiments demonstrate an autoregulation of estradiol signaling in astrocytes that may represent a common signaling mechanism in the CNS.

Materials and Methods

Animals and cultures. Primary hypothalamic astrocyte cultures were prepared from 40-d-old Long–Evans female rats (Charles River) and from 60-d-old female mice [C57BL/6 wild-type (WT) and ERα knock-out (ERαKO); a gift from Dr. D. Lubahn, University of Missouri, Columbia, MO] as described previously (Micevych et al., 2007). All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California at Los Angeles. Briefly, a hypothalamic block was isolated with the following boundaries: the rostral extent of the optic chiasm, rostral extent of the mammillary bodies, lateral edges of the tuber cinereum, and top of the third ventricle. Hypothalamic tissue was dissociated with 2.5% trypsin (Invitrogen) and a fire-polished glass Pasteur pipette. Cultures were maintained at 37°C with 5% CO_2 and grown in DMEM/F-12 medium (Mediatech) with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin–streptomycin (PS; Mediatech) for 14 d.

Biotinylation. Astrocytes were pretreated for 12 h with 5% charcoal-stripped FBS DMEM/F-12 medium and incubated with vehicle or in the presence 1 nM 17β-estradiol (Sigma-Aldrich) for 0, 5, and 30 min and 1, 2, 24, and 48 h at 37°C. Cells then were washed three times with ice-cold PBS buffer and incubated with freshly prepared 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS at 4°C for 30 min with gentle agitation. Excess biotin reagent was quenched by rinsing the cells three times with ice-cold glycine buffer (50 mM glycine in PBS). Then the cells were scraped into 10 ml of PBS solution, transferred into a 50 ml conical tube, and centrifuged at 500 × *g* for 3 min. The pellet was washed twice with ice-cold PBS and resuspended in 200 ml of RIPA Lysis Buffer (Santa Cruz Biotechnology) containing the following proteases inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM sodium orthovanadate (all inhibitors were from Santa Cruz Biotechnology). The cells were homogenized by passing them through a 25 gauge needle. The cell extract was centrifuged at 10,000 × *g* for 2 min at 4°C, and the protein concentration of the supernatant was determined using the Bradford Assay (Bio-Rad). Two hundred microliters of each sample with 1500 μg/ml protein concentration (rat astrocytes) or 950 μg/ml protein concentration (mouse astrocytes) was incubated with 200 μl of Immobilized NeutrAvidin Gel (Pierce) for 2 h at room temperature and spun for 1 min at 1000 × *g*. The beads were washed four times with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology) containing the same proteases inhibitors mentioned previously. The bound proteins were eluted with SDS-PAGE sample buffer supplemented with 50 mM dithiothreitol (DTT) for 1 h at 37°C.

Internalization. Internalization of ERα was analyzed using a reversible biotinylation technique and was modified from the methods described previously. Cells were preincubated for 12 h with charcoal-stripped medium and cooled to 4°C by washing three times with ice-cold PBS. Then cells were biotinylated with freshly prepared 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin in PBS for 30 min at 4°C, and the free biotinylating reagent was quenched with glycine buffer. Cells were incubated with 1 nM 17β-estradiol for 0, 5, and 30 min and 1, 2, 24, and 48 h. At the end of each time point, the reagents were removed, and the cells were washed with prechilled medium. The remaining biotinylated proteins on the cell surface were stripped with glutathione buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH in H_2O). Cells were then washed twice for 15 min at 4°C with iodoacetamide buffer (50 mM iodoacetamide in PBS, pH 7.4) to quench residual glutathione. The internalized proteins labeled with biotin were protected from stripping, and the subsequent analysis provided an assay for protein internalization. For isolation, the biotinylated proteins in the supernatant of the cell lysate were mixed with Immobilized NeutrAvidin Gel (Pierce). To define the total amount of biotinylated receptors, one flask was not stripped with glutathione buffer and directly processed for extraction, followed by isolation with NeutrAvidin Gel. After incubation for 2 h at room temperature, the beads were washed four times with RIPA buffer (Santa Cruz Biotechnology) and eluted with SDS-PAGE sample buffer supplemented with 50 mM DTT for 1 h at 37°C. All the proteins were resolved by SDS-PAGE.

To study ER trafficking to the membrane, astrocytes were (1) treated with 1 nM β-estradiol 6-(*O*-carboxy-methyl) oxime BSA (E-6-BSA; Sigma-Aldrich) for 0, 5, and 30 min and 1, 2, 24, and 48 h before biotinylation to test whether ERα trafficking required estradiol access to the cell interior; (2) incubated with the ER antagonist ICI 182,780 (1 μM; Tocris) for 1 h before and during the treatment with 1 nM estradiol to confirm that activation of ERα is essential for estradiol signaling; and (3) incubated with the mGluR1a antagonist LY 367385 (50 μM; Tocris) for 1 h before and during the treatment with estradiol to confirm the necessity of ERα–mGluR1a interaction in ERα trafficking. The plasma membrane and internalized concentrations of the respective proteins were determined.

Western blotting. Samples were separated on 10% Tris-HCl Ready Gels (Bio-Rad), and the proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare). For determination of the molecular weight of the proteins, samples were run with Biotinylated Protein Ladder (Cell Signaling). To detect the COOH terminal of ERα, a rabbit polyclonal ERα antibody (C1355; 1:1000; Millipore) and a rabbit polyclonal ERα antibody (MC-20; 1:1000; Santa Cruz Biotechnology) were used. To detect the NH_2 terminal of ERα, a rabbit polyclonal ERα antibody (ab3574; 1:1000; Abcam) and a rabbit polyclonal ERα antibody (H-184; 1:1000; Santa Cruz Biotechnology) were used. For GPR30, a rabbit polyclonal GPR30 antibody (1:250; Abcam) was used. To detect the cytoplasmic protein LIM domain kinase 1 (LIMK1), a rabbit polyclonal LIMK1 antibody (1:1000; Abcam) was used. For mGluR1a, a rabbit polyclonal mGluR1a antibody (1:1000; Millipore) was used. β-Actin was used as a loading control and detected by a rabbit polyclonal β-actin anitbody (1:5000; Abcam). A secondary donkey anti-rabbit IgG (H+L) antibody (1:5000; Jackson ImmunoResearch) and a goat anti-biotin, HRP-linked antibody (1:1000; Cell Signaling) were then used (1.5 h incubation). Bands were visualized using an enhanced chemiluminescence (ECL) kit and ECL hyperfilm (GE Healthcare). Routine exposures varied from 0.5 to 2 min. For detection of the 66 kDa ERα band, the exposure time was 2 h.

Intracellular Ca^{2+} measurements. Primary astrocyte cultures were grown in DMEM/F-12 medium with 10% FBS and 1% PS. The astrocytes were washed with HBSS (Mediatech), dissociated with a 2.5% trypsin solution, and resuspended in 5 ml of DMEM/F-12 medium with 10% FBS. These cells were centrifuged for 3 min at 80 × *g*, plated onto poly-D-lysine (0.1 mg/ml; Sigma-Aldrich)-coated 15 mm glass coverslips, and incubated for 48–72 h *in vitro* before Ca^{2+} imaging. Cultures were routinely checked for purity using immunocytochemistry for glial fibrillary acidic protein (Millipore) and Hoechst 3342 (Sigma-Aldrich) nuclear stain. Cultures were determined to be >95% pure astrocytes as reported previously (Sinchak et al., 2003; Micevych et al., 2007; Kuo et al., 2009).

Before experimentation, astrocytes were either nonsteroid starved or steroid starved for 6 or 12 h by incubating in DMEM/F-12 medium with 5% charcoal-stripped FBS at 37°C. Astrocytes were loaded with the calcium indicator Fluo-4 AM (4.5 μM; Invitrogen), dissolved in dimethyl sulfoxide and methanol, and diluted in HBSS. The cells were then washed three times with HBSS to remove the excess Fluo-4 AM. For the calcium imaging after preexposure to estradiol, astrocytes were steroid starved for 12 h, loaded with Fluo-4 AM, and exposed to 1 nM cyclodextrin-encapsulated 17β-estradiol (Sigma-Aldrich) for 0, 5, and 30 min and 1 or 2 h before stimulation with 10 nM cyclodextrin-encapsulated 17β-estradiol in HBSS.

The coverslips containing astrocytes were mounted into a 50 mm RC-61T-01 chamber insert (Warner Instruments) fixed into a 60 × 15 mm cell culture dish (Corning) and placed into a QE-2 quick exchange platform (Warner Instruments). Test solutions were delivered by gravity perfusion through PE160 tubing and MP series perfusion manifold (Warner Instruments) and removed by vacuum suction at the opposite end of the perfusion chamber. Fluo-4 AM imaging was performed using an Axioplan2-LSM 510 Meta confocal microscope (Zeiss) with an IR-Achroplan 40×/0.80 water-immersion objective (Zeiss) with 488 nm

laser excitation and emission monitored through a low-pass filter with a cutoff at 505 nm.

Statistics. For the Western blotting of surface biotinylation/internalization experiments, the optical density of each immunoreactive band was determined. Levels of β-actin were compared to ensure uniformity in protein loading for each experiment. Immunoreactive ERα was normalized to β-actin for each sample and multiplied by 100 to obtain the percentage of protein ratio. Results are presented as SEM of at least four trials. The ratios were then compared with the 0 min time point as described below. For the Ca^{2+} imaging experiments, data are presented as mean ± SEM in relative fluorescent units (RFU). The change in relative Ca^{2+} fluorescence ($\Delta F\ Ca^{2+}$) was calculated as the difference between baseline fluorescence and peak response to drug stimulation. For all experimental results, statistical comparisons were performed with one-way ANOVA with the Student-Newman-Keuls *post hoc* test using SigmaStat 3.5 (Systat Software). For all experiments, differences at the $p < 0.05$ level were considered significant.

Results

Surface biotinylation

To determine whether ERα is associated with the plasma membrane (Chaban et al., 2004) and regulated at the cell membrane by estradiol, astrocytes were surface biotinylated. With the C1355 ERα antibody, which is directed toward the COOH terminal, two ERα-immunoreactive bands were observed: a major band at 52 kDa seen after a 2 min exposure and a lower-intensity band at 66 kDa seen after a 2 h exposure to film (Fig. 1). Both bands were also observed with MC-20, ab3574, and H-184 antibodies directed against the COOH and NH_2 terminals of ERα. Immunostaining for LIMK1, a cytoplasmic protein, was negative and verified the purity of the biotinylated sample. Antibodies directed against GPR30 were used to determine whether this putative membrane estradiol-binding protein was present in the membrane and could be surface biotinylated. However, no GPR30-immunoreactive bands were identified (Fig. 1*A*).

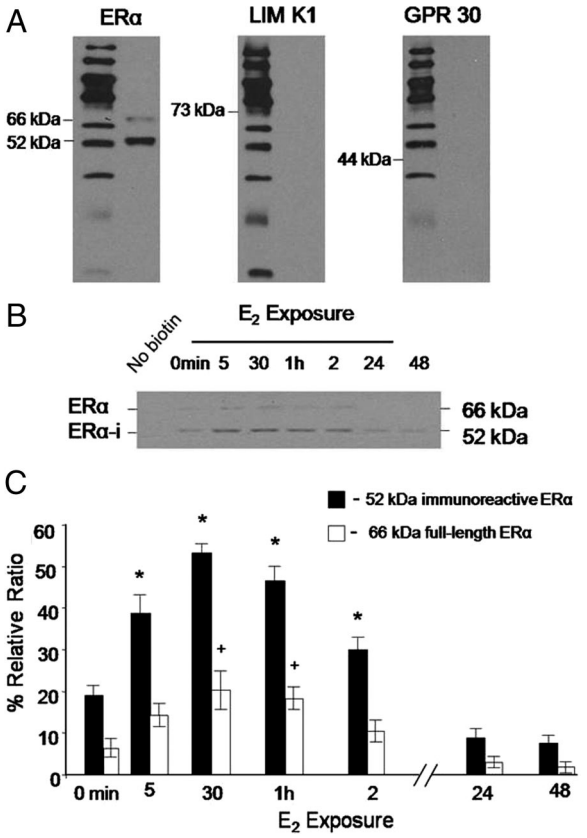

Figure 1. Postpubertal hypothalamic astrocytes were incubated with vehicle (0 min) or in the presence of 1 nM estradiol for 5 and 30 min and 1, 2, 24, and 48 h. Astrocytes were then biotinylated, the biotin was removed, and the labeled proteins were separated and detected with ERα, GPR30, and LIM K1 antibodies. ***A***, Two ERα-immunoreactive (ERα-i) bands were identified: 66 and 52 kDa. The cytoplasmic protein LIM K1 and the putative membrane estrogen receptor GPR30 were not labeled with surface biotinylation. ***B***, Estradiol treatment increased both the 66 and 52 kDa ERα-i bands. In the first lane, cells were not surface biotinylated (no biotin), thus no biotinylated ERα-i were labeled. Detection of the 66 kDA ERα required a 2 h exposure compared with a 1–2 min exposure for the 52 kDa ERα-i. ***C***, Quantification of the 66 and 52 kDa ERα-i bands was calculated by comparing optical density of the ERα-i bands with the optical density of the β-actin bands. Both 66 and 52 kDa ERα-i bands are regulated in parallel by estradiol treatment, but the amount of 66 kDa ERα was much less at each time point. Data are mean ± SEM ($n = 4$). $^{*,+}$Statistical differences at the $p < 0.05$ level compared with 0 min for each molecular-weight species. E_2, Estradiol.

ERαKO mouse experiment

Surface biotinylation using astrocytes from ERαKO mice demonstrated an absence of both the 66 and 52 kDa ERα bands in the plasma membrane. WT mice astrocytes revealed the same pattern of membrane ERα expression as rat astrocytes: a major 52 kDa band and a minor 66 kDa band. Both of these ERα-immunoreactive bands were not detectable in the ERαKO astrocytes (supplemental material, available at www.jneurosci.org).

Estradiol regulation of ERα trafficking

Estradiol transiently increased the amount ERα in the plasma membrane. Both the 66 and 52 kDa ERα bands responded in parallel to estradiol treatment and thus the 52 kDa ERα band was quantified (Figs. 1*B*,*C*, 2*A*). Initially, the levels of membrane ERα were low (19 ± 4%; $n = 4$), but estradiol treatment for 5 min significantly increased ERα levels to 38 ± 6% ($n = 4$; $p < 0.05$). Maximal membrane levels were seen at 30 min (54 ± 3%; $n = 4$; $p < 0.05$) of estradiol treatment and remained elevated for 1 h (42 ± 6%; $n = 4$; $p < 0.05$) before declining by 2 h (32 ± 3%; $n = 4$; $p < 0.05$). The levels of ERα were minimal at 24–48 h of estradiol treatment despite the continuous presence of estradiol (10 ± 3 and 9 ± 3%; $n = 4$; $p > 0.05$). Interestingly, the membrane estrogen receptor GPR30 was not detected by surface biotinylation and/or estradiol treatment (Fig. 2*A*).

Internalization is part of the mechanism of receptor desensitization and follows the binding of a natural ligand to its receptor. To test whether estradiol treatment would induce receptor internalization, cells were biotinylated and treated with estradiol for varying lengths of time, and then the biotin was stripped off the surface with glutathione. Therefore, only internalized ER would retain the biotin tag. Internalization paralleled the increase of ERα in the membrane. The amount of internalized ERα increased from 4 ± 2% ($n = 4$) at 0 min to 20 ± 3% at 5 min ($n = 4$; $p < 0.05$) and was maximal between 30 min and 1 h (47 ± 4 and 52 ± 4%, respectively; $n = 4$; $p < 0.05$). Although the amount of internalized ERα was still significantly elevated at the 2 h time point (35 ± 5%; $n = 4$; $p < 0.05$), it was beginning to decrease compared with its peak concentration. By 24 h, the levels of internalized receptor reached basal levels (10 ± 2%; $n = 4$; $p > 0.05$) (Fig. 2*B*) and remained low at the 48 h time point (12 ± 3%; $n = 4$; $p > 0.05$).

To test whether ERα trafficking to the membrane depends on membrane or intracellular actions of estradiol, membrane-impermeable E-6-BSA was used. Cells were incubated with 1 nM E-6-BSA for the experimental time course before biotinylation. Membrane-limited estradiol treatments did not change the levels of biotinylated 52 kDa ERα ($n = 4$; $p > 0.05$) (Fig. 3*A*). In fact,

levels throughout the course of the experiment differed by <5% and were not statistically different than the level measured at 0 min (28 ± 2%; $n = 4$) (Fig. 3*A*). These results demonstrate that intracellular estradiol is required to induce trafficking of the ERα to the membrane. Treatment with ICI 182,780 prevented ERα trafficking to the membrane and estradiol-induced internalization (Fig. 3*B*,*C*). As with E-6-BSA, ERα levels throughout the experiment did not change (Fig. 3*A*–*C*). Since GPR30 has been suggested to be associated with intracellular membranes, we tested the idea that estradiol was activating GPR30 to induce trafficking of ERα to the membrane. Astrocytes were incubated with the GPR30 agonist G-1. However, G-1 treatment failed to alter levels of biotinylated 52 kDa ERα in the membrane. The amount of surface-biotinylated ERα varied from 16 ± 2% (0 min) to 21 ± 3% at 1 h (data not shown). None of the levels of ERα were statistically different ($n = 4$; $p > 0.05$ vs 0 min).

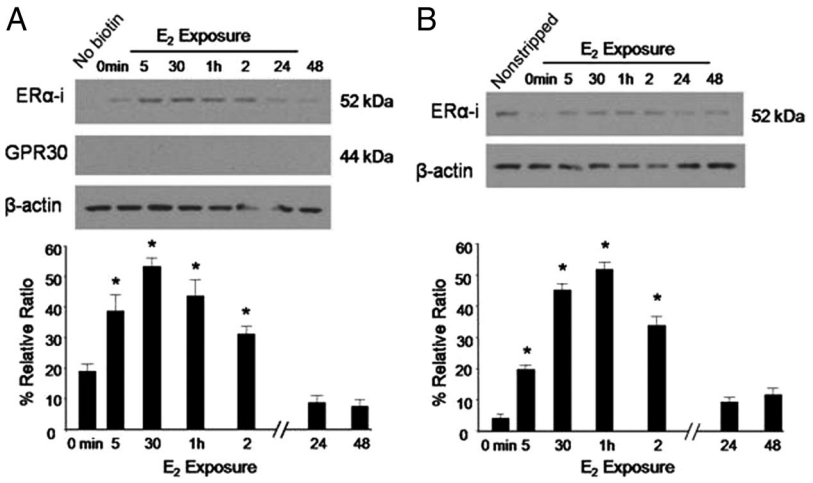

Figure 2. ***A***, Estradiol treatment transiently increases membrane ERα-immunoreactive (ERα-i) bands in postpubertal hypothalamic astrocytes. Basal levels of ERα-i bands were observed before estradiol treatment (0 min). These levels were rapidly increased (5 min time point) (*$p < 0.05$), with a maximum at 30 min (*$p < 0.05$) and a slight depression after 1 h (*$p < 0.05$), and remained elevated for 2 h of estradiol stimulation (*$p < 0.05$). After 24–48 h of estradiol treatment, ERα-i bands returned to basal levels ($p > 0.05$). GPR30 was not biotinylated on the surface despite incubating the astrocytes with estradiol for up to 48 h. ***B***, To track internalization, astrocytes were biotinylated and treated with estradiol, and the biotin was stripped from the surface of the cells with glutathione. Under these conditions, ERα-i bands are biotinylated, but after glutathione treatment, the only biotinylated receptors remaining are those that were internalized. The time course of internalization matched the time course of the estradiol-induced trafficking to the membrane. In the first lane, the biotin was not removed by glutathione (nonstripped). The amount of internalized ERα-i bands, with varying estradiol treatment, began increasing at 5 min (*$p < 0.05$) and reached its maximum at 30 min to 1 h (*$p < 0.05$). After 2 h of estradiol incubation, the level of internalized ERα decreased compared with the maximum but was still statistically significant from the 0 min time point (*$p < 0.05$). At the 24–48 h time points, internalized ERα-i levels reached basal levels comparable to 0 min ($p > 0.05$). All the data are mean ± SEM ($n = 4$). *Statistical differences at the $p < 0.05$ level compared with 0 min for each experiment. E_2, Estradiol.

mGluR1a- and ERα-immunoreactive trafficking

Previously, we and others have reported that ERα interacts with mGluR1a on the plasma membrane to initiate cell signaling (Boulware et al., 2005; Dewing et al., 2007, 2008; Micevych and Mermelstein, 2008; Kuo et al., 2009). We tested whether mGluR1a is also trafficked to the membrane by estradiol. After estradiol treatment, astrocytes were biotinylated, and mGluR1a levels were detected by Western blotting. Estradiol increased the levels of mGluR1a on the membrane (Fig. 4*A*), but the kinetics of mGluR1a trafficking to the membrane showed a delayed pattern compared with the estradiol-induced increase in ERα. A statistically significant increase of mGluR1a was first observed at the 30 min time point (56 ± 8%; $n = 4$; $p < 0.05$ vs 0 min) (Fig. 4*A*) compared with ERα, which was significantly increased at 5 min (Fig. 2*A*). Levels of mGluR1a remained elevated at 1 and 2 h of estradiol treatment (52 ± 7 and 48 ± 5%, respectively; $n = 4$; $p < 0.05$) and slowly decreased by 24 and 48 h (36 ± 8 and 34 ± 4%, respectively; $n = 4$; $p < 0.05$ vs 0 min) (Fig. 4*A*) but still remained elevated compared with basal levels. Treating the astrocytes with the mGluR1a antagonist LY 367385 prevented the estradiol-induced ERα trafficking to the membrane ($n = 4$; $p > 0.05$) (Fig. 4*B*). Similarly, LY 367385 treatment blocked ERα internalization regardless of the duration of estradiol treatment ($n = 4$; $p > 0.05$ vs 0 min) (Fig. 4*C*).

Steroid-starvation effects the $[Ca^{2+}]_i$ response in astrocytes

Estradiol induces a rapid and robust $[Ca^{2+}]_i$ flux in hypothalamic astrocytes (Micevych et al., 2007; Kuo et al., 2009). Furthermore, the $[Ca^{2+}]_i$ response to estradiol at concentrations between 1 and 100 nM are similar (Kuo et al., 2009). Without prior steroid-starvation, estradiol at 10 nM induced a moderate increase in $[Ca^{2+}]_i$ flux ($\Delta F\,Ca^{2+} = 339 \pm 18$ RFU; $n = 27$) (Fig. 5*A*). Steroid-starvation increased the $[Ca^{2+}]_i$ response to estradiol stimulation. After steroid-starvation for 6 or 12 h, estradiol at 10 nM significantly increased the $[Ca^{2+}]_i$ response ($\Delta F\,Ca^{2+} = 503 \pm 19$ RFU, $n = 22$, $p < 0.001$ vs no steroid-starvation; and $\Delta F\,Ca^{2+} = 534 \pm 10$ RFU, $n = 26$, $p < 0.001$ vs no steroid-starvation, respectively) (Fig. 5*A*). The prolonged absence of sex steroids increased the estradiol-induced $[Ca^{2+}]_i$ flux in hypothalamic astrocytes.

Duration of estradiol exposure influences estradiol-induced $[Ca^{2+}]_i$ flux

Incubation of hypothalamic astrocytes in 1 nM estradiol influenced their subsequent response to estradiol stimulation at 10 nM. The 10 nM estradiol-induced $[Ca^{2+}]_i$ flux significantly increased after incubation with 1 nM estradiol for 30 min ($\Delta F\,Ca^{2+} = 594 \pm 17$ RFU; $n = 29$; $p < 0.05$) compared with no previous exposure to estradiol ($\Delta F\,Ca^{2+} = 534 \pm 10$ RFU; $n = 26$) (Fig. 5*B*). The response to 10 nM estradiol stimulation did not increase significantly after previous exposure to estradiol at 1 nM for 5 min ($\Delta F\,Ca^{2+} = 582 \pm 15$ RFU; $n = 23$; $p > 0.05$), 1 h ($\Delta F\,Ca^{2+} = 558 \pm 20$ RFU; $n = 21$; $p > 0.05$), or 2 h ($\Delta F\,Ca^{2+} = 547 \pm 11$ RFU; $n = 21$; $p > 0.05$) compared with no previous incubation with estradiol at 1 nM (Fig. 5*B*). All astrocytes were steroid-starved for a total of 12 h before stimulation with 10 nM estradiol. Previous exposure to estradiol increased the $[Ca^{2+}]_i$ response to subsequent estradiol stimulation with the maximum effect occurring after 30 min of estradiol exposure. The estradiol-induced $[Ca^{2+}]_i$ flux then decreased with increasing duration of estradiol incubation beyond 30 min.

Discussion

Membrane ERα has been associated with rapid estradiol-induced $[Ca^{2+}]_i$ flux, regulation of progesterone synthesis, and interaction with the mGluR1a in neurons and astrocytes (Dewing et al., 2007; Micevych et al., 2007, 2009; Kuo et al., 2009). The major

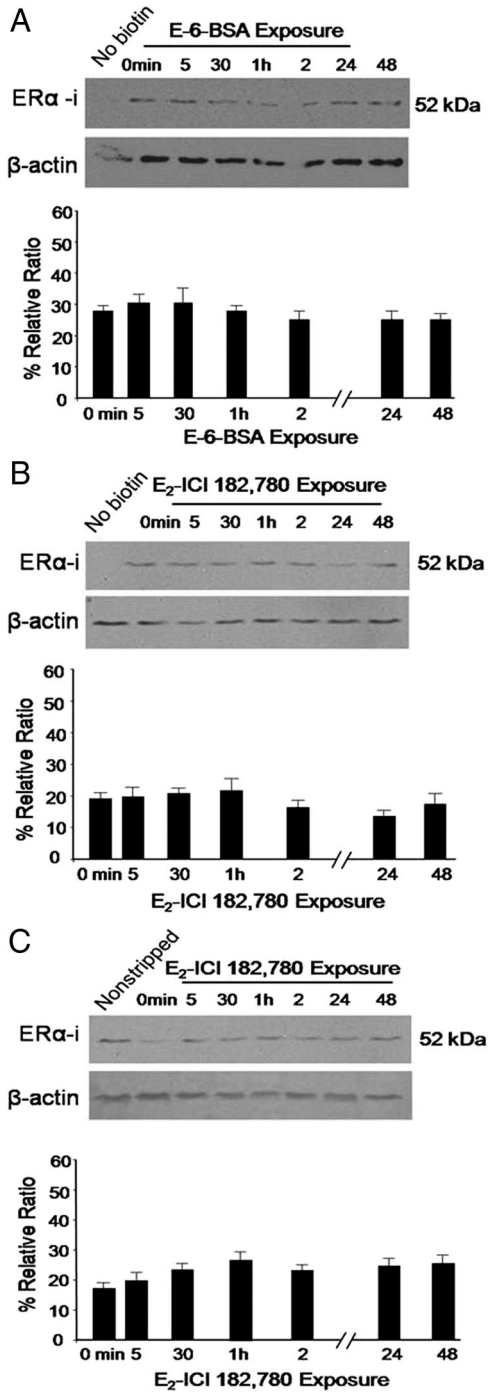

Figure 3. ***A***, Postpubertal hypothalamic astrocytes were incubated with 1 nM E-6-BSA before biotinylation. The membrane-constrained estradiol did not change membrane levels of ERα-immunoreactive (ERα-i) bands, indicating that estradiol acts within the cell to regulate ERα-i band trafficking to the membrane. ***B***, To study the effect of estradiol on ERα-i band trafficking to the membrane, cells were preincubated with charcoal-stripped medium containing 1 μM ICI 182,780, an ER antagonist, for 1 h before and during 1 nM estradiol treatment. ICI 182,780 prevented trafficking of ERα-i bands to the membrane. ***C***, To study the effect of estradiol on ERα-i band internalization, cells were surface biotinylated and treated with estradiol (1 nM) and ICI 182,780 (1 μM), which prevented internalization of ERα-i band. All the data are mean $\pm$ SEM ($n = 4$). *Differences at the $p < 0.05$ level compared with 0 min for each experiment. E_2, Estradiol.

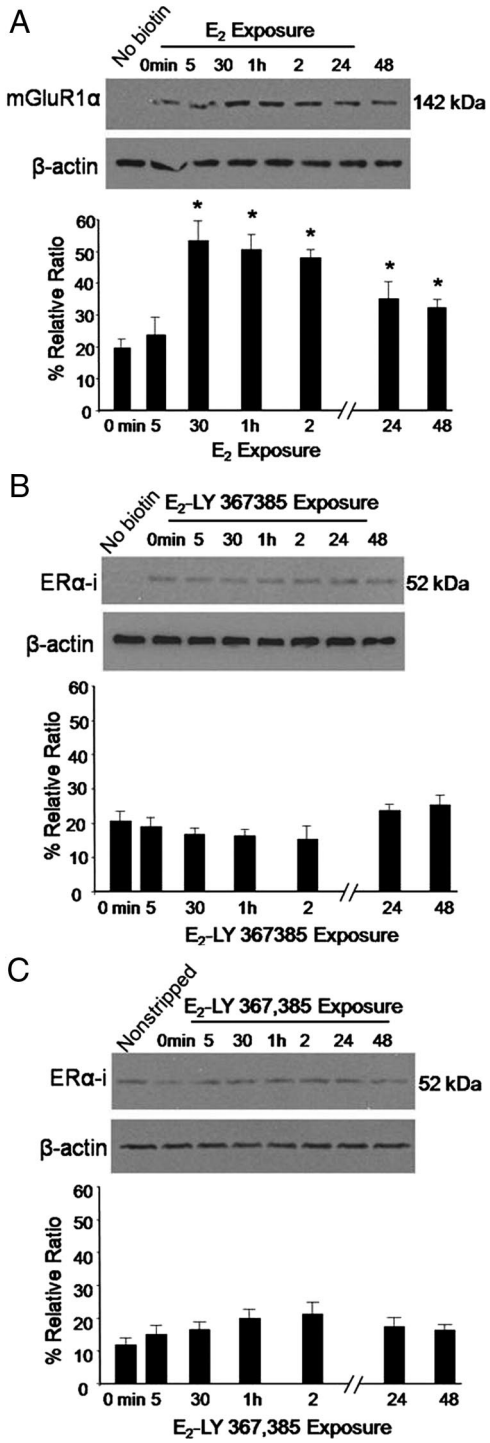

Figure 4. ***A***, To detect whether estradiol regulates mGluR1a trafficking, postpubertal hypothalamic astrocytes were treated with estradiol (1 nM) for the indicated times and surface biotinylated. Levels of membrane mGluR1a were increased by estradiol treatment. By 30 min, mGluR1a levels were significantly higher than no previous estradiol treatment (0 min; *$p < 0.05$). Membrane levels of mGluR1a did not return to basal levels during the experiment (48 h). ***B***, The mGluR1a antagonist LY 367385 (50 μM) blocked the estradiol-induced ERα-immunoreactive (ERα-i) trafficking to the membrane. ***C***, Similarly, 50 μM LY 367385 prevented the estradiol-induced ERα-i internalization. All the data are mean $\pm$ SEM ($n = 4$). *Differences at the $p < 0.05$ level compared with 0 min for each experiment. E_2, Estradiol.

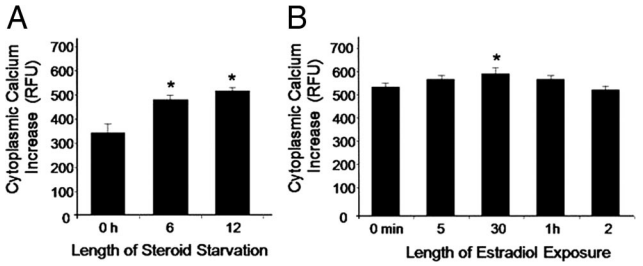

Figure 5. ***A***, Duration of steroid-starvation increased the estradiol-induced $[Ca^{2+}]_i$ flux in postpubertal hypothalamic astrocytes. Estradiol at 10 nM induced a greater $[Ca^{2+}]_i$ response after a 6 h (*$p < 0.001$) and 12 h (*$p < 0.001$) steroid-starvation compared with no prior (0 h) steroid-starvation. ***B***, Prior estradiol exposure transiently increased $[Ca^{2+}]_i$ flux in astrocytes. After a 12 h steroid-starvation without previous estradiol incubation (0 min), estradiol at 10 nM induced a robust $[Ca^{2+}]_i$ flux. The 10 nM estradiol-induced $[Ca^{2+}]_i$ flux increased after exposure to 1 nM estradiol for 30 min (*$p < 0.05$). However, incubation with 1 nM estradiol for 5 min, 1 h, or 2 h ($p > 0.05$) was no different than no prior incubation with estradiol (0 min). All the data are mean ± SEM. *Differences at the $p < 0.05$ level compared with 0 min.

finding of this study is that estradiol regulates ERα trafficking into and out of the plasma membrane. Surface biotinylation revealed two ERα-immunoreactive bands: a full-length 66 kDa and a smaller 52 kDa ERα, both of which are regulated in parallel by estradiol. Trafficking to the membrane was not induced by E-6-BSA and was blocked by the ER antagonist ICI 182,780 and the mGluR1a antagonist LY 367385. Similarly, internalization, a measure of receptor activation, was blocked by ICI 182,780 and LY 367385. As a measure of ERα signaling, $[Ca^{2+}]_i$ flux was also shown to be maximal when the amount of biotinylated ERα in membrane was highest. These results are consistent with the hypothesis that ERα is a membrane receptor with an extracellular portion whose levels are regulated by estradiol, temporally limiting membrane-initiated cell signaling.

A number of different techniques have been used to demonstrate membrane association of ERs. A particularly useful method has been to use membrane-constrained estradiol constructs such as E-6-BSA or estradiol conjugated to biotin (E-biotin). The membrane-impermeable conjugates of estradiol has been shown to elicit rapid activation of cell signaling pathways (Beyer et al., 2003; Chaban et al., 2004; Kelly and Ronnekleiv, 2008; Mermelstein and Micevych, 2008). Western blotting revealed ERα and ERβ in membrane fractions of neurons and astrocytes (Azcoitia et al., 1999; Chaban et al., 2004; Pawlak et al., 2005). Furthermore, coimmunoprecipitation experiments showed an interaction of 66 kDa ERα with mGluR1a in membrane fractions of astrocytes and neurons (Dewing et al., 2007; Kuo et al., 2009). This interaction is necessary for estradiol cell signaling (Boulware et al., 2005; Dewing et al., 2007, 2008; Kuo et al., 2009). Surface biotinylation with the Sulfo-NHS-SS-Biotin reagent is a convenient method for the identification of cell-surface proteins and can be used to study membrane protein trafficking. An advantage is that Sulfo-NHS-Biotin is membrane impermeable and labels only proteins that have extracellular lysine residues. This was verified by showing that LIMK1, a cytoplasmic protein, was not labeled (Fig. 1), whereas mGluR1a, a typical seven-membrane pass G-protein-coupled receptor, was labeled (Fig. 4). Treatment with estradiol not only increased trafficking of ERα to the membrane, but also its internalization (Fig. 2). The current observations of receptor internalization complement studies using a membrane-impermeable E-6-BSA-FITC construct, which binds to and labels membrane ERs (Dominguez et al., 2009; Micevych and Dominguez, 2009). GPR30, a putative G-protein-coupled ER, was not detected in surface-biotinylated fractions from astrocytes (Figs. 1, 2), confirming previous results that suggest that GPR30 may not be present on the cell membrane (Gorosito et al., 2008; Matsuda et al., 2008; Otto et al., 2008).

Hydropathicity analysis of ERα suggests a potential transmembrane domain near the NH_2 terminal (SOSUI, TMpred program). This supports potential extracellular ERα binding sites. Our current biotinylation experiments did not indicate which part of the ERα extends through the membrane but did suggest that there is an extracellular portion whose characterization will require additional study. Interestingly, the present surface-biotinylation studies identified two ERα forms: a 66 kDa and a 52 kDa ERα band. A similar, 50/55 kDa ERα-immunoreactive band was surface biotinylated in embryonic hypothalamic neurons, but no 66 kDa bands were detected (Gorosito et al., 2008). In oligodendrocytes, the MC-20 (anti-ERα antibody) labeled a 66 kDa band and an ~52 kDa doublet (Gorosito et al., 2008). Interestingly, both full-length 66 kDa ERα and 55 kDa ERα were identified in an endothelial cell line derived from the hypothalamus (Deecher et al., 2003). However, only the full-length ERα could bind estradiol and increase $[Ca^{2+}]_i$ flux, suggesting that the 55 kDa band may have been a proteolytic/degradation fragment. In the present study, the 52 kDa may also be a proteolytic/degradation product, but without further characterization, this would be a premature conclusion. Every precaution was taken to prevent degradation during our experiments. All procedures were done at 4°C and in the presence of several protease inhibitors. Moreover, the 52 kDa band was labeled with COOH- and NH_2-teminally directed antibodies.

Previous studies have discovered various splice variants of ERα in different tissues, including the CNS (Couse et al., 1995; Bollig and Miksicek, 2000; Kos et al., 2002; Shughrue et al., 2002; Herynk and Fuqua, 2004; Nethrapalli et al., 2005; Perlman et al., 2005; Dominguez-Salazar et al., 2006). Using an ERαKO mouse model, we confirmed that both ERα-immunoreactive proteins identified in our WT mice and rat experiments are derived from the ERα gene (supplemental material, available at www.jneurosci.org). It is likely that the 52 kDa protein is a splice variant. mRNAs missing exon 7 (ERαΔ7) are predicted to code for a protein of ~52 kDa, but this protein is missing the COOH terminal. However, we were able to identify the 52 kDa protein with antibodies directed against both the NH_2 and COOH termini, suggesting that this protein is not derived from ERαΔ7, but rather another slice variant. A potential splice variant is ERαΔ4, which codes for an ~54 kDa protein that has both the NH_2 and COOH termini conserved and could interact with our antibodies (Fuqua et al., 1992; Bollig and Miksicek, 2000; Deecher et al., 2003; Perlman et al., 2005). At the present time, the identity of this lower-weight, 52 kDa ERα-immunoreactive protein is not known and will require further characterization.

In previous studies, only the 66 kDa ERα coimmunoprecipitated with mGluR1a, which in astrocytes is needed for the estradiol-induced $[Ca^{2+}]_i$ flux (Chaban et al., 2004; Kuo et al., 2009). In the present study, the estradiol-induced increase of $[Ca^{2+}]_i$ flux paralleled the levels of the 66 kDa form and did not coincide with the large changes in the 52 kDa level.

Regardless, the 52 kDa ERα is a useful marker for membrane ERα because it is regulated in parallel to 66 kDa ERα, and the 52 kDa ERα is easier to detect. Therefore, the 52 kDa form was quantified for the trafficking and internalization studies. Estradiol treatment had a biphasic effect on levels of surface-biotinylated ERα. Within 5 min, estradiol acting within the cell, in an ICI 182,780-dependent manner, dramatically increased

membrane ERα trafficking and internalization. Interestingly, treatment with the mGluR1a antagonist LY 367385 also prevented ERα trafficking to the membrane and internalization, consistent with the idea of a direct ERα-mGluR1a interaction.

The response of ERα to estradiol stimulation is interesting because it suggests an auto-regulation of MISS. These results indicate that there is a 2 h window during which estradiol induces membrane trafficking and internalization of ERα. The rapidity of the trafficking to the membrane suggests that *de novo* synthesis of ERα is probably not occurring. Estradiol is inducing exocytosis of vesicles that have ERα inserted in the membrane as has been reported on neuronal vesicles in hippocampal neurons (Milner et al., 2001; Hart et al., 2007) and in pituitary cells (Gonzalez et al., 2008). ERs are targeted to membranes through palmitoylation and in association with caveolin proteins that also regulate the interaction with mGluRs (Acconcia et al., 2005; Boulware et al., 2007; Pedram et al., 2007; Micevych and Mermelstein, 2008). When ERα vesicles fuse with the cell membrane, they deliver the ERα–mGluR complex to the cell surface. Similarly, internalization also has been linked to the formation of endocytic vesicles in which E-6-BSA-FITC is sequestered (Micevych and Dominguez, 2009). Once receptors are internalized and release their ligand, they can either be recycled to the cell surface or degraded. Our results suggest that for several hours ERα was recycled since sequestration of the receptor paralleled the highest levels of ERα in the membrane (Fig. 2). Continuous treatment with estradiol for 24–48 h leads to a decreased concentration in plasma membrane and internalized levels of ERα, suggesting that estradiol exposure eventually leads to degradation of the receptor and downregulation. This indicates that rapid estradiol signaling is temporally restricted. *In vivo*, only membrane-constrained E-6-BSA given before estradiol was facilitatory (Kow and Pfaff, 2004).

Understanding the regulation of ERα levels in the plasma membrane is fundamental for the understanding of estradiol signaling. Levels of membrane ERα result from the balance between exocytosis and endocytosis, which we demonstrate as being regulated by estradiol acting in astrocytes. Thus, the present study indicates a novel autoregulation of ERα and MISS by estradiol.

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