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Estrogen Actions on Neuroendocrine Glia

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Key Words
Astrocytes · Surface biotinylation · Estrogen receptor-alpha trafficking

Abstract
Astrocytes are the most abundant cells in the central nervous system (CNS). It appears that astrocytes are as diverse as neurons, having different phenotypes in various regions throughout the brain and participating in intercellular communication that involves signaling to neurons. It is not surprising then that astrocytes in the hypothalamus have an active role in the CNS regulation of reproduction. In addition to the traditional mechanism involving ensheathment of neurons and processes, astrocytes may have a critical role in regulating estrogen-positive feedback. Work in our laboratory has focused on the relationship between circulating estradiol and progesterone synthesized de novo in the brain. We have demonstrated that circulating estradiol stimulates the synthesis of progesterone in adult hypothalamic astrocytes, and this neuroprogesterone is critical for initiating the LH surge. Estradiol cell signaling is initiated at the cell membrane and involves the transactivation of metabotropic glutamate receptor type 1a (mGluR1a) leading to the release of intracellular stores of calcium. We used surface biotinylation to demonstrate that estrogen receptor-α (ERα) is present in the cell membrane and has an extracellular portion. Like other membrane receptors, ERα is inserted into the membrane and removed via internalization after agonist stimulation. This trafficking is directly regulated by estradiol, which rapidly and transiently increases the levels of membrane ERα, and upon activation, increases internalization that finally leads to ERα degradation. This autoregulation temporally limits membrane-initiated estradiol cell signaling. Thus, neuroprogesterone, the necessary signal for the LH surge, is released when circulating levels of estradiol peak on proestrus and activate progesterone receptors whose expression has been induced by the gradual rise of estradiol during follicular development.

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Introduction

Astrocytes are found in abundance throughout the central nervous system (CNS). The relative percentage of astrocytes varies by species with an increasing astrocyte-to-neuron ratio with increasing brain size and complexity [1]. Initially astrocytes were named for their stellate appearance and distribution: protoplasmic in the grey matter, fibrous in the white matter and adult forms of radial glia (i.e. tanicytes of the median eminence, pituicytes of the posterior pituitary, Müller cells of the retina and Bergmann glia of the cerebellar cortex). Later, astrocytes

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were typically identified by their expression of the intermediate filament, glial fibrillary acidic protein (GFAP). Traditionally, astrocytes have been considered to provide the structural framework that organizes neurons and helps maintain the blood-brain barrier. Over the years, our view of astrocytes has evolved beyond a static role as the CNS stroma. Astrocytes are vital for the maintenance of local ionic concentration, neurotransmitter milieu, and regulation of electrical and chemical synaptic transmission through dynamically changing ensheathment of neuronal somata and processes [2]. One of the best-studied examples of the changing relationship between astrocytes and neurons is in the supraoptic nucleus of the hypothalamus. Activation of oxytocin magnocellular neurons leads to decreased astrocytic coverage allowing electrical coupling and synchronous firing that results in the pulsatile release of oxytocin [3–9]. Similar effects of dynamic astrocytic ensheathment have been reported in the arcuate nucleus and median eminence of the hypothalamus in response to fluctuations of sex steroid hormones [10, 11]. These changes in astrocytic coverage appear to coincide with an increase in dendritic spines and their synapses [12–14].

It has become clear that astrocytes express a wide diversity of phenotypes. As with neurons, astrocytes appear to have different phenotypes throughout the brain [10, 15–21], including differential expression of receptors for extracellular signaling molecules such as estradiol, ATP, glutamate, melanocortin, and norepinephrine [22–30]. This variation of receptors allows astrocytes to sense and respond to appropriate extracellular signals in specific brain regions leading to the modulation of calcium and other signaling pathways [22, 31, 32].

A variety of transmitters increase free cytosolic calcium ($[Ca^{2+}]_i$) through the release of intracellular calcium stores in astrocytes. In distinction to neurons, however, astrocytes are able to propagate Ca^{2+} waves over long distances since they are coupled through gap junctions [33–39]. Calcium waves activate glial synaptic mechanisms to trigger the release of glial transmitters (e.g. glutamate, serine, ATP and taurine), which has been termed 'gliotransmission' [9, 40–43]. Thus, by responding to neurotransmitters and releasing their own gliotransmitters, astrocytes engage in a dynamic bidirectional cross talk with neurons and participate in the intercellular signaling of the CNS [9, 44].

It is not surprising then that astrocytes have an active role in CNS regulation of reproductive physiology. Similar to the other types of glial-neuronal interaction, astrocytes help regulate the function of gonadotropin-releasing hormone (GnRH) neurons, including modulation of synaptic input [14, 45–49] and access of GnRH terminals to the hypothalamo-hypophyseal portal capillaries in the median eminence [50–52]. In addition, astrocytes secrete a number of factors that stimulate GnRH release, including TGF-α, TGF-β, IGF-1, progesterone and 3α,5α-tetrahydro-progesterone (3α, 5α-THP) [52–55, reviewed in 11, 56]. We have been interested in studying how circulating estradiol regulates the activity of astrocytes that are involved in the central control of reproduction.

Astrocytes and Reproduction in Females

The Luteinizing Hormone Surge

The central event in female reproduction is the surge release of LH from anterior pituitary gonadotropes. These cells are controlled by a network of GnRH neurons that project to the median eminence where they make a neurohemal contact with portal capillaries serving the anterior pituitary. The resulting surge release of LH induces ovulation and subsequent luteinization of the ruptured ovarian follicles. The hormonal event that triggers increased GnRH activity to signal the surge release of LH is estrogen-positive feedback. This is a well-known but somewhat ill-defined process through which increasing levels of circulating estradiol, derived from developing follicles, act on the hypothalamus to induce the surge release of GnRH. This is dependent upon high levels of estradiol and progesterone, which activate their respective cognate receptors to stimulate the GnRH network [57–61, reviewed in 43, 62].

Since GnRH neurons do not express ERα, estradiol must activate a CNS network of astrocytes and neurons, which then indirectly stimulate GnRH neurons and the surge release of LH. A large number of peptide and non-peptide transmitters have been implicated in this process, but they do not appear critical [63, reviewed in 64]. The exception may be kisspeptin, a neuropeptide that has been suggested as the proximal neuronal signal for GnRH release [65–67]. Indeed, kisspeptin-containing neurons express ERα, progesterone receptor (PR), and the G protein-coupled receptor (GPCR) GPR54, a receptor shown to be essential for normal GnRH secretion [65, 68, 69].

While progesterone has been demonstrated to be necessary for the LH surge, its source has been debated. A telling experiment showed that in ovariectomized and adrenalectomized (ovx/adx) rats, estradiol priming without exogenous progesterone induced a physiological LH

surge [61, 70]. However, the LH surge was blocked if progesterone synthesis was disrupted with trilostane, a 3β-hydroxysteroid dehydrogenase inhibitor, in ovx/adx rats. When progesterone was given back to the estradiol-primed, trilostane-treated ovx/adx rats, the LH surge was restored. These results are congruent with a critical role for progesterone in the LH surge [71–74], and suggested that in ovx/adx rats progesterone synthesis continued – most likely in the CNS. This was verified by measuring an estradiol-induced increase of hypothalamic progesterone in the face of low to undetectable circulating levels of progesterone. Since the early 1980s, it has been accepted that the brain is a steroidogenic organ [reviewed in 75, 76] and that astrocytes may be the most active steroidogenic cells in the CNS [77, 78].

Work in our laboratory has focused on the relationship between circulating estradiol and neuroprogesterone, progesterone synthesized de novo in the brain. To demonstrate the importance of hypothalamic progesterone, a P450 side-chain cleavage (P450scc) enzyme inhibitor, aminoglutethimide (AGT), was infused into the third ventricle on the morning of proestrus to block hypothalamic steroidogenesis in gonadally intact rats with normal estrous cycles. Central AGT prevented the LH surge and ovulation, but did not disrupt peripheral steroidogenesis [78]. In AGT-treated rats, hypothalamic levels of neuroprogesterone were significantly reduced compared to vehicle treated rats – who continued to cycle as assessed by vaginal cytology. Moreover, the AGT-treated rats did not transition from proestrus to estrus, which requires the LH surge. When the AGT was metabolized after several days, the rats ovulated and resumed their estrous cycle. These results suggested a sequence of events in which peripheral estradiol (of ovarian origin) stimulates neuroprogesterone synthesis [79, 80]. In the intact rat, as follicles develop in the ovary, circulating levels of estradiol increase and induce PRs in hypothalamic neurons, including kisspeptin neurons that lie along the rostral third ventricle [81]. When estradiol peaks during proestrus, it induces progesterone synthesis in hypothalamic astrocytes. This hypothalamic neuroprogesterone then acts as a trigger: stimulating estrogen-induced PRs, activating kisspeptin neurons that excite GnRH neurons, and initiating the LH surge [81, 82]. Based on such observations of neuroprogesterone action, progesterone synthesized in the brain, like other neurosteroids, is a fourth-generation transmitter. Such fourth-generation transmitters are a family of diverse molecules that are regulated at the level of synthesis rather than release. Other examples include nitric oxide, carbon monoxide, prostaglandins and endocannabinoids [78].

Astrocytes and Neuroprogesterone

Neurons, oligodendrocytes and astrocytes are all capable of steroidogenesis [77]. However, the most probable source of neuroprogesterone is from astrocytes [32, 55, reviewed in 43]. Both whole hypothalamus in vivo and hypothalamic astrocyte cultures from postpubertal female rats express the enzymes and associated proteins needed for progesterone synthesis: P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), steroid acute regulatory protein (StAR), and sterol carrier protein-2 (SCP-2) [32, 59]. Estradiol has been shown to stimulate progesterone synthesis in hypothalamic astrocytes and whole hypothalamus, which corresponded with an increased expression of 3β-HSD mRNA and enzyme activity [reviewed in 83]. Interestingly, estradiol did not increase hypothalamic progesterone levels in acyclic female rats with persistent estrus suggesting that reproductive aging may be the result of a lack of estrogen-induced neuroprogesterone synthesis [83]. Furthermore, no increase in hypothalamic progesterone was measured in male rats, consistent with the inability of male rodents to display an estrogen-positive feedback surge of LH [61].

Parallel studies in vitro showed that estradiol rapidly increased $[Ca^{2+}]_i$ flux in astrocytes through a phospholipase C (PLC)/inositol triphosphate (IP_3) receptor-mediated pathway [22]. This effect was stereospecific, reproduced with E-6-BSA and blocked with ICI 182,780, indicating that estradiol is signaling via a classical ER associated with the cell membrane. Further evidence for classical ER-mediated membrane signaling is supported by astrocytic expression of ERα and ERβ in both the cytoplasmic and membrane fractions. In subsequent experiments, physiological levels of estradiol or E-6-BSA increased $[Ca^{2+}]_i$ flux through membrane ER activation [43]. Since calcium is a general signal in astrocytes, we needed to determined whether the increase in $[Ca^{2+}]_i$ flux was the intracellular signal through which estradiol acted to rapidly increase progesterone synthesis. To this end, thapsigargin, a Ca^{2+}-ATPase inhibitor that mobilizes IP_3 receptor-sensitive calcium stores, was used [32]. Thapsigargin was as effective as estradiol at facilitating progesterone synthesis in astrocytes, indicating that de novo progesterone synthesis in astrocytes is dependent on the estradiol-induced $[Ca^{2+}]_i$ flux.

A great deal of evidence now exists that the same ER proteins that interact with the estrogen response element

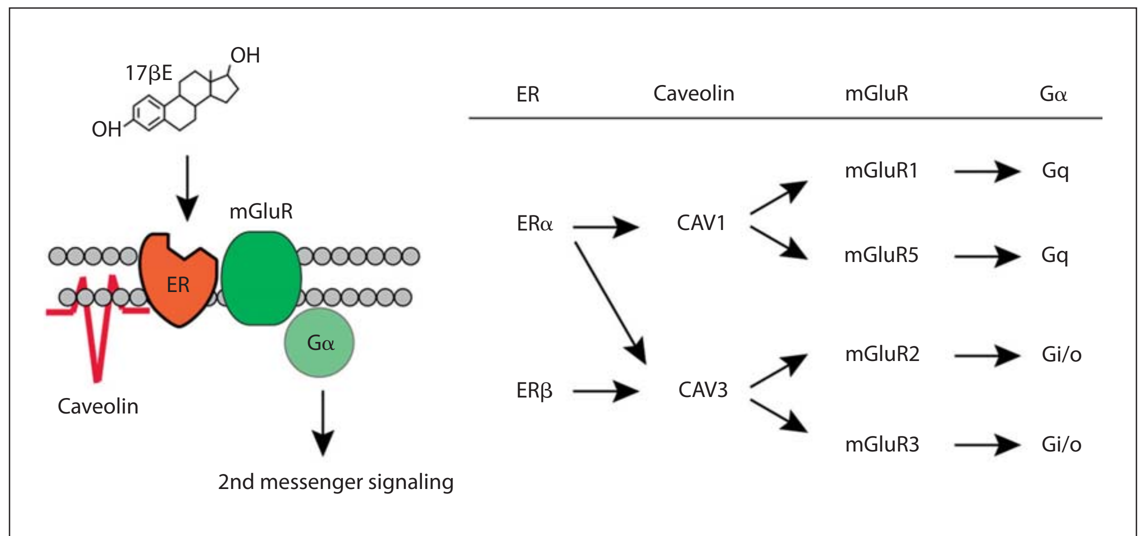


Fig. 1. ER activation of mGluR signaling through interactions with caveolin proteins. **a** Model framework of estradiol-induced activation of mGluRs via caveolin-based caveolae. **b** Summary of previous findings demonstrating ER activation of group I (mGluR1/5) and group II (mGluR2/3) metabotropic glutamate receptors is mediated by caveolin 1 and caveolin 3, respectively. From Micevych and Mermelstein [75].

(ERE) on DNA in the nucleus is also trafficked to and associated with the cell membrane. In addition to the classic ERα and ERβ, several other estrogen-binding proteins have been proposed including ER-X, GPR30 and a STX-activated protein [reviewed in 84]. To date, we have concentrated on ERα and ERβ because: (1) transfection of ER-negative cells with ERα or ERβ mRNA produce cognate proteins in the membrane fraction that are functional [85]; (2) Western blots with ER-specific antibodies demonstrate both full-length and splice variants of the ER in the membrane fraction of neurons and astrocytes [22, 86, 87]; (3) the binding affinity (K_D) for the membrane ER and nuclear ER is similar, but there are fewer ERs on the membrane (B_{MAX}) [88], and (4) ERα and ERβ proteins are trafficked and attached to the membrane through palmitoylation and in association with calveolin proteins [89–93].

Membrane-initiated estradiol signaling can activate a number of GPCR associated pathways, including $[Ca^{2+}]_i$ flux, cAMP, DAG, IP_3, protein kinase C (PKC), PKA, MAPK/ERK and phosphorylation of cAMP response element-binding protein (CREB) [94–96, reviewed in 97]. ERα and ERβ activate intracellular signaling cascades by interacting with bona fide membrane receptors such as growth factor tyrosine kinase receptors or metabotropic glutamate receptors (mGluRs) to initiate cell signaling [98–104, reviewed in 75, 79]. In the CNS, membrane ERs interaction with mGluRs has been reported in many different brain regions [reviewed in 79]. The mGluRs are glutamate-binding proteins grouped according to sequence homology and second messenger linkage: mGluR1 and mGluR5, coupled to Gq, are group I mGluRs; mGluR2 and mGluR3, activating Gi/Go signaling, are group II mGluRs; and mGluR4, mGluR6, mGluR7 and mGluR8, also Gi/Go coupled, comprise group III mGluRs. Co-immunoprecipitation demonstrated the probable physiological interaction of ERs and mGluRs in both neurons and astrocytes (fig. 1) [99, 100, 105].

While the details of the transactivation of mGluRs by ERs have not been elucidated, the downstream actions of estradiol-initiated transactivation of mGluR2/3 leads to inhibition of Ca^{2+} influx through the L-type voltage-gated calcium channel (VGCC) [35, 98, 106, 107], while ERα transactivation of the mGluR1a activates the PLC pathway, increasing $[Ca^{2+}]_i$ flux, activating PKC, and phosphorylating CREB [98, 100, 105]. In terms of reproduction, ERα interacts with mGluR1a to initiate lordosis behavior though activation of a novel PKC and increases neuroprogesterone synthesis, which is a necessary step for estrogen positive feedback [100, 108]. Membrane-initiated activation of cell signaling can be blocked with the specific ER antagonist ICI 182,780 or the selective mGluR1a antagonist LY367385. Activating the mGluR1a with

(S)-3,5-dihydroxyphenylglycine (DHPG) without estradiol mimicked the steroid-induced $[Ca^{2+}]_i$ flux [100].

ERα in the Membrane

Evidence that reproduction is dependent on ERα, including the membrane ERα, is very strong [60, 61, 109, 110, but see 111]. Membrane impermeable estradiol constructs (e.g. E-6-BSA) and studies showing ERα protein in the membrane fraction of native or transfected cells indicate an ERα association with the membrane [35, 85, 99, 105, 106, 112–118]. Since ERα does not have the typical structure of a classic membrane protein, this association has remained unresolved. It is not clear whether ERα is anchored to the inner leaflet of the membrane or an integral membrane protein with an exposed extracellular portion. A method that has been useful in identifying integral membrane proteins is surface biotinylation. Proteins that have an extracellular portion are labeled with biotin, subsequently concentrated using an avidin column, eluted and characterized by Western blotting [119].

Using this technique, membrane ERs were labeled with the membrane-impermeable biotin reagent in primary cultures of hypothalamic neurons and astrocytes (fig. 2) [86, 87, 120]. These studies demonstrated surface biotinylated ERα-immunoreactive proteins, indicating that a portion of the ERα is exposed on the cell surface and implying that ERα is a membrane protein. Together with the functional E-6-BSA studies that showed activation of PKC and $[Ca^{2+}]_i$ flux, these results suggest that the exposed portion of the ERα contained the ligand-binding site. Interestingly, while both groups identified a lower molecular weight (MW) form (50–55 kDa), as the major ERα-immunoreactive protein in the membrane, only our group detected a 66-kDa protein that was assumed to be the full-length ERα (fig. 2).

To determine whether these proteins were derived from the ERα gene, astrocytes from wild-type and ERα-disrupted (ERKO) mice were surface biotinylated [87]. As in the experiments conducted with rat astrocytes, both 52- and 66-kDa ERα proteins were detected in wild-type mouse astrocytes. However, ERKO astrocytes had neither the 52- nor the 66-kDa proteins, indicating that both proteins were derived from the ERα gene. It is likely that the 66-kDa protein is the full-length ERα, but the identity of the 52-kDa protein has not been resolved. The 52-kDa protein may potentially be an alternatively spliced form of ERα. Numerous alternatively spliced forms of ERα mRNA have been identified in a variety of estrogen-sensitive tissues, including the brain [88, 121–126]. Some of these alternatively spliced mRNAs are translated into proteins [88, 121, 127–130]. Based on the MW of the predominant membrane ERα, the most probable splice variant is the exon 7-deleted form (ERαΔ7), with a predicted MW of 52 kDa. The resulting protein, however, is truncated and missing the COOH-terminal end of the full-length ERα, which includes part of the ligand-binding domain [88, 122, 131, 132]. Both NH_2-terminal-directed, H-184, and COOH-terminal directed, MC-20, antibodies recognized the 52-kDa protein, and E-6-BSA stimulated cell signaling indicating that it is unlikely to be the truncated ERαΔ7 product [87]. Another possible splice variant, ERαΔ4, has an apparent MW of 54 kDa. This splice variant is missing exon 4 of the full-length ERα, which codes for the DNA-binding domain and the hinge region, but retains the COOH-terminal amino acid sequence recognized by MC-20. In addition to our studies, other groups have also identified ERα splice variants using specific antibodies directed against both the NH_2- and COOH-terminal ERα domains [88, 122, 131, 132], but mass spectrometry studies (MALDI-TOF) focusing on identifying the membrane ERα have not been successful [93, 133].

Regardless of the identity of the ERα splice variant, it is important to note that the full-length ERα was demonstrated in the membrane since it is probably the ERα needed for signaling. A previous study identified both full-length 66- and 55-kDa ERα in an endothelial cell line from the hypothalamus, but only the full-length ERα-bound estradiol and increased $[Ca^{2+}]_i$ flux [88]. Based on co-immunoprecipitation with mGluR1a, membrane-initiated estrogen signaling in astrocytes is mediated by the 66-kDa ERα [100]. No lower MW ERα variants co-immunoprecipitated with mGluR1a. Currently, the identity of the 52-kDa ERα is unknown and will require further experimentation to understand its function. Interestingly, GPR30, a putative G protein-coupled membrane ER, was not detected in surface-biotinylated fractions from astrocytes (fig. 2) [87], confirming previous results suggesting that GPR30 may not be present on the cell membrane [120, 134, 135].

ER Trafficking to and from the Cell Membrane

If ERα is a membrane receptor, its levels would be modulated at the cell surface. Membrane receptor populations are dynamic: inserted into and removed from the membrane. In addition, membrane receptors are massively internalized following agonist binding. To ascertain if ERα was trafficked to and/or internalized from the membrane, astrocyte cultures were exposed to estradiol for increasing intervals and then surface biotinylated. Es-

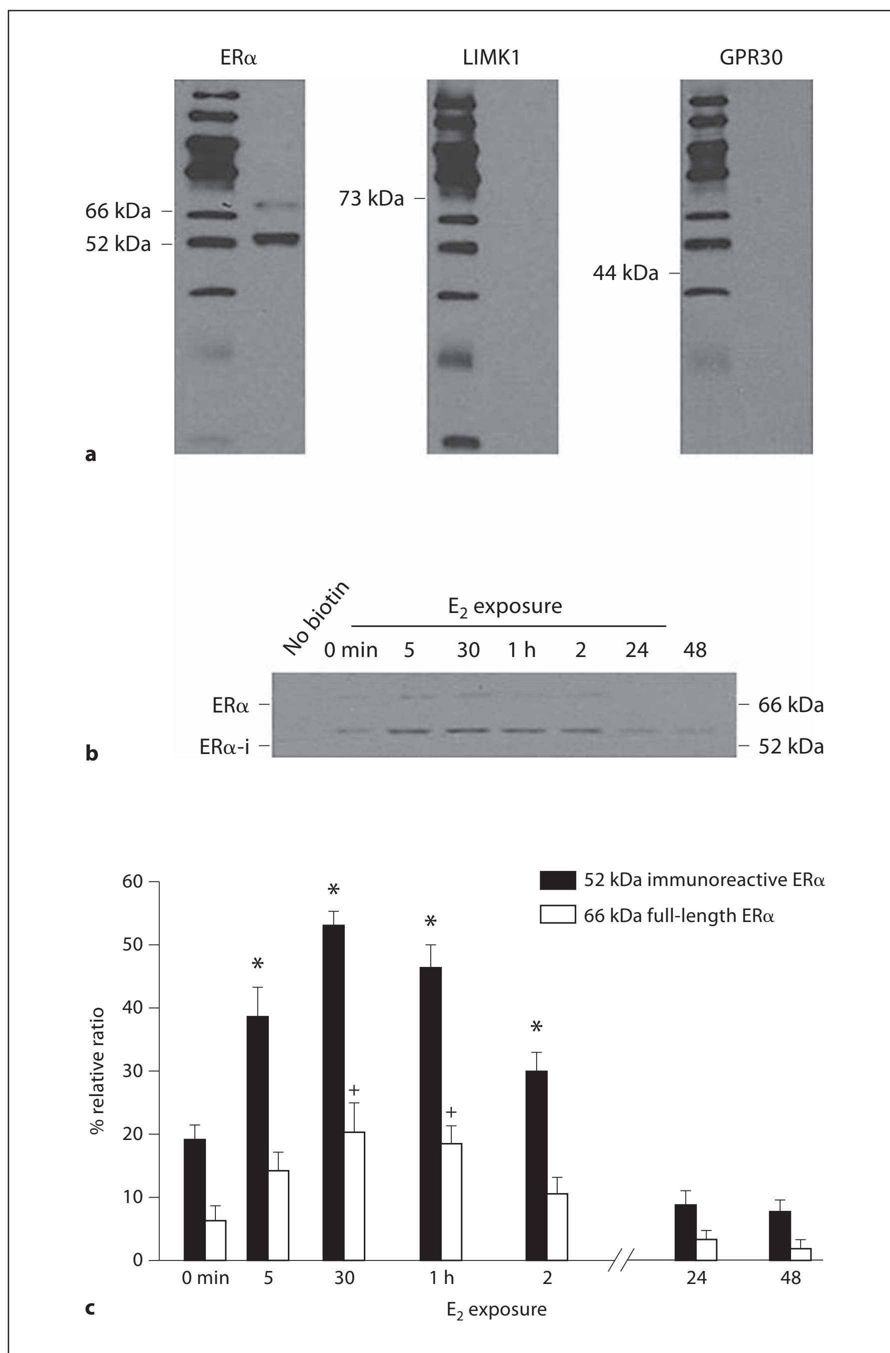
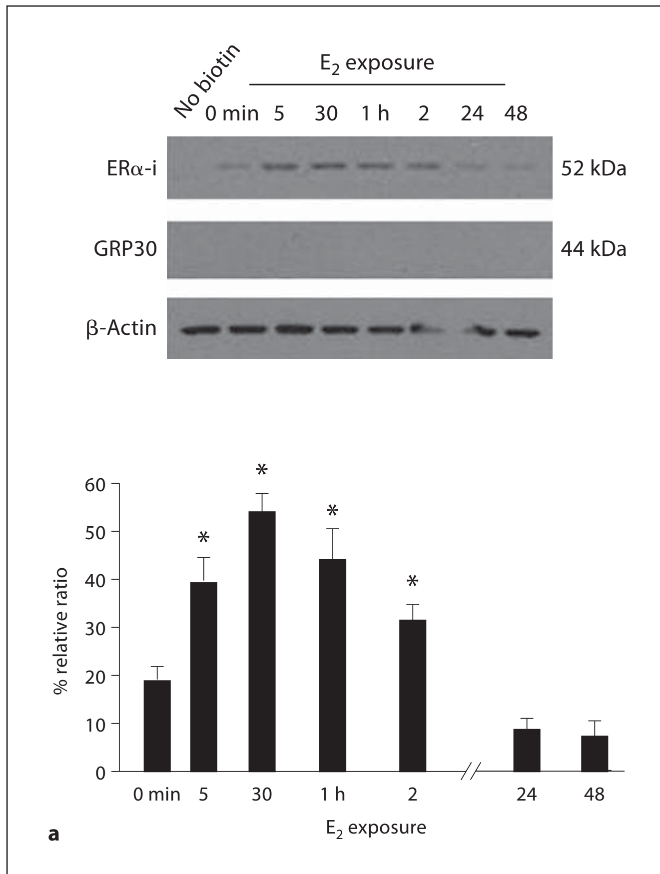


Fig. 2. Postpubertal hypothalamic astrocytes were incubated with vehicle (0 min) or in the presence of 1 nM estradiol (E_2) for 5, 30 min, 1, 2, 24 and 48 h. Astrocytes were then surface biotinylated, excess biotin removed, and the labeled proteins separated and detected with ERα, GPR30 and LIM domain kinase 1 (LIMK1) antibodies. **a** Two ERα-immunoreactive (ERα-i) ER bands were identified: 66 and 52 kDa. The cytoplasmic protein LIMK1 and the putative membrane ER GPR30 were not labeled with surface biotinylation. **b** Estradiol treatment (1 nM) increased both the 66- and 52-kDa ERα-i. In the first lane, cells were not surface biotinylated (no biotin), thus no biotinylated ERα-i was labeled. Detection of the 66-kDA ERα required a 2-hour exposure compared with a 1- to 2-min exposure for the 52-kDa ERα-i. **c** Quantification of the 66- and 52-kDa ERα-i was calculated by comparing the optical density of the ERα-i bands with that of the β-actin bands. Both 66- and 52-kDa ERα-i are regulated in parallel by E_2 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. From Bondar et al. [87].

tradiol treatment modulated the 52- and 66-kDa ERα proteins in parallel. In untreated astrocytes, levels of ERα in the membrane were low, but 5 min of estradiol treatment significantly increased the amount of ERα trafficked to the membrane and peak levels were reached after 30 min of estradiol exposure. Levels remained elevated with up to 2 h of estradiol exposure, but decreased to below basal concentration by 24 h, where they remained for the duration of the experiment (fig. 3) [87]. This transient increase of membrane ERα was dependent on estradiol as demonstrated by inhibition of trafficking with the ER antagonist ICI 182,780.

Estradiol not only increased trafficking of ERα to the membrane, but also internalization (fig. 3) [87]. In this



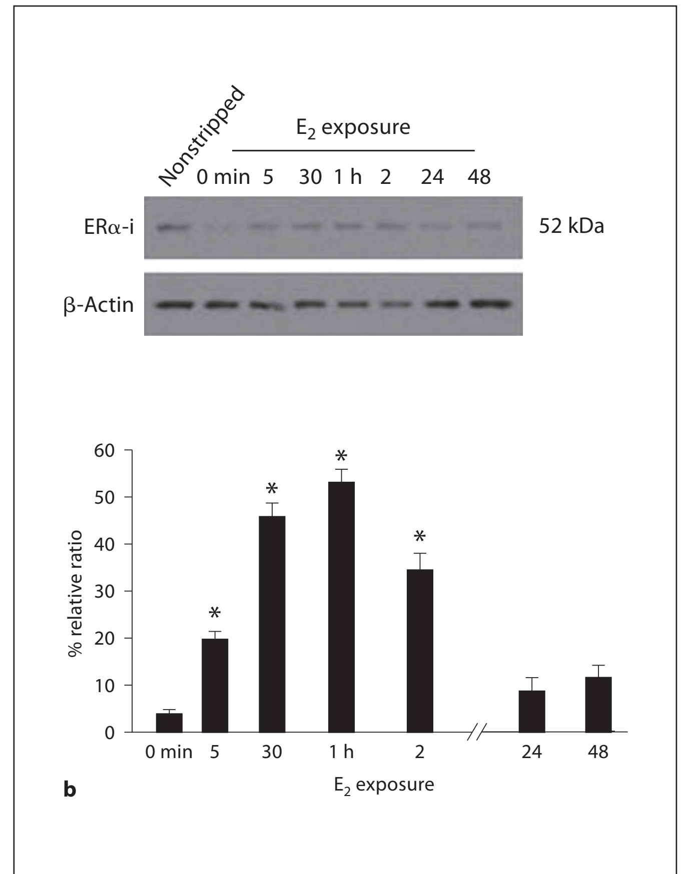
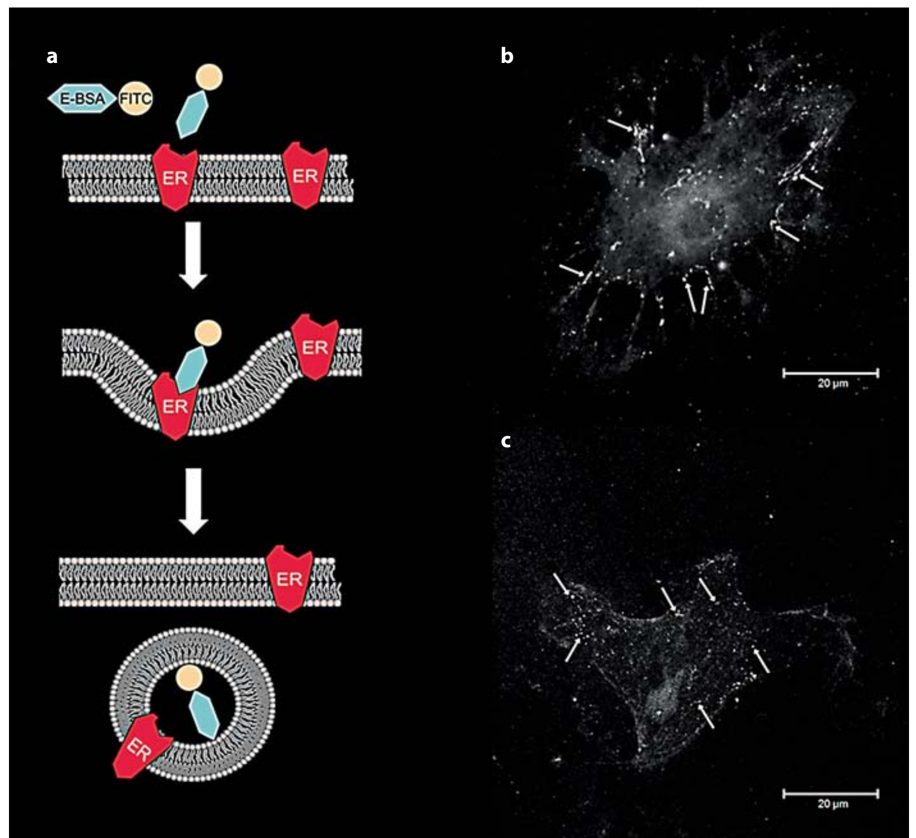


Fig. 3. a Estradiol (E_2) treatment transiently increases membrane ERα-i in postpubertal hypothalamic astrocytes. Basal levels of ERα-i were observed prior to E_2 treatment (0 min). These levels were rapidly increased (5 min time point) (* $p < 0.05$), with a maximum at 30 min (* $p < 0.05$) and a slight depression after 1 h (* $p < 0.05$), and remained elevated at 2 h with 1 nM E_2 stimulation (* $p < 0.05$). After 24–48 h of E_2 treatment, ERα-i returned to basal levels ($p > 0.05$). GPR30 was not surface biotinylated despite incubating the astrocytes with estradiol for up to 48 h. **b** To track internalization, astrocytes were biotinylated, treated with estradiol (1 nM), and then biotin stripped from the cell surface with glutathione. Under these conditions, ERα-i is 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, with varying estradiol (1 nM) 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. From Bondar et al. [87].

experiment, astrocytes were surface biotinylated, treated with estradiol and then incubated with glutathione to remove the biotin from the cell surface. Any remaining biotinylated ERα must have been internalized and thus, protected from the glutathione. The time course of internalization, a marker for membrane receptor activation [reviewed in 136], mimicked the pattern of membrane ERα insertion: estradiol increased the amount of internalized ERα at 5 min and remained elevated after 2 h. By 24 h, the amount of internalized ERα was below prestimulation levels. These observations of receptor internalization complement studies using a membrane-impermeable E-6-BSA-FITC [β-estradiol-6-(O-carboxymethyl) oxime-bovine serum albumin conjugated with fluorescein isothiocyanate] construct, which binds to and labels membrane ERs [84, 114]. When cells are allowed to interact for a period of time (≥5 min) with the E-6-BSA-FITC, the fluorescent marker is seen within intracellular vesi-

Fig. 4. E-6-BSA-FITC is internalized in primary cultures of adult hypothalamic astrocytes. **a** Experiment in which binding of the membrane-impermeable E-6-BSA-FITC complex to membrane ER induces internalization. Estradiol-bound ERs are internalized and transported to endosomes in which the highly acidic environment facilitates the disassociation of the ligand from its receptor. **b**, **c** Confocal images of primary hypothalamic astrocytes grown on glass coverslips and treated with 100 μg/ml E-6-BSA-FITC for 5 min (**b**) and 30 min (**c**) at 37°C, then fixed with 4% paraformaldehyde. Arrows indicate binding of the E-6-BSA-FITC to ER on the cell membrane (**b**) and the internalized complex (**c**).

Fig. 5. Schematic diagram illustrating estradiol-mediated ERα trafficking at the membrane. ERα-mGluR1a (blue-orange) complexes are inserted into the membrane of an exocytotic vesicle (**a**). These are associated through interactions with calveolin as depicted in figure 1. Estradiol (red spheres) induces vesicle docking with the membrane and insertion of the ERα -mGluR1a complex into the membrane (**b**). Colors refer to the online version only. Exposed to the extracellular space, estradiol binds to ERα (**c**), transactivating mGluR1a and initiating cell signaling. Estradiol-activated ERα-mGluR1a complexes are then internalized via a clathrin-mediated process (**d**) and fuse with early endosomes (**e**) where the estradiol is released from ERα. The ERα-mGluR1a complex can then be recycled to the membrane or degraded. Initially, there appears to be a recycling of the ERα-mGluR1a complex, but with continued stimulation, the process shifts toward degradation (fig. 2, 3).



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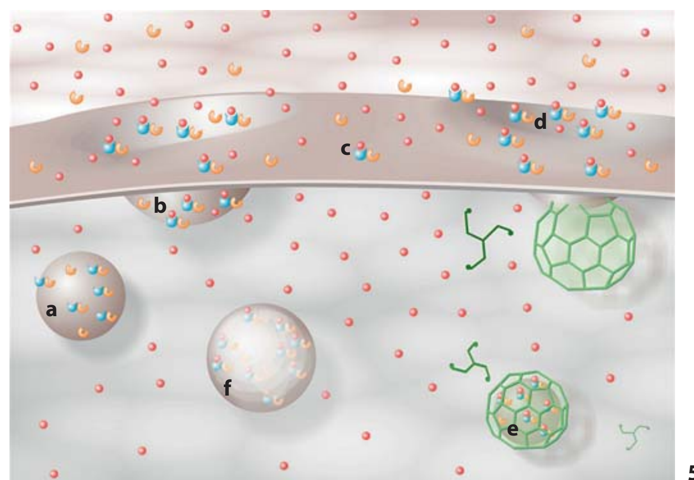

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cles thought to be early endosomes, which is suggestive of internalization and is a morphological equivalent of the surface biotinylation experiments (fig. 4).

Proximal events in membrane-initiated cell signaling involve an ERα-mGluR1a interaction. The available evidence suggests that the ER and the mGluR are inserted together into the cell membrane as a complex [89]. To confirm this, we tested whether estradiol regulated ERα and mGluR1a trafficking in astrocytes. Indeed, ERα and the mGluR1a were trafficked to the cell membrane and internalized in tandem [87]. The rapidity of the estradiol-induced insertion into the cell membrane suggests a delivery mechanism consisting of exocytic vesicles loaded with the ERα-mGluR1a complex. In support of this, es-

tradiol induces exocytosis of ERα-immunoreactive vesicles in hippocampal neurons and pituitary cells [137]. Trafficking of ERα to the membrane requires palmitoylation and association with calveolin proteins [91, 92]. Calveolin proteins determine the association of ERα and ERβ with specific mGluRs (fig. 1) [75, 89]. For example, ERα interaction with either mGluR1 or mGluR2/3 is dependent upon either caveolin 1 or caveolin 3, respectively [reviewed in 75]. Disrupting calveolin synthesis prevented insertion into the membrane. Similarly, antagonizing mGluR1a with LY 367385 or ERα with ICI 182,780 prevented trafficking of both the mGluR1a and ERα.

In summary, these results indicate that astrocytes have an important function within the CNS network that regulates reproduction. These cells express a functional membrane ERα that associates with mGluR1a to activate GPCR cell signaling pathways. Circulating estradiol regulates the levels of membrane ERα on the astrocyte membrane thereby modulating its own membrane-initiated cell signaling [87]. In the context of estrogen-positive feedback, rising estradiol levels increase the concentration of membrane ERα and mGluR1a in hypothalamic astrocytes (fig. 5). The ERα-mGluR1a complex is activated by spiking estradiol levels on the morning of proestrus, releasing intracellular calcium stores that stimulate neuroprogesterone synthesis. The transient increase of neuroprogesterone stimulates local estradiol-induced PRs initiating the LH surge. Rapid estradiol signaling and progesterone synthesis are constrained by the internalization and eventual degradation of ERα, preventing continuous signaling. Thus, membrane-initiated estradiol signaling in astrocytes is an important step in the regulation of reproduction.

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