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# Membrane estrogen receptors activate metabotropic glutamate receptors to influence nervous system physiology

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## Abstract

Until recently, the idea that estradiol could affect cellular processes independent of nuclear estrogen receptors was often dismissed as artifact. This in spite of a large number of carefully controlled studies performed both within and outside the nervous system demonstrating estrogens regulate various intracellular signaling pathways by acting at the membrane surface of cells and/or at biological rates incompatible with the time course of genomic-initiated events. The concept that estradiol can act on surface membrane receptors to regulate nervous system function is now far less controversial. However, there is evidence that there may be multiple types of estrogen receptors on the membrane surface of cells. Determining the physiological relevance of each of these receptors is currently underway. Two important membrane estrogen receptors are in fact the classical estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) proteins, which is somewhat surprising based upon their well-established role in nuclear gene transcription. This review will focus on the mechanism by which surface-localized ERα and ERβ stimulate intracellular signaling events in cells of the nervous system through activation of metabotropic glutamate receptors (mGluRs). This mechanism of estrogen receptor function also requires caveolin proteins, which provide the subcellular compartmentalization of the particular signaling components required for appropriate cell stimulation. The review will conclude with several examples of physiological processes under the apparent regulation of ER/mGluR signaling.

### Keywords

Estradiol; MGluR; Lordosis; Nociception; Membrane; Rapid actions

## 1. Classical estrogen receptor signaling

Researchers have studied the effects of gonadal hormones on brain function for quite some time [1]. Across the lifespan of an organism, hormones such as estrogens affect nervous system function through alterations in anatomy and/or physiology. Principle roles of estrogens include its regulation of sexual development, maturation and reproductive behaviors. Following the cloning of the first estrogen receptor [2,3], ERα was determined to be a ligand-regulated transcription factor [4,5]. This was consistent with previous work demonstrating the actions of estradiol were dependent on the translation of new protein [6,7]. Furthermore, the distribution of ERα [8,9] was tightly correlated with steroid autoradiography studies [10,11], which found the highest levels of estrogen binding in brain regions critical for reproductive success. A simple, single model for estrogen action continued to be developed as ERα was



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found to be located primarily in the nucleus [12,13], where it would bind DNA at estrogen response elements (EREs) as a dimer once bound to steroid [14].

These ERα-mediated changes in gene expression and protein synthesis are referred to as the classical mechanism of estrogen action. The complexity of ER-mediated gene expression has expanded with the finding of a second estrogen receptor, ERβ [15] and diverse ER interactions with various co-activators and other transcription factors [16–18]. This interplay between ERs and various other nuclear machinery involved with gene transcription account for the diversity of estrogen-regulated genes, including those which lack EREs. In addition, ERs can be activated in the absence of estrogens [19–24], making the classical model of estrogen action in brain far from simple. Yet, even with the many adaptations required to expand the original model of estrogen action to fit these additional findings, major support was lacking within the field of neuroendocrinology for estradiol affecting cell function outside of a transcriptionally-initiated event.

## 2. Classical versus novel mechanisms of estrogen action

Along with the evidence that the classical effects of estradiol in brain went far beyond the simple model of ERα-induced gene expression, another paradigm shift (i.e. estrogens act at the surface membrane to regulate neuronal function) attempted to gain traction. This was in response to three novel, but related, themes in the literature. The first being multiple discoveries of estrogen action within areas of the nervous system not associated with reproduction and a corresponding revelation that various non-reproductive behaviors are affected by estrogens. The second focus was that many of the effects of estradiol affecting neuronal function occur on a time scale too rapid to be accounted for by the classical mechanism of action. Third, many of these rapid effects appear to be initiated by estradiol acting at the surface of the neuronal membrane.

In neurons, Kelly et al. were the first main proponents of estradiol having rapid effects. They showed that within seconds, the hormone altered the electrical activity of preoptic and septal neurons [25]. Of note, rapid actions of estrogens were observed not just within the nervous system, but also in various other tissues. For example, one of the first reported non-classical effects of estrogens was on the accumulation of cAMP in uterine tissue. Szego and Davis reported that concentrations of cAMP increased within 15 s of estrogen application [26]. Regardless of the preparation, the work these investigators and others studying rapid actions of steroid hormones was initially met with tremendous skepticism. However, with time and continued experimental resilience, initial skepticism was gradually replaced with a general agreement that estrogens could indeed act at the neuronal membrane to affect cellular function.

Recent studies have demonstrated estrogen modification of cell excitability through modulation of ion channels in many other brain regions [27–29]. Various intracellular signaling proteins are also affected by membrane estrogen signaling, including activation of protein kinase A (PKA), protein kinase B (AKT), protein kinase C (PKC), phospholipase C (PLC), inositol triphosphate (IP3), and mitogen-activated protein kinase (MAPK) [30–41]. Not surprisingly then, estradiol through novel mechanisms can affect gene expression and protein synthesis through the activation of transcription factors such as cAMP response element-binding protein (CREB) [34,42–44]. These rapid actions of estrogens have been found relevant to a whole host of behaviors, such as learning and memory, motor control, mood and pain perception [45–48]. Interestingly, the regions of the nervous system critical to these behaviors were originally thought to have little or no expression of the classical ERs, ERα and ERβ. Thus, the question was raised as to the mechanism by which estradiol was able to exert these novel effects.

While novel information was escalating regarding rapid effects of estradiol in the central nervous system, the theories of possible underlying mechanisms remained very much controversial. This was by and large due to the fact that many of the reported rapid effects appeared to be initiated at the membrane surface, determined through the utilization of membrane impermeable estrogen analogs [49,50]. The persistence of a novel action of estradiol following the intracellular dialysis of a cell with the steroid also supports this mechanism of action [29]. Indeed, estradiol had been shown to bind to the membrane of endothelial cells as early as 1977 [51]. However, due to the fact that a membrane estrogen receptor had not yet been identified, these results remained controversial.

In the 1980s, researchers began testing the hypothesis that the classical ER, ERα, could localize to the membrane surface [52,53]. These reports were often improperly discredited, on the claim that these findings were due to a technical artifact; i.e. contamination of membrane fractions with transposed receptors from the nucleus during the isolation procedure. In addition, the known structure of these classical receptors provided no clue as to how they would be membrane-localized, as well as be able to activate intracellular signaling even if they were trafficked to this region of the cell. However, in 1999, overexpression of ERα and ERβ revealed that a portion of the classic ER protein was targeted to the membrane and activated intracellular signaling [54]. This simple and elegant experiment demonstrated that the same protein is capable of mediating both intracellular and membrane actions of estradiol. And while membrane-localized ERα and ERβ still maintains some controversy within the nervous system, in other cell types it is clearly well established and virtually undisputed (for numerous examples, see the other articles in this special issue).

## 3. Estrogen receptor interactions with mGluRs

One often studied indication of rapid estradiol action has been the phosphorylation (activation) of CREB [34,35,42,44,55]. Phosphorylation of CREB is an important node in cell signaling, critically involved in various forms of neuronal plasticity. Several laboratories have found that the activation of surface estrogen receptors leads to CREB phosphorylation via stimulation of the MAPK/ERK signaling pathway. In turn, activated CREB regulates gene expression though interaction with DNA at CREB response elements (CREs). These and other novel estradiol actions are blocked by the ER antagonist ICI 178,820, whereas ERα and ERβ agonists frequently mimic the actions of the steroid [42,56]. Such results provided pharmacological evidence that classical ERs play a role in the novel actions of estradiol. While these data still provide room to argue for a unique membrane estrogen receptor, debate whether classical estrogen receptors were at least partially responsible for mediating some of the reported rapid effects essentially ended when Herbison and colleagues, using ER knock-out mice, determined that the rapid actions of estradiol on phosphorylation of CREB and MAPK were dependent on ERα and ERβ [57]. However, with the end of one controversy, another quickly surfaced: that is, how do classical ERs initiate cell signaling when localized to the membrane? Moreover, how are ERs trafficked to the membrane in the first place?

Clues to the mechanism by which membrane-localized ERα and ERβ exert effects on cell function included numerous reports that describe estrogen action to be sensitive to G-protein manipulation [29,41]. Based upon these data, a relatively straightforward hypothesis, i.e. ERα and ERβ directly bind and activate G-proteins, was put forth [58,59]. In support of this mechanism, ERα can directly interact with G protein subunits [60]. Yet, the diverse array of signaling pathways regulated by membrane estrogen receptors suggests additional mechanisms of action. Outside of the nervous system, membrane estrogen receptors have been found to directly bind and activate surface receptors linked to various second messenger systems [61–63]. In parallel, we find membrane-localized ERα and ERβ capable of activating various metabotropic glutamate receptors (mGluRs).

Our initial experiments performed in cultured hippocampal neurons found that estradiol stimulation of ERα resulted in increased CREB phosphorylation through activation of mGluR1 [42]. mGluR1 and mGluR5 comprise the group I mGluRs, which are Gq linked and were previously shown capable of activating CREB [64,65]. Mechanistically, mGluR1 stimulation leads to MAPK-dependent CREB phosphorylation via activation of phospholipase C (PLC), protein kinase C (PKC) and inositol trisphosphate (IP3) signaling. Interestingly, the activation of ERα by estradiol was only effective in triggering CREB phosphorylation in cultures derived from female, and not male, hippocampus. The underlying cause for this sex difference is currently being investigated.

The actions of ERα on mGluR1 required only picomolar concentrations of estradiol. Furthermore, CREB phosphorylation was observed within 30 s of steroid application (with maximal responses at 2 min following estradiol administration). With the additional evidence that non-permeable estrogen analogs and ERα agonists mimicked the response of estradiol and that the pure estrogen receptor antagonist ICI 182,780 blocked the actions of estradiol, we concluded ERα at the membrane was responsible for triggering CREB phosphorylation.

As mentioned, estradiol has been observed to stimulate a variety of intracellular signaling cascades. In striatal neurons, we had previously reported that estradiol, through activation of a G-protein coupled receptor, could decrease L-type calcium channel currents [29]. This has subsequently been confirmed in various other neuronal systems [66,67]. This is of particular importance because calcium entry through L-type calcium channels can rapidly trigger CREB phosphorylation through activation of calcium calmodulin-dependent protein kinase IV (CaMKIV). Consequently, we found estradiol to also decrease L-type calcium channel-dependent CREB phosphorylation. Estrogen inhibition of L-type calcium channel-dependent CREB phosphorylation was dependent upon activation of the group II mGluRs, mGluR2 and/or mGluR3 [42]. These mGluRs are functionally linked to Gi/o second messenger signaling. The only major difference between estradiol activation of mGluR1 versus mGluR2/3 was that the latter was triggered by both ERα as well as ERβ.

It was of particular interest that we observed the bidirectional affects (i.e. activation of both mGluR1 and mGluR2/3 signaling) of estradiol upon CREB phosphorylation within the same population of cells. Isolation of one pathway versus the other was first achieved through pharmacological manipulation. However, in a follow up study, we were able to independently disrupt one signaling pathway or the other through modification of caveolin expression and/or function [68]. Caveolin proteins are membrane proteins that organize signal transduction machinery [69]. Originally described outside of the nervous system, caveolin proteins were found essential for various membrane ERα responses (reviewed in Ref. [70]). In hippocampal neurons, the caveolin-1 protein (CAV1) is essential for the functional coupling of ERα with mGluR1. Conversely, caveolin-3 (CAV3) is necessary for ERα and ERβ activation of mGluR2/3 [68]. To our knowledge, this is the first demonstration of functionally discrete microdomains within the same cell being generated by different caveolin proteins.

Our recent studies have examined putative ER/mGluR interactions in other brain regions. We have focused our efforts in striatum, where rapid effects of estrogens have been reported for some time [71]. Analogous to our results in hippocampal tissue, activation of ERα leads to the phosphorylation of CREB, whereas ERα and ERβ attenuate L-type calcium channel-dependent CREB phosphorylation. Both pathways were similarly dependent upon CAV1 and CAV3. To our surprise, however, the mGluRs responsible for estrogen signaling to the nucleus were different. While ERα is coupled to mGluR1 in hippocampus, it is mGluR5 in striatum. Likewise, recent findings suggest ERα/ERβ activates mGluR2 signaling in hippocampus but mGluR3 in striatum [72]. These results are particularly intriguing as all four mGluRs are expressed in both hippocampus and striatum. Regardless of the mechanism for differential

pairing of ERs and mGluRs across neuronal subtypes, these data suggest ERs may be coupled to various GPCRs, and not a select population. This may account for the widespread estrogen action within the nervous system. At the very least, ERs can have diverse effects on neuronal cell excitability through pairing with different caveolin and mGluR proteins (Fig. 1).

## 4. Physiological relevance of ER/mGluR signaling

While our work has focused on elucidating the mechanisms by which membrane ERs regulate neuronal function, and thus have used a more reductionist approach, it is essential to demonstrate these same signaling pathways are present and relevant in more intact preparations, and thus show physiological relevance. The laboratory of Micevych has examined three separate model systems previously demonstrated to be regulated by membrane estrogen receptors. In each of these systems, ER/mGluR coupling was deemed essential.

The first series of experiments examined estradiol signaling in the arcuate nucleus and its role in female sexual receptivity. In the female rat, estradiol acts on a limbic-hypothalamic circuit to allow the expression of lordosis, a stereotypic sexually receptive behavior [73,74]. It has been known for almost 30 years that estradiol-induced lordosis behavior is dependent on the transcription of new proteins [7,75]. However, priming animals with a membrane-constrained estradiol (E-6-BSA) followed with a subthreshold dose of estradiol was as efficacious as two estradiol injections [76]. Thus membrane actions of the steroid are also important for lordosis. Previous work by the Micevych lab had determined a critical, estrogen-sensitive pathway from the arcuate nucleus to the medial preoptic area [77,78]. In the arcuate nucleus, ERα was found to be co-localized with mGluR1a. In addition, estrogen action in the arcuate nucleus upon lordosis behavior was critically dependent on mGluR1 signaling [79].

This first demonstration of ER/mGluR coupling in hypothalamic neurons was followed by an equally elegant demonstration in hypothalamic glial cells. Previous studies have shown that estradiol administration results in an increase in intracellular calcium within these cells. The resulting rise in calcium is believed to be critical in the synthesis of neuroprogesterone and the LH surge [80]. Similar to the findings in neurons, the estrogen-dependent rise in intracellular calcium within glial cells was reliant upon interactions between membrane ER and mGluR1a. The estradiol-induced increase in intracellular calcium flux was blocked by both the classical ER antagonist, ICI 182,780 and the mGluR1a antagonist LY 367385 [81]. Additionally, ERα and mGluR1a were found to co-immunoprecipitate in hypothalamic glial cell membrane fractions, suggesting a direct interaction between these two receptors at the membrane surface [81].

In a third preparation, the functional coupling of ERs to mGluR2/3 was observed. The cell bodies of primary visceral spinal afferent neurons are located in the DRG. These cells transmit nociceptive information to the spinal cord. One such activator of these DRG cells is ATP, which has emerged as a putative signal for visceral pain. Noxious stimuli such as distention of the viscera or tissue damage release ATP [82], which then transduces noxious stimuli by activating purinergic, ATP-gated P2X receptors on primary afferent fibers [83]. Opening of P2X channels results in membrane depolarization sufficient to trigger action potentials and calcium influx through voltage-gated calcium channels associated with nociception [84]. The vast majority of ATP-sensitive DRG neurons respond to estradiol [66]. Estradiol was found to inhibit ATP-mediated calcium influx in small diameter DRG neurons, though inhibition of L-type calcium channels. Further, estradiol inhibition of L-type calcium channels was dependent on mGluR2/3 [85].

## 5. Conclusions

It has now become widely accepted that the actions of estrogens within the brain far exceed its classical effects upon sexual behavior and maturation. Indeed, estradiol has been shown to induce a multitude of rapid, membrane-initiated events within various regions of the brain. These novel effects have been demonstrated to play a crucial role in diverse behaviors such as learning and memory, motor control, mood and pain perception. Although this has become a burgeoning and exciting field of research to many, there is still much dispute regarding the underlying cellular mechanism(s) by which the steroid hormone elicits these novel actions. By further examining the interactions between membrane-localized ERs and other proteins such as mGluRs and caveolins, our understanding of the wide-ranging effects of this steroid hormone on brain function may be greatly expanded.

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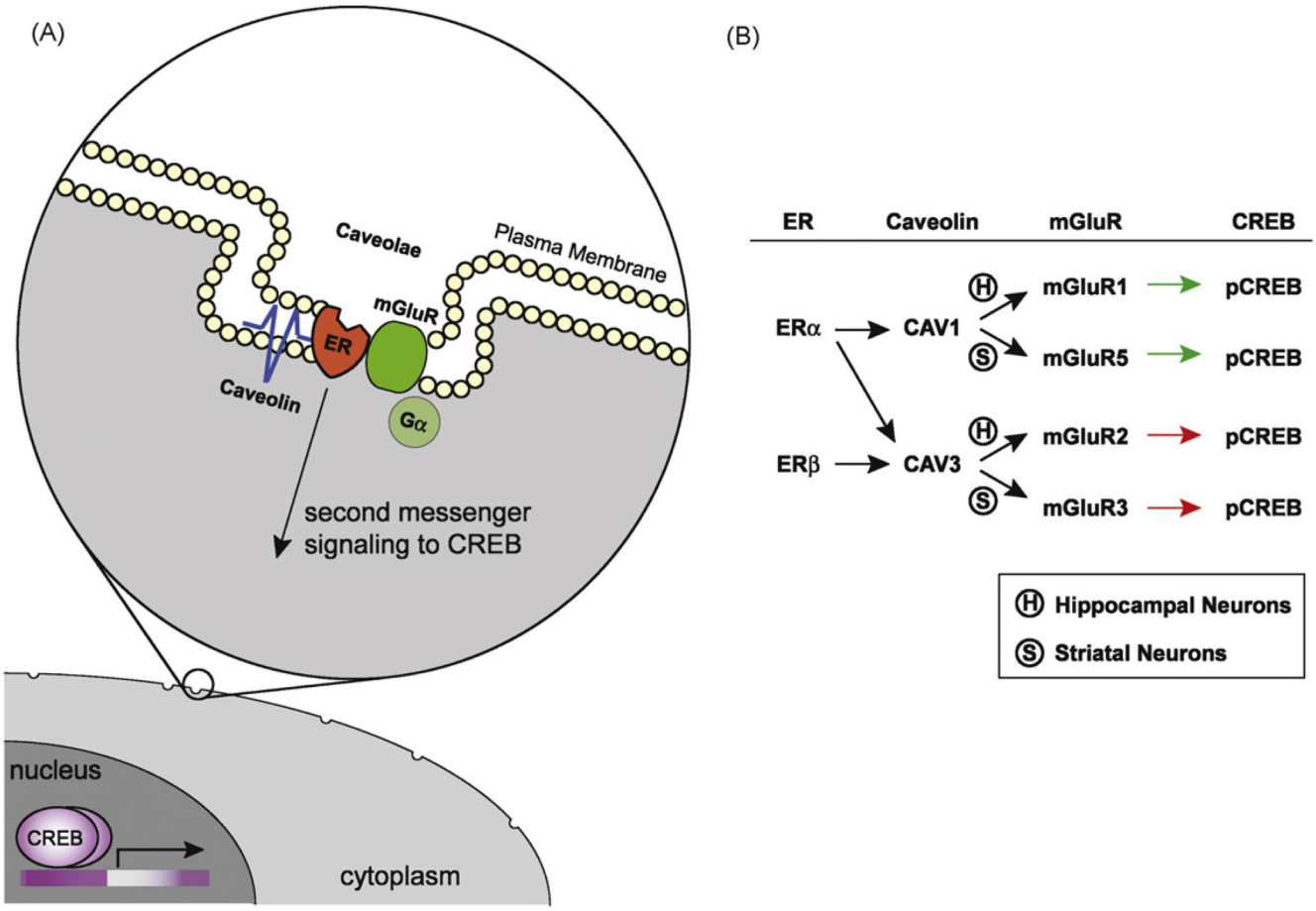


**Fig. 1.**

Estrogen receptor activation of mGluR signaling through interactions with caveolin proteins. (A) Model system of estradiol-induced activation of mGluRs via caveolin-based caveolae. (B) Summary of distinct signaling pathways by which ERα and ERβ can affect CREB phosphorylation.