Mol Neurobiol (2008) 38:66–77
DOI 10.1007/s12035-008-8034-z

Membrane Estrogen Receptors Acting Through Metabotropic Glutamate Receptors: An Emerging Mechanism of Estrogen Action in Brain

Paul E. Micevych · Paul G. Mermelstein

Received: 29 April 2008 / Accepted: 18 June 2008 / Published online: 2 August 2008

Abstract It has been over 60years since the first studies have been published describing the effects of steroid hormones on brain function. For over 30years, estrogen has been presumed to directly affect gene expression and protein synthesis through a specific receptor. More than 20years ago, the first estrogen receptor was cloned and identified as a transcription factor. Yet, throughout their course of study, estrogens have also been observed to affect nervous system function via mechanisms independent of intracellular receptor regulation of gene expression. Up until recently, the membrane estrogen receptors responsible for these rapid actions have remained elusive. Recent studies have demonstrated that a large number of these rapid, membrane-initiated actions of estradiol are due to surface expression of classical estrogen receptors. This review focuses on the importance of membrane estrogen receptor interactions with metabotropic glutamate receptors for understanding rapid estradiol signaling mechanisms and downstream effectors, as well as their significance in a variety of physiological processes.

Keywords Estradiol · mGluR · Lordosis · Nociception · Pain · Estrous cycle · Membrane · Rapid actions

P. E. Micevych (✉)
Department of Neurobiology and Laboratory
of Neuroendocrinology of the Brain Research Institute,
David Geffen School of Medicine at UCLA,
Los Angeles, CA 90095, USA
e-mail: pmicevych@mednet.ucla.edu

P. G. Mermelstein (✉)
Department of Neuroscience, University of Minnesota,
6-145 Jackson Hall, 321 Church St. S.E.,
Minneapolis, MN 55455, USA
e-mail: pmerm@umn.edu

Introduction

For more than 60years, researchers have studied the effects of steroid hormones on brain function [1]. From the outset, it was clear that hormones such as estrogens could profoundly affect development as well as adult behavior. To understand the mechanism(s) by which estrogens act within the central nervous system, initial experimenters utilized steroid autoradiography techniques [2, 3]. This method identified brain regions in which the steroid would bind, thus presumptive sites of estrogen receptor expression. Areas of the brain identified by this technique included the hypothalamus, pituitary, and preoptic area, amongst others, which, based on a series of lesion and stimulation studies, were known to affect physiology and behaviors related to endocrine function [4]. The yet to be characterized estrogen receptor was presumed to affect gene expression, as estrogen action on the central control of reproduction was dependent on the transcription of new protein [5, 6]. With the cloning of the first estrogen receptor [7, 8], our ability to study estrogen receptor function greatly expanded.

The distribution of estrogen receptor messenger RNA (mRNA), especially the receptor now known as estrogen receptor-alpha (ERα), within the nervous system [9, 10] confirmed steroid autoradiography studies [2, 3]. Furthermore, ERα was characterized as an intracellular, ligand-regulated transcription factor [11]. As such, ERα is located primarily in the nucleus [12, 13]. Once bound to estradiol, ERα dimers were shown to regulate gene expression via action at estrogen response elements (EREs) [14].

These ERα-mediated changes in gene expression and protein synthesis are commonly referred to as the classical mechanism of estrogen action. Following a series of discoveries, this classical mechanism required further

refinement, including the identification of a second, but structurally related, estrogen receptor (estrogen receptor-β, ERβ) [15]; ER regulation of transcription factor and co-factor interactions [16, 17]; and ER regulation of gene transcription independent of EREs. This latter finding provided an explanation for why the selective estrogen receptor mediator, tamoxifen, had both antagonistic and agonistic properties, albeit in different tissues. ERs can stabilize the binding of Fos and Jun to the AP-1 site [18]. In such tissues, tamoxifen acts as a receptor agonist. When tamoxifen acts as an antagonist, a classic ER–ERE interaction is present [18]. Finally, estrogen receptors have also been shown to act in the absence of estrogens [19, 20], adding another layer of transcriptional complexity.

Classical Versus Novel Mechanisms of Estrogen Action

With the identification of a clearly defined receptor that localized to brain regions that are both sensitive to estradiol as well as being critical to neuroendocrine function, the fundamental question of how estrogen acts on the brain seemed to be answered. Further supporting this notion of estradiol action in the nervous system were the parallel findings in endocrine tissue [21, 22]. However, as far back as 30–40years, studies described phenomena that could not be explained by the classical mechanism of estrogen action. When the first reports of extremely rapid effects of estrogens were published, they were met with great skepticism. And while today there is general agreement that these novel actions of estradiol are real, there are still many questions and controversies that remain regarding the exact mechanism(s) by which these effects occur and their physiological relevance.

As alluded to in the previous paragraph, for many years, the principal defining feature of a non-classical or novel effect of estrogen was the timing in which a response to steroid application could first be measured. One of the first reported, non-classical effects of estrogens was on the accumulation of cAMP in uterine tissue. Clara Szego and June Davis reported that concentrations of cAMP increased within 15s of estrogen application [23]. In neurons, Robert Moss and Martin Kelly were the main proponents of estradiol having rapid effects. They showed that within seconds, the hormone altered the electrical activity of preoptic and septal neurons [24]. Since then, modification of cell excitability through modulation of ion channels has been observed in various other brain regions [25–27]. Another term often used to distinguish between classical and novel actions of estradiol has been genomic versus non-genomic effects. This definition was once popular because novel actions of estrogens were thought to be limited to transient alterations in cell excitability. Today, we know this not to be the case, as 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) [28–31]. It should also be mentioned that, while the timing of estradiol action was also once thought to clearly differentiate between classical and novel effects of the hormone, this line has also been blurred. It was previously considered that classical actions of estradiol required hours for the onset of an observable effect, with durations of action lasting for days. In contrast, novel effects appeared to be much more rapid with effects observable in seconds to minutes and present only for the duration of hormone application. With more powerful and sensitive scientific techniques, classical actions of estradiol can be observed within minutes. A similar convergence has occurred with estradiol action ascribed to novel receptors. Novel estradiol actions are now known to activate transcriptional events that have long-term consequences on nervous system function that far exceed the duration of estradiol application.

A common criticism of membrane actions is that they often require higher concentrations of estradiol than the traditional transcriptional regulation. These estradiol levels have been characterized as non-physiological or, less charitably, pharmacological and even toxicological. The issue of steroid concentration needed to activate membrane receptors is a difficult one to deal with since estradiol concentrations needed to activate membrane receptors is usually two to three orders of magnitude above peak circulating levels in vivo. There are several possible explanations for this. One idea suggests that, while in the membrane, the estrogen receptor steroid binding site is constrained, requiring higher levels of estradiol for binding [32]. Another proposes that the membrane ER may act as a sensor to detect changes in estradiol concentration rather than the absolute level. Still another idea based on classic G protein-coupled receptor (GPCR) physiology is that, although the binding affinity of a receptor may be relatively low, the concentration of ligand needed to elicit a cellular response is somewhat less than predicted by binding studies due to the non-linear nature of GPCR signaling [33]. However, as methods for examining these membrane effects of estradiol become more refined, the concentrations needed to elicit an effect have approached or even matched the K_d of the intracellular receptor [27, 28, 34, 35]. Finally, this discussion may be moot since brain concentrations of estradiol are not actually known. Indeed, there are suggestions that local estradiol concentrations in hypothalamus are much higher than in the circulation [36]. The lipid-rich brain environment may depot estradiol that is slowly released, and testosterone may be aromatized locally—raising local estradiol concentration into the range that stimulates various membrane ER processes.

Along with the 'classical' and 'novel' nomenclature used to classify these two modes of estradiol action, there is a second, more descriptive terminology. While classical effects require the activation of intracellular receptors, novel actions appear to be initiated at the membrane surface. This has been typically determined through the utilization of membrane impermeable estrogen analogs [37, 38]. The persistence of a novel action of estradiol following the intracellular dialysis of a cell with the steroid also supports this mechanism of action [27]. Following this logic, estradiol would require the expression of an estrogen receptor on the cell membrane. Pietras and Szago [39] first demonstrated estradiol binding to endothelial cell membranes, suggesting a putative membrane ER, but because there was no indication that a novel molecule bound estradiol at the membrane, these results languished. Since this initial investigation, the numerous reports of membrane localization of ERα and ERβ were argued to be artifact and thus often dismissed out of hand. The idea behind such arguments was that an ER in the membrane must be a completely different protein. 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 [40]. This simple and elegant experiment demonstrated that the same protein is capable of mediating both intracellular and membrane actions of estradiol.

One often studied indication of rapid estradiol action has been the phosphorylation (activation) of CREB [29, 31, 41, 42]. 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 mitogen-activated protein kinase (MAPK) or externally regulated kinase 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 [28, 43]. 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 Alan Herbison and colleagues, using ER knockout mice, determined that the rapid actions of estradiol on phosphorylation of CREB and MAPK were dependent on ERα or ERβ [44]. However, with the end of one controversy, another quickly surfaced: That is, how do classical ERs, known to be transcription factors, initiate cell signaling when inserted in the membrane? Moreover, how are ERs trafficked and inserted into the membrane?

Estrogen Receptor Interactions with mGluRs

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. Based upon these data, a relatively straightforward hypothesis, i.e., ERα and ERβ directly bind and activate G-proteins, was put forth [45, 46]. Although temptingly straightforward, this hypothesis does not address the myriad of cell signaling cascades activated by estradiol. Moreover, G protein receptors as a family are characterized as integral membrane proteins with a conserved structure that includes seven transmembrane helices. The structure of estrogen receptors does not resemble the consensus structure of GPCRs. Based on these issues, we chose to examine another possibility. What if ERs interact with and activate known GPCRs. While in some respects this just alters the basic deficiency in our knowledge (i.e., how estrogen receptors activate GPCRs), there is accumulating evidence that estrogen receptors are capable of trans-activating surface membrane proteins.

Our recent findings provide evidence for such an alternative explanation for ERα and ERβ regulation of G-protein signaling. In various cells of the nervous system, we find ERα and ERβ to activate metabotropic glutamate receptors (mGluRs). mGluRs are a family of GPCRs that, when binding glutamate, initiate G protein signaling. Three groups of mGluRs have been identified based on sequence homology and second messenger linkage. mGluR1 and mGluR5 comprise the group I mGluRs and are coupled to Gq. In contrast, group II mGluRs are the mGluR2 and mGluR3 receptors that activate Gi/Go signaling. mGluR4, mGluR6, mGluR7, and mGluR8 comprise the group III mGluRs, which are also Gi/Go-coupled. Activation of group I mGluRs has been demonstrated to phosphorylate CREB through the same signaling pathway as estradiol [47, 48]. Consequently, in cultured hippocampal neurons, estradiol-induced MAPK-dependent CREB phosphorylation was dependent on membrane estrogen receptor stimulation of mGluR1. In addition, L-type calcium channel-dependent CREB phosphorylation (in this case, CREB is phosphorylated by calmodulin-dependent protein kinase IV, CaMKIV) was attenuated by estrogen receptor activation of group II mGluRs [28]. This action of estradiol in hippocampal tissue was only found in neurons generated from female and not male tissue.

We subsequently determined that estrogen receptor interactions with mGluRs are dependent upon caveolin proteins [34], known to be essential for the trafficking and clustering of signaling molecules. The importance of caveolin proteins in the trafficking of ERα to the membrane was first demonstrated outside of the nervous system [49] and in which palmitoylation of the ER was also required [50, 51]. ER interactions with caveolins and mGluRs do not

appear random. In hippocampal neurons, ERα interaction with either mGluR1 or mGluR2/3 was dependent upon either caveolin 1 (CAV1) or caveolin 3 (CAV3), respectively. Conversely, ERβ only interacted with mGluR2/3 via CAV3. The pairing of particular ERs with mGluRs is observed in various other brain regions. For example, ERα is associated with mGluR1 in hypothalamic neurons in vivo [52], whereas ERβ activation of mGluR2/3 has been observed in dorsal root ganglia [53]. Similarly, rapid estradiol signaling in astrocytes is also dependent on mGluR1a [54].

More recently, we have found that ERα via CAV1 activates mGluR5-based signaling in striatal neurons [55]. This is of particular interest because striatal neurons express both group I mGluRs, mGluR1 and mGluR5. In striatal neurons, mGluR1 signaling was unaffected by estradiol. Upon further study, hippocampal neurons also demonstrate functional coupling of mGluR1 and mGluR5 to CREB. Yet, estradiol does not affect mGluR5 signaling in the hippocampus (Boulware and Mermelstein, unpublished). It remains to be determined why, across different neuronal populations, ERs would be paired with discrete types of mGluRs that are known to activate the same second messenger systems.

Functional isolation of different ERs with mGluRs suggests a diverse array of potential estrogen-sensitive signaling pathways at the disposal of individual cells (Fig. 1). The generation of specific ER/mGluR pairs via caveolin function may eventually be found to be responsible for many of the diverse observations of novel estrogen signaling in the central nervous system. And while the question as to how ERα and ERβ activate GPCRs remains to be answered, there are previous precedents that provide the basis of a potential mechanism.

It is our working hypothesis that ERs activate mGluRs through a direct protein–protein interaction. Upon estradiol binding to the ER, a conformational change, similar to what occurs when glutamate binds the receptor, results in mGluR activation of the G-protein and initiation of second messenger signaling. In non-neuronal tissue, estrogen receptors have been found to directly bind tyrosine kinase receptors [56], which are in turn activated following estrogen application [57]. This common mechanism of ER to surface receptor activation may account for the novel actions of estradiol outside of the brain, as mGluR expression is nervous system-specific. Notably, this does not preclude the converse possibility, i.e., that ERs interact with tyrosine kinase receptors in the central nervous system (CNS) as well. Indeed, these interactions occur in the hypothalamus to influence reproduction [58] as well as in the substantia nigra where it appears to mediate neuroprotection [59]. In terms of evidence for this putative mechanism, co-immunoprecipitation experiments suggest that ERs can directly interact with mGluRs [52] (Fig. 2a). In vivo experiments utilizing an mGluR agonist and an estrogen receptor antagonist strongly suggest that an ER/mGluR protein–protein interaction can alter the function of mGluR signaling. Figure 2b examines CREB phosphorylation following the activation of mGluR1 with (*S*)-3,5-dihydroxyphenylglycine (DHPG), a selective group I mGluR agonist. Activation of CREB by DHPG is attenuated following the application of the estrogen receptor antagonist. This action of ICI 182,780 does not appear due to a direct effect on mGluR1; as in cultures from male animals that lack an estrogen response, ICI does not influence the actions of DHPG. Future research will need to determine more definitively the mechanism by which ERs activate mGluRs.

Physiological Aspects of Rapid Estradiol Signaling

One of the continuing controversies of rapid, membrane-initiated actions of estradiol relates to their physiological significance. That is, it has been argued that the classical mechanism of estradiol action can account for all estrogen-regulated behaviors. Yet, here we describe three separate estrogen-sensitive processes that require a "novel" mechanism

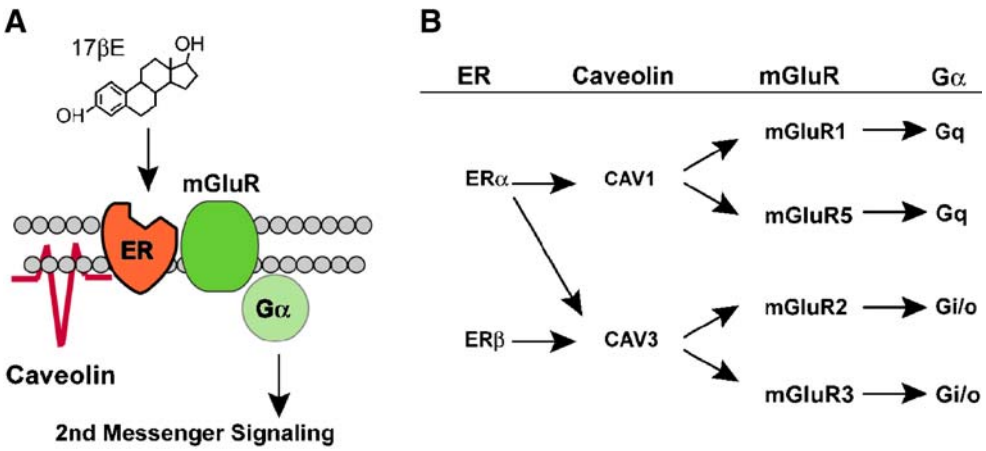

Fig. 1 Estrogen receptor activation of mGluR signaling through interactions with caveolin proteins. **a** Model framework of estradiol-induced activation of mGluRs via caveolin-based caveolae. **b** Summary of previous findings demonstrating estrogen receptor activation of group I (mGluR1/5) and group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

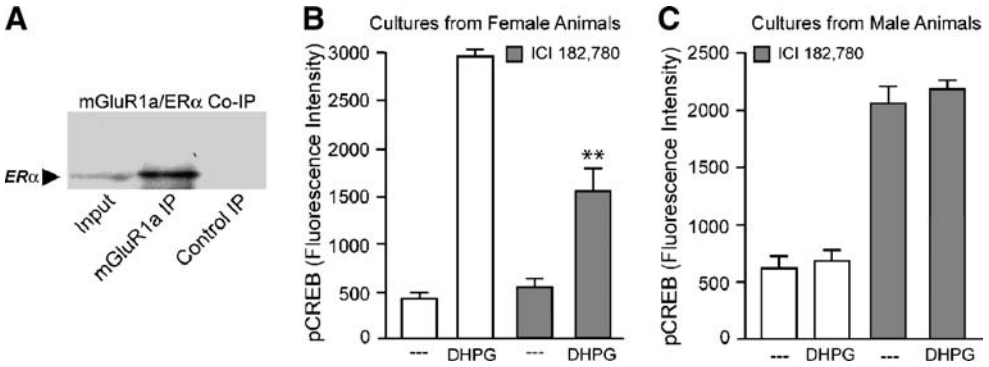

Fig. 2 Direct ER/mGluR interactions may underlie estradiol-induced activation of mGluRs. **a** Co-immunoprecipitation of endogenous ERα with mGluR1a in cultured hippocampal neurons. **b** Antagonism of ERs with ICI 182,780 attenuates CREB phosphorylation following the application of the group I mGluR agonist DHPG. **c** ICI 182,780 has no effect on CREB phosphorylation in cultures generated from male tissue, which lack the mGluR-mediated estrogen response

of estrogen action: regulation of lordosis, neuroprogesterone synthesis and its influence on the hypothalamic–pituitary–gonadal (HPG) axis, and signaling in dorsal root ganglion (DRG) neurons associated with nociception. In all three systems, we find a rapid component of estradiol that is dependent upon ER/mGluR signaling.

Sexual Receptivity Arguably, the best studied and most robust actions of estradiol in the brain have been on neural circuits controlling the HPG axis that regulate reproduction. Thus, it is appropriate to study the physiology of rapid estradiol actions in the brain within the context of reproduction. In the female rat, estradiol acts on a limbic–hypothalamic circuit to allow the expression of lordosis, a stereotypic sexually receptive behavior [60, 61]. Although lordosis can be elicited by implanting estradiol directly into the hypothalamus [62, 63], attempts to induce lordosis behavior exclusively through membrane actions of estradiol have not been successful. The assumption is that gene transcription is needed to elicit lordosis behavior. During the estrous cycle, estradiol rises slowly over several days before peaking on the afternoon of proestrus in rodents prior to the onset of sexual receptivity. Estradiol given at relatively low doses, without additional supplemental estradiol, will induce lordosis behavior approximately 48h later. It has been known for almost 30years that estradiol-induced lordosis behavior is dependent on the transcription of new proteins [6, 64]. As expected, a host of reproductively important neuropeptide mRNAs and proteins have been shown to be increased by estradiol [65–71]. To a first approximation, estradiol induction of female sexually receptive behavior has a time course that strongly implicates transcriptional actions of estradiol. 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 [72]. The interpretation favored by those authors is that rapid estradiol signaling facilitates nuclear ER-stimulated transcriptional events, suggesting cooperation between nuclear- and membrane-initiated actions of estradiol. These results indicated that rapid actions participated in the control of sexual receptivity but did not offer a mechanism through which these rapid actions were initiated.

To examine the rapid estradiol component involved in the facilitation of lordosis, we studied estradiol activation as a part of the extensive limbic–hypothalamic lordosis regulating circuit, involving a β-endorphin projection from the arcuate nucleus to the medial preoptic nucleus [73]. A hallmark of this circuit being activated is the rapid internalization of μ-opioid receptors (MOR) in the medial preoptic area that are associated with the regulation of lordosis behavior [74, 75]. Without MOR activation, lordosis is significantly attenuated [76, 77]. Further indication that this was a membrane-initiated estradiol action was obtained by facilitating lordosis behavior with administration of E-6-BSA directly into the arcuate nucleus. In the arcuate nucleus, ERα was colocalized with mGluR1a. Furthermore, the membrane fraction of ERα co-immunoprecipitated with mGluR1a, suggesting a potential functional association between these receptors [52]. When mGluR1a is blocked, estradiol-induced lordosis is attenuated. Interestingly, this blockade is only effective at the time of estradiol treatment and not several hours after estradiol, further suggesting that mGluR1 signaling is directly related to the rapid action of estradiol.

Our understanding of how the ER/mGluR interaction mediates behavior is illustrated in Fig. 3. Low systemic estradiol levels are insufficient to elicit lordosis behavior. The arcuate–medial preoptic circuit is quiescent. Membrane ERs in the arcuate nucleus are not stimulated, which is reflected by the cellular distribution of MOR in the medial preoptic area. The majority of MORs are localized on the cell membrane, indicating that these receptors have not been activated. In these conditions, the female rat is not sexually receptive (Fig. 3a). However, when systemic

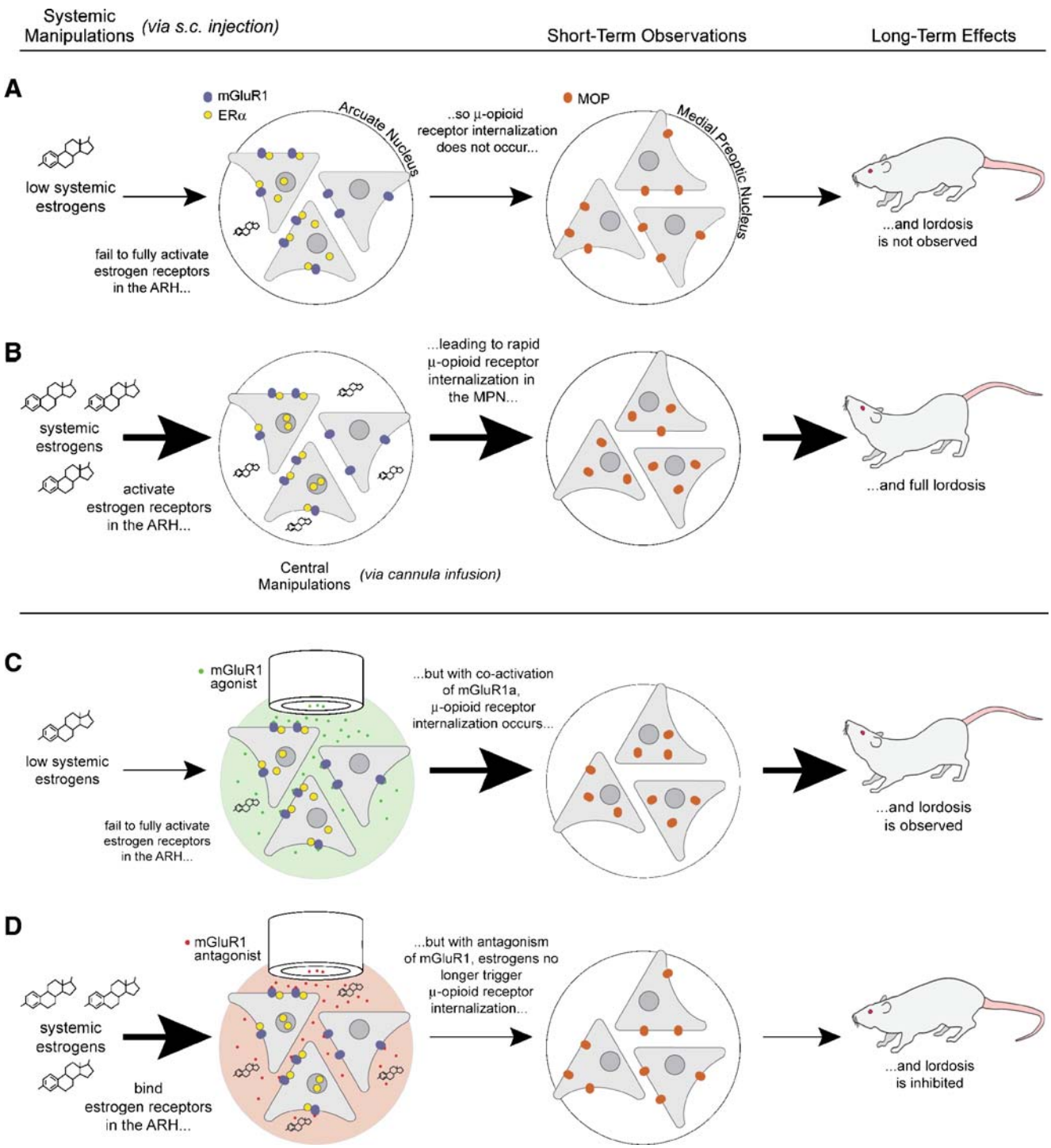

Fig. 3 ER/mGluR signaling in the arcuate nucleus (ARH)–medial preoptic nucleus (MPN) circuit regulates female sexual receptivity. **a** When circulating levels of estradiol are low, the circuit is not activated and the rat is not sexually receptive. **b** When circulating levels of estradiol are increased, estrogen receptors in the ARH are stimulated. A population of estrogen receptors, located on the plasma membrane, induces the release of β-endorphin in the MPN from ARH projection neurons activating and internalizing MORs, producing lordosis. **c** If mGluR1a is stimulated in the presence of low circulating estradiol concentrations, the ARH–MPN projection is activated: MORs are activated/internalized and lordosis is displayed. **d** Conversely, when mGluR1a is antagonized in the presence of high levels of circulating estrogen, the ARH–MPN circuit is not activated: MORs are not internalized and lordosis is attenuated

estradiol reaches levels that induce behavior, membrane ERs in the arcuate nucleus are activated, leading to MOR internalization and subsequent full lordosis behavior (Fig. 3b). Accordingly, when mGluR1a is directly stimulated with an agonist under low estradiol conditions, membrane ERs can be bypassed, resulting in MOR internalization and facilitation of lordosis (Fig. 3c). Conversely, under high estrogenic conditions, antagonizing mGluR1a blocks estrogen-induced MOR internalization and attenuates sexual behavior (Fig. 3d). These data are consistent with the in vitro demonstration of ERα/mGluR1a signaling in hippocampal neurons and provided the first in vivo evidence that estradiol can signal through activation of mGluR1a. By demonstrating that lordosis behavior, a classical assay of estradiol action, has a rapid non-genomic component underscores the importance of ER/mGluR interactions in the brain [52].

Neuroprogesterone Synthesis With respect to the LH surge, the most effective estradiol replacement strategies rely on a long time course, in excess of 48h [78], which is not compatible with rapid actions of estradiol. However, a number of tantalizing hints that the rapid effects of estradiol influence the estrous cycle have emerged over the years. Kelly and his colleagues have identified a number of rapid actions of estradiol, including regulation of potassium channels, activation of protein kinase C and protein kinase A pathways, and uncoupling μ-opioid receptors from $GABA_B$ receptors in guinea pig GnRH neurons [79]. Whether these actions are related to GnRH release is not yet known. In addition to these possible interactions, we have proposed a neurosteroid-based hypothesis to help understand the interaction between circulating estradiol and neuroprogesterone, progesterone synthesized de novo from cholesterol by the brain [80].

The brain, like the gonads and adrenal cortex, is a steroidogenic organ. All of the necessary steroidogenic enzymes and activities needed to synthesize sex steroids from cholesterol have been isolated in various parts of the brain [81]. One of the most intriguing steroids synthesized in the brain is neuroprogesterone. In addition to the myriad of progesterone-mediated actions, its metabolite, allopregnenalone, has profound effects on neuronal excitation through actions at the $GABA_A$ receptor [82, 83]. Neuroprogesterone itself has been shown to control aspects of reproduction [80]. The capacity of steroidogenesis in the cells of the CNS is widespread, but different cell types appear to preferentially produce specific steroids [84]. Progesterone is the preferred product of astrocytes [85, 86]. Interestingly, the synthesis of progesterone is widely distributed in the rat brain, with measurable levels in the hypothalamus, cortex, hippocampus, medulla, and cerebellum. Within the adult female rat hypothalamus, estradiol stimulates the synthesis of progesterone [87]. Since we were interested in understanding the interaction between circulating estradiol and neuroprogesterone synthesis, we treated primary astrocyte cultures with estradiol. Estradiol rapidly increased free cytoplasmic calcium influx by releasing intracellular stores of calcium [25]. This calcium influx is dependent on activation of the phospholipase C/inositol trisphosphate (PLC/IP_3) pathway and was blocked by an inhibitor of the IP_3 receptor. Similarly, estradiol increased the synthesis of progesterone that was dependent on a robust calcium influx [85]. To mimic the actions of estradiol on releasing IP_3 receptor-sensitive intracellular calcium stores, thapsigargin was used to induce the release of intracellular calcium. The effect was a stimulation of progesterone synthesis that was as robust as estradiol. Moreover, the increase of progesterone synthesis was seen after 1h of treatment, the earliest time point examined [85]. Thus, in astrocytes, like in ovarian cells, the stimulation of progesterone synthesis is dependent on calcium.

In the ovary, G protein-coupled gonadotropin receptors induce the calcium influx [88–91], but in the brain, it is estradiol that mobilizes calcium [25]. As in the arcuate nucleus, the mGluR1a antagonist LY367385 blocked estradiol-induced calcium influx, suggesting that, in astrocytes, the same ER-mGluR1a signaling was the result of an interaction between membrane ER and mGluR1a [54]. Further evidence of such an interaction is the co-immunoprecipitation of ERα and mGluR1a in the membrane fraction of astrocytes. Interestingly, while both estradiol and DHPG (a group I mGluR agonist) alone produced a robust influx of calcium, neither was as effective alone as they were together, indicating that for maximal signaling in astrocytes both glutamate and estradiol need to be present. These results suggest that estradiol may act most effectively on astrocytes that are near active glutamatergic terminals (Fig. 4).

Adenosine Triphosphate Signaling in DRG Neurons The cell bodies of primary visceral spinal afferent neurons are located in the DRG. Primary afferents transmit information about chemical or mechanical stimulation from the periphery to the spinal cord. Nociceptors are small to medium size DRG neurons whose peripheral processes detect potentially damaging physical and chemical stimuli. One such signal is adenosine triphosphate (ATP), which has emerged as a putative signal for visceral pain. Noxious stimuli such as distention of the viscera or tissue damage release ATP [92]. ATP transduces noxious stimuli by activating purinergic, ATP-gated P2X receptors on primary afferent fibers [93]. Opening of P2X channels results in membrane depolarization sufficient to trigger action potentials and calcium influx through voltage-gated calcium channels (VGCC) associated with nociception [94]. According to this theory, the pain of

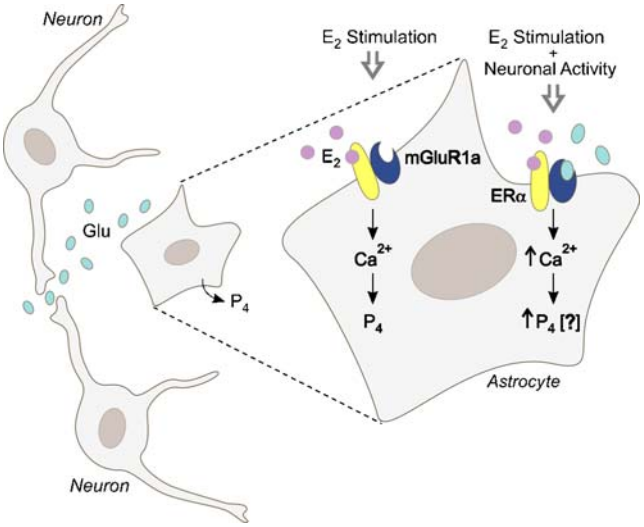

Fig. 4 Proposed mechanism through which estradiol signaling in astrocytes is integrated with local neuronal activity involved in the synthesis of neuroprogesterone. Estradiol (*E2*), typically of ovarian origin, binds to membrane ERα and activates the mGluR1a. This increases levels of free cytoplasmic calcium (Ca^2) through the inositol trisphosphate (IP_3) receptor-mediated release of intracellular stores of calcium. Elevated levels of intracellular Ca^2 are needed for neuroprogesterone (P_4) synthesis in astrocytes. Studies in vitro demonstrate that E2 alone or an mGluR1a agonist alone increase intracellular calcium levels. However, when both an mGluR1a agonist and E2 are applied to astrocytes, the resulting Ca^2 flux is significantly greater, suggesting that P4 synthesis is also augmented. We propose that in vivo when E2-stimulated astrocytes are in the proximity of active nerve terminals, the released glutamate (*Glu*) activates astrocyte mGluR1a, resulting in significantly greater Ca^2 responses. This elevated Ca^2 response is hypothesized to produce a greater P4 synthesis in astrocytes

tissue irritation (mechanical distortion or inflammation) is due to ATP activation of high threshold nociceptors [92, 95]. The predominant ATP receptor in small diameter nociceptive DRG neurons is the $P2X_3$ [96, 97]. $P2X_3$-null mice have reduced pain-related behavior in response to noxious stimuli [98, 99].

Several lines of evidence indicate that estradiol directly influences the function of primary afferent neurons. Both ERα and ERβ are present in DRG neurons including the small- to medium-diameter putative nociceptors [100]. In vitro, 85% of the ATP-sensitive DRG neurons respond to estradiol [101], which correlates well with the idea that visceral afferents are estradiol-sensitive. Evidence for this supposition include (1) visceral pain is affected by hormonal levels in cycling females [102–104], (2) there are sex differences in the prevalence of functional disorders involving the viscera [105, 106], and (3) putative visceral afferents [107] fit into the population of DRG neurons that are sensitive to estradiol [101]. These data suggest that, in addition to central actions of estradiol [108], estradiol can also act in the periphery to modulate nociception. ERs are distributed in regions of the central and peripheral nervous system that mediate nociception. For example, ERs are expressed in dorsal horn neurons of the spinal cord [109, 110] and DRG neurons [100, 111, 112]. Estradiol acts in neurons to modulate L-type VGCC [27, 101, 113]. Recent studies demonstrate that estradiol has a significant role in modulating visceral sensitivity, indicating that estradiol alterations in sensory processing may underlie sex-based differences in functional pain symptoms [114]. However, reports of estradiol modulation of visceral and somatic nociceptive sensitivity are inconsistent. For example, elevated estradiol levels have been reported to increase the threshold to cutaneous stimuli [115] but decrease the percentage of escape responses to ureteral calculosis [116]. On the other hand, nociceptive sensitivity appears to increase when estradiol levels are elevated [117]. Indeed, in most clinical studies, women report more severe pain levels, more frequent pain, and longer duration of pain than men [118, 119].

In a primary culture of DRG neurons, estradiol inhibited the ATP-mediated calcium influx in 85% small-diameter ATP-responsive DRG neurons. A dose–response curve demonstrated that the estradiol action was stereospecific and inhibited by ER antagonists tamoxifen and ICI 182,780. The cellular calcium response initiated by ATP has two components. The initial ion flux is through activated P2X channels, and the secondary response is the opening of VGCC in response to membrane depolarization [94]. The entire calcium transient was blocked with the purine receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, but the calcium response was only partially inhibited by estradiol, suggesting that estradiol did not directly antagonize P2X receptors. Specific VGCC channel blockers were used to characterize the VGCC blocked by estradiol. Nifedipine, an L-type VGCC blocker significantly attenuated the ATP-induced calcium influx. Furthermore, estradiol treatment did not result in additional inhibition, suggesting that estradiol acts on L-type VGCC. This result is consistent with an estradiol blockade of L-type calcium channels in PC-12 cells [120], neostriatal, and hippocampal neurons [27, 121]. ERα is needed for the estradiol attenuation of ATP-induced calcium influx as demonstrated by a lack of response to estradiol in DRG neurons from ERα knockout (ERαKO) mice. The estradiol response is retained in ERβ knockout (ERβKO) mice [122].

ERα interacts, in a tissue-specific manner, with mGluR2/3 [28, 34]. To determine if the estradiol inhibition of ATP-induced Ca^2 signaling required mGluR2/3, astrocytes were stimulated with ATP, washed, and then re-stimulated in the presence of estradiol or estradiol and the mGluR2/3 antagonist LY341495 [53]. The estradiol attenuation of ATP-mediated calcium influx was blocked by

LY341495. Thus, rapid estradiol inhibition of calcium influx in DRG neurons is dependent on mGluR2/3.

Conclusions

Clearly, we are in the initial stages of understanding when and how rapid estradiol actions fit into the physiologic context of estradiol actions in the nervous system. Yet, recent findings suggest a mechanism by which ERs in the membrane can elicit changes in cell signaling and several different systems in which the actions of estradiol on membrane ERs require an interaction with mGluRs. This mechanism of ER–mGluR interactions provides a framework through which the pleiomorphic membrane-initiated estradiol actions can be understood and used to elucidate various means by which ERs play an important role in brain function.

Acknowledgment This work was supported by NIH grants DA013185 and HD042635 (PEM) and NS41302 (PGM). The authors would like to thank Dr. Marissa Boulware for providing the experimental data found within this review and Jessie Luoma for her technical assistance regarding the preparation of this manuscript.

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