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Physiology of Membrane Estrogen Receptor Signaling in Reproduction

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Abstract

The best characterized estrogen receptors that are responsible for membrane initiated estradiol signaling are the classic estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). When in the nucleus, these proteins are estradiol activated transcription factors, but when trafficked to the cell membrane, ERα and ERβ rapidly activate protein kinase pathways, alter membrane electrical properties, modulate ion flux and can mediate long-term effects through gene expression. To initiate cell signaling, membrane ERs transactivate metabotropic glutamate receptors (mGluRs) to stimulate Gq signaling through pathways using PKC and calcium. In this review, we will discuss the interaction of membrane ERα with metabotropic glutamate receptor 1a (mGluR1a) to initiate rapid estradiol cell signaling and its critical roles in female reproduction – sexual behavior and estrogen positive feedback of the luteinizing hormone (LH) surge. Although long considered to be regulated by long term actions of estradiol on gene transcription, new results indicate that membrane estradiol cell signaling is vital for full display of sexual receptivity. Similarly, the source of preovulatory progesterone necessary for initiating the LH surge is hypothalamic astrocytes. Estradiol rapidly amplifies progesterone synthesis through the release of intracellular calcium stores. The ERα-mGluR1a interaction is necessary for critical calcium flux. These two examples provide support for the hypothesis that membrane ERs are not themselves G protein receptors, rather they use mGluRs to signal.

Keywords

estradiol; calcium signaling; lordosis; neurosteroids; estrogen positive feedback

Introduction

The best characterized estrogen receptors are estrogen receptor-α (ERα) and estrogen receptor-β (ERβ), which were cloned in the 1980's and 1990's (1,2). These molecules were thought to act exclusively as ligand-activated transcription factors whose primary function was modulation of gene expression. In the brain, the actions of steroids were also considered to mediate only long-term effects that required transcriptional regulation. Some of these actions were said to act during the perinatal period to organize the brain and then again during adulthood to activate estrogen-sensitive circuits (3). The most obvious effects of estrogens in

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the brain are those that regulate reproduction: sexual behaviors and the secretion of gonadotropins. These actions had long time-courses and were blocked by transcription inhibitors (e.g., actinomycin D) or translation inhibitors (e.g., cycloheximide; (4–6)). There is no doubt that such long-term estrogen actions are vital for driving reproduction, but more recent evidence suggests that along with gene regulation, estrogen also mediates more rapid cellular effects. Although rapid actions of estradiol have been observed for decades, only more recently have these actions been accepted (7–12). These membrane estrogen receptors (ERs) rapidly activate protein kinase pathways, alter membrane electrical properties and modulate ion flux (13–18), but suggestions about their physiological roles, especially in reproduction, have only recently been elucidated (12,19–22).

In addition to the rapid timeframe of some responses to estradiol, membrane-initiated action could be mimicked with membrane-constrained estradiol conjugates. For example: 17β-estradiol 6-(O-carboxymethyl)oxime-bovine serum albumin (E2-6-BSA), 17β-estradiol-horseradish peroxidase (E2-HRP) and 17β-estradiol-biotin are all compounds that prevent estradiol from entering cells due to the large size and charge properties of the conjugated molecules, and all elicit rapid cell signaling events (17,20,23–25). Similarly, dialysis of estradiol into the interior of neurons did not mimic the rapid effects of estradiol even though intracellular ERs were activated (13). Finally, overexpression of ERα and ERβ were shown to be targeted to the membrane in ER naïve cells, demonstrating that the same proteins could function as membrane and nuclear receptors (26). Over the ensuing years the preponderance of evidence indicates that ERα and ERβ are the membrane receptors that activate mitogen-activated protein kinase (MAPK), extracellular regulated kinase (ERK) pathways and cytoplasmic free calcium ($[Ca^{2+}]_i$) flux (reviewed in (10–12)).

This is not to say the question of the membrane ER has been solved. In hippocampal neurons from ERα knockout mice, estradiol induced membrane currents that were not inhibited by ICI 182, 780, suggesting a mechanism independent from the classical ER (27). Several other candidates have been proposed as membrane ERs based on sequence homology or estrogen binding: most notably ER-X, STX-binding protein and GPR30 (28–30). Thus, there may be a cornucopia of ERs or our definition of an ER is too lax. Perhaps what is needed is a minimum definition of an ER. Exactly what that those criteria should include is open to debate. To initiate the discussion, we propose that an ER should include stereospecificity and common antagonism. For the definition of an ER, we propose that an ER be antagonized by ICI 182,780, and respond to 17β-but not 17α-estradiol. If the definition were strictly enforced, ER-X would be excluded since it is not antagonized by ICI 182,780 and is not stereospecific. On the other hand its sequence homology with classic ERα and ERβ (28) make the ER-X situation similar to the opioid receptor-like protein (ORL-1), which is has a sequence homology with opioid receptors but is not antagonized by naloxone, the *sine qua non* of an opioid receptor (31,32). GPR30 is a G protein-coupled receptor (GPCR) with seven transmembrane hydrophobic domains through which estradiol induces Erk-1/-2 activation (33). ICI 182,780 binds to GPR30, but in some assays it does not antagonize the receptor ((34); but see (30)). GRP30 appears to be localized to Golgi apparatus and endoplasmic reticulum membranes, but not the cell membrane and thus, represents a novel intracellular ER (35). Finally, an intriguing putative ER is activated by the diphenylacrylamide, STX (36). The STX receptor attenuates the outward (GIRK) current induced by the $GABA_B$ receptor agonist baclofen, mimicking estradiol (29). The STX-activity remains in $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ double knockout animals where STX is antagonized by ICI 182, 780. The STX-binding protein has not been cloned nor has its structure been determined.

ERα and ERβ are present in the membrane (17), but these molecules are not seven membrane-pass, GPCR proteins. To explain how ERα and ERβ activation initiates G protein signaling two hypotheses have been proposed. The first is that ERα and ERβ are GPCRs. The second

hypothesis is that membrane ERα interacts with another receptor that is a GPCR. Estradiol binding allows ER transactivation of a GPCR that in turn initiates cell signaling. Both in the periphery and in the nervous system, membrane ERα and ERβ have been shown to interact with other receptors to initiate cell signaling. The topic is well-reviewed by Mermelstein et al. in the current issue. Briefly, in the nervous system, membrane ERα and ERβ, associate with metabotropic glutamate receptors (mGluRs; (20,37–39)). In the present review, we discuss the effects of the ERα and mGluR1a interaction on female sexual behavior and estrogen positive feedback.

Sexual Receptivity

Integration of relevant interoceptive and exteroceptive information leading to the sexually receptive behaviors involves an extensive limbic and hypothalamic circuit (for reviews see (40,41)). Part of this lordosis regulating circuit is the projection from the arcuate nucleus of the hypothalamus to the medial preoptic nucleus (MPN). Estradiol activates microcircuits in the ARH that lead to the release of β-endorphin (β-END) in the MPN. Briefly, when estradiol activates ERα in the arcuate nucleus, neuropeptide Y (NPY) is released stimulating NPY-Y1 receptors on β-END neurons that project to the MPN (42–44). The release of β-END in the MPN activates μ-opioid receptors (MORs), which modulate the display of lordosis behavior, a measure of female sexual receptivity. As with other membrane receptors, following activation the MOR is internalized (45). Internalization is a process involved in desensitization of receptors and has been used to track the activation of specific circuits by estradiol (20,42,44). In this case, activation of the MOR and its internalization occur rapidly (within 30 mins of estradiol treatment) and is correlated with the concurrent inhibition of lordosis. This transient, estradiol-induced inhibition is necessary for full sexual receptivity measured 30 hours after treatment. For example, pharmacologic blockade of MOR activation/internalization with MOR antagonists such as H-d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) or removing the MOR, as in MOR-KO mice, results in a greatly diminished lordosis response even in the presence of estradiol (42,46). The time course of estradiol activation of the arcuate nucleus and subsequently the MPN suggests that these actions are mediated by cell signaling events and not by transcription-translational regulation.

Microinfusion of estradiol-biotin into the arcuate nucleus to stimulate only membrane associated ERs results in both an increase in phosphorylated CREB (pCREB) and an increase in MOR internalization (20). Taken together, this implies that a membrane associated ER has rapid actions in the ARH that are likely mediated by a G-protein. ERα and mGluR1a are co-expressed in a population of arcuate neurons (20) and these molecules can interact in the membrane as demonstrated by co-immunoprecipitation using membrane fractions obtained from the arcuate nucleus. We showed that membrane initiated ERα-mediated estradiol regulation of MOR internalization and lordosis behavior was dependent on mGluR1a. Antagonizing mGluR1 with LY367385 in the arcuate nucleus prevented the estradiol induced MOR internalization and the lordosis behavior. If mGluR1a was needed for the estradiol action, activating the glutamate receptor should mimic estradiol. The selective mGluR1a agonist (S)-3, 5-dihydroxyphenylglycine (DHPG) infused into the arcuate nucleus induced the activation/internalization of MOR in the medial preoptic nucleus. Rats pretreated with a dose of estradiol too low to initiate lordosis behavior alone and then supplemented with an intra-arcuate nucleus infusion of DHPG were sexually receptive. This result indicated that activation of mGluR1a was a necessary downstream event in the estradiol activation of the arcuate to MPN projection and the regulation of lordosis behavior (Fig. 1A).

More recent work has begun to uncover the cell signaling activated by the ERα-mGluR1 interaction. It has been well-known that estradiol treatment can result in the activation of several different protein kinases. Using E2-6-BSA, both protein kinase A (PKA) and protein kinase C

(PKC) been shown to be necessary components for rapid estradiol signaling in a number of systems (19,47–50). Both PKC and PKA have previously been associated with estradiol's rapid stimulation of the lordosis reflex (16,19,51). Also mGluR1a is primarily coupled to members of the Gq family resulting in activation of PLC and DAG, suggesting the activation of a PKC (51–53).

While the mechanisms responsible for mERα-mGluR1a mediated activation of lordosis behavior are likely to involve many players, an important role for PKC signaling has recently been elucidated (16,38). PKCs are a family of serine/threonine kinases that have a conserved catalytic ATP-binding site and kinase domain as well as a conserved N-terminal domain for membrane targeting. The PKCs are divided into three classes based upon their method of activation: (1) conventional PKCs (α, β and γ) which require diacylglycerol (DAG) or phosphotidylserine and calcium for activation; (2) novel PKCs (δ, ε, η, μ and θ) which require only DAG or phorbol esters; and (3) atypical PKCs (ι, ζ and λ) which depend on neither DAG nor calcium for their activation although they may be activated by phosphotidylserine, inositol lipids or phosphatidic acid (54). A protein microarray targeted at cell signaling pathways, identified a number of activated signaling molecules including PKA and PKCθ. The increased phosphorylation of PKA was not confirmed by western blots of estradiol treated arcuate nucleus, but phosphoPKCθ upregulation was confirmed (38). In our experiments, the ARH-MPN portion of the limbic-hypothalamic lordosis regulating circuit was not modulated by activation of PKA. Pharmacologically antagonizing PKA activation with [N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89) did not prevent estradiol-induced lordosis, even at very high doses. We cannot conclude, however, that PKA does not regulate other parts of the CNS that modulate lordosis, such as the periaquaductial gray or ventromedial nucleus of the hypothalamus (55) or in other estradiol activated reproductive functions in the hypothalamus (51,56–61).

Bisindolymaleimide (BIS), a blocker of PKC activation, injected into the arcuate nucleus attenuated the estradiol-induced MOR internalization/activation and lordosis. These results were similar to those seen after antagonizing of GluR1a in the arcuate nucleus. Blocking PKC prevented MOR internalization induced by the mGluR1a agonist DHPG, suggesting that PKC activation was downstream of the activation of mGluR1a. To further test this idea, phorbol 12, 13-dibutyrate (PDBu), an activator of PKC, was used to internalize MOR in the absence of estradiol. Not only did PDBu cause internalization of MOR, but it also facilitated lordosis when the rats were treated with a subthreshold dose of estradiol. These studies along with the pathway array results strongly implicate PKCθ activation as a critical step in estradiol membrane initiated cell signaling regulating sexual receptivity.

Both nuclear initiated and membrane initiated actions of estradiol described here are vital for activation of lordosis behavior. Transcription may be activated by nuclear receptors or by membrane to nuclear signaling cascades (20,37). The available evidence is that membrane initiated signaling is not sufficient by itself to induce sexual receptivity. Membrane ERa potentiate transcriptional events that regulate lordosis (19,20). In spite of this fact, it is clear that the internalization of MOR in the MPN critically depends upon the interaction of ERa and mGluR1 at the membrane and that this interaction allows the phosphorylation and activation of PKC, all of which is necessary for the full display of lordosis behavior (20,38,42,46). Estradiol treatment always precedes lordosis behavior by 30 hours and antagonizing mGluR1a or PKC only at the time of estradiol treatment attenuates lordosis behavior. This implies that these are rapid, transient actions of estradiol. Moreover, the membrane constrained construct estradiol-biotin, induces MOR internalization seen when free estradiol is systemically injected (20).

Estrogen Positive Feedback and Neurosteroids

Ovulation is a key event in mammalian reproduction. On the afternoon of proestrus, rising levels of estradiol from maturing ovarian follicles activate the hypothalamic-pituitary-gonadal axis to release a surge of luteinizing hormone (LH). Unlike the negative feedback effects of estradiol during other times of the estrous cycle, spiking levels of estradiol become stimulator. This is known as estrogen positive feedback (62). It is well established that an estrogen positive feedback mechanism is essential for induction of the LH surge that leads to ovulation and subsequent luteinization of the postovulatory ovarian follicle. In addition to elevated levels of estradiol, a pre-ovulatory rise in progesterone and progesterone receptors has been shown to be essential for the LH surge (63–68). Specifically, both transcription and activation of progesterone receptors in the hypothalamus is an obligatory event in the stimulation of the GnRH and LH surges in estradiol-primed, ovariectomized (OVX) rats (68). Furthermore, treatment with trilostane, a blocker of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) that catalyzes the conversion of pregnenolone to progesterone, inhibits the LH surge, indicating that progesterone synthesis is critical for estrogen-induced positive feedback in an OVX and adrenalectomized (ADX) rats (69). Therefore, a pre-ovulatory increase in both progesterone and estradiol as well as synthesis and activation of PRs are all essential for inducing the LH surge.

Very little progesterone is detectable in the systemic circulation prior to the LH surge, indicating that pre-ovulatory progesterone needed for the LH surge may not be synthesized peripherally (70–72), but produced locally within the hypothalamus. Consistent with such an idea, ovariectomized/adrenalectomized rats injected with 17β-estradiol have been shown to produce a robust LH surge and have elevated levels of hypothalamic progesterone levels (69, 73). The importance of *de novo* hypothalamic progesterone synthesis was further demonstrated in gonadally intact rats. These rats had normal four-day estrous cycles, but blocking steroidogenesis in the hypothalamus with aminoglutethemide (AGT), a P450scc enzyme inhibitor, on the morning of proestrus prevented the LH surge, ovulation and luteinization (74). In these rats, peripheral steroidogenesis was not disrupted since estradiol levels in the AGT treated rats were the same as cycling controls. After several days, AGT treated rats resumed their cycles, indicating that the treatment had not damaged the estrogen positive mechanism. In hypothalamus, estradiol stimulated progesterone synthesis in astrocytes (21, 75). The mechanism of estradiol regulation of progesterone synthesis was examined using primary cultures of post-pubertal hypothalamic astrocytes.

In vitro, estradiol increases free cytoplasmic calcium levels ($[Ca^{2+}]_{i;}$; (17,21)). The rapid estradiol action (< 30 sec) is mediated by a membrane ER, a conclusion based on results with the universal ER antagonist ICI182,780 and membrane-constrained E2-6-BSA that induced a statistically similar $[Ca^{2+}]_i$ flux compared with estradiol. Subnanomolar doses of estradiol (ED50 = 0.15 nM) increase $[Ca^{2+}]_i$ flux, indicating that the mER responds to physiological levels of estradiol, which are achieved during the proestrus surge in rats (76). ERα and ERβ are found in astrocytes (17,77–79) and in astrocyte membrane fractions (17). Removing calcium from the media did not alter the estradiol-induced $[Ca^{2+}]_i$ flux indicating the release of intracellular stores. Using a series of pharmacological agents, this was confirmed: membrane-initiated, estradiol-induced $[Ca^{2+}]_i$ flux activated the phospholipase C/inositol trisphosphate (PLC/IP_3) pathway leading to the release of IP_3 receptor sensitive calcium stores (80). To demonstrate the relationship between the rapid estradiol-induced $[Ca^{2+}]_i$ flux and the estradiol amplification of progesterone synthesis, thapsigargin was used to release IP_3 receptor sensitive internal stores of calcium (21). Thapsigargin, a Ca^{2+}-ATPase inhibitor that initially induces a massive release of intracellular calcium, was as effective as estradiol at inducing the *de novo* synthesis of progesterone (*neuro*progesterone) in hypothalamic astrocytes cultured

from the post-pubertal females, suggesting that the estradiol-induced progesterone synthesis involves rapid increases in $[Ca^{2+}]_i$ flux (21).

Several lines of evidence indicate that the membrane-initiated estradiol actions utilize the same mechanism in astrocytes as does ERα in neurons (20,37,76). First, the selective ERα agonist, propylpyrazole triole (PPT), induced a robust $[Ca^{2+}]_i$ flux, but the selective ERβ agonist, diarylpropionitrile (DPN), did not, supporting a role for ERα in the membrane initiation of estradiol signaling. Second, hypothalamic astrocytes express mGluR1a receptors (76). Third, ERα, but not ERβ, co-immunoprecipitates with mGluR1a, suggesting that these two receptors can directly interact. Fourth and finally, the estradiol-induced $[Ca^{2+}]_i$ flux was blocked by LY367385, a mGluR1a antagonist, suggesting that ERs localized to the plasma membrane do not interact directly with G proteins; rather, estradiol bound mERs activate mGluRs, which then serve as the intermediate to activate G proteins (20,37,81).

In primary cultures of hypothalamic astrocytes, activation of the mGluR1a without estradiol induced a $[Ca^{2+}]_i$ flux, but required a high dose of DHPG (100 nM to 50 μM). This result was consistent with our experiments in vivo where high doses of DHPG mimicked the rapid actions of estradiol on MOR internalization/activation and modulation of sexual receptivity. Interestingly, a combined treatment of estradiol and DHPG stimulated a greater $[Ca^{2+}]_i$ flux than the maximal response of estradiol or DHPG alone (Fig. 1B), indicating that estradiol not only increases the maximal response of mGluR1a activation, but also lowers the threshold concentration of DHPG required to obtain a maximal response. These results suggest that for a subpopulation of mGluR1a optimal signaling requires an interaction with membrane ERα. One possible explanation is that the ERα associated with mGluR1a is in a conformation that does not allow optimal activation of intracellular signaling pathways. Without the estradiol bound to ERα the associated mGluR1a does not become fully stimulated and signaling is moderated. When estradiol excites the membrane ERα, the conformation change activates the mGluR1a to initiate Gq signaling. This could account for the additive effects of DHPG during estradiol stimulation and the inhibition of estradiol signaling with LY367385.

Intracellular calcium regulation and homeostasis is crucial for regulation of gene expression, development and survival in astrocytes (82). In hypothalamic astrocytes, ERa interaction with mGluR1a has been demonstrated to mediate the $[Ca^{2+}]_i$ flux required for neuroprogesterone synthesis and critical for the LH surge and ovulation. Maximal calcium signaling in astrocytes requires both glutamate and estradiol, suggesting that estradiol may act most effectively on astrocytes that are near active glutaminergic nerve terminals. This $[Ca^{2+}]_i$ wave can also be propagated between astrocytes (83) to extend the activation over a long distance. Further experiments are needed to define whether mERα-mGluR1a interactions can also stimulate other pathways associated with reproduction, such as DAG, PKA, PKC, CREB, mitogen-activated protein kinase (MAPK) and steroid acute regulatory protein (StAR). Additionally, experiments with ER knock-out mice are currently underway to confirm the critical role of ERα in the regulation of neuroprogesterone synthesis in astrocytes.

Conclusion

Membrane initiated estradiol signaling regulates rapid cell signaling in at least two physiologically relevant mechanisms that control reproduction: the control of sexual receptivity and estrogen positive feedback regulating the LH surge. While a number of aspects remain to be verified, the general idea is that rapid estradiol signaling requires the transactivation of ERα, trafficked to the cell membrane, with mGluR1a, a GPCR. That the mGluR1a initiates Gq signaling that leads to the release on internal calcium stores in astrocytes and phosphorylation of PKCθ in neurons points to the commonality of this mechanism in nervous tissue and its importance in the physiology of estradiol signaling.

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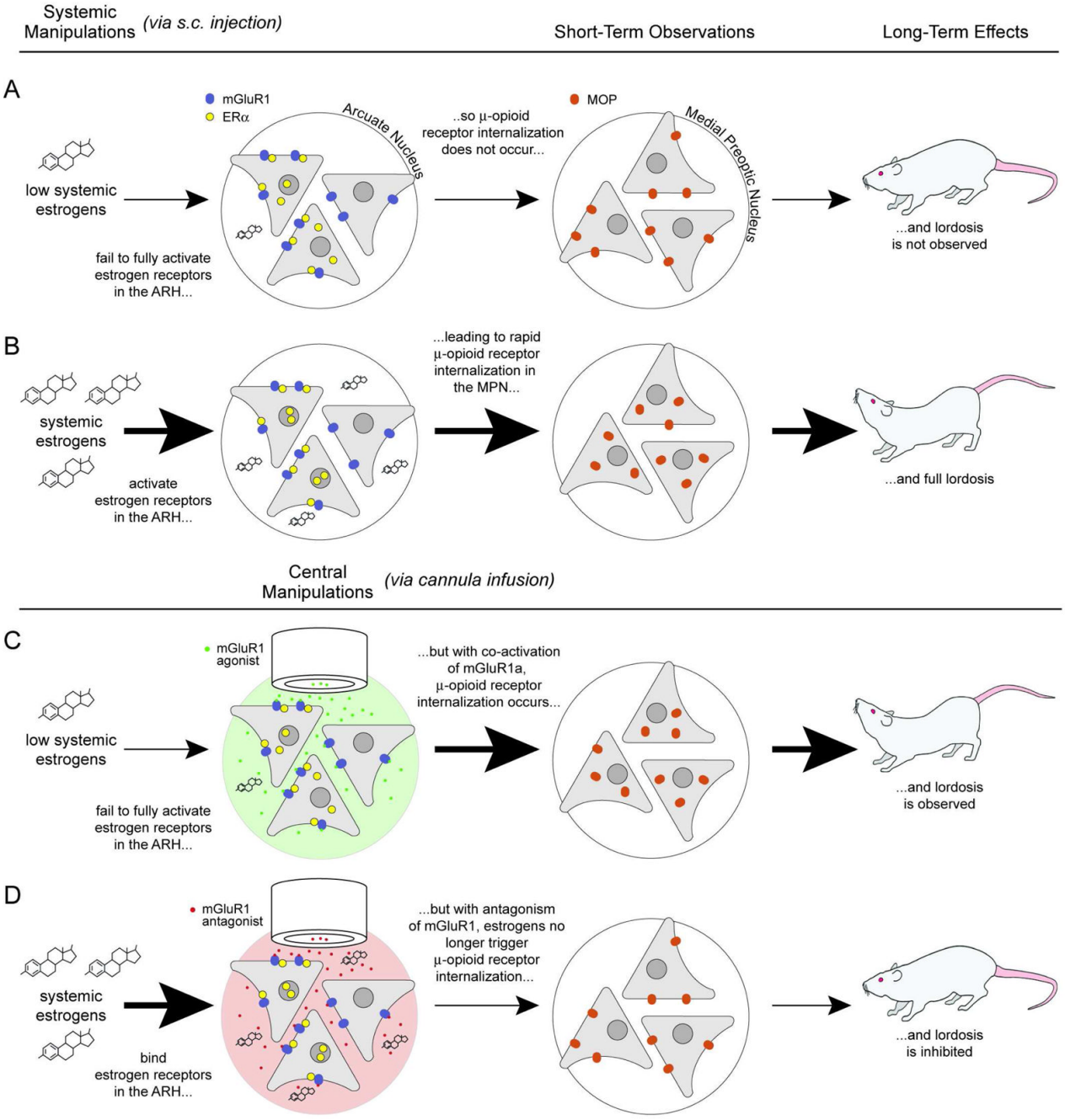

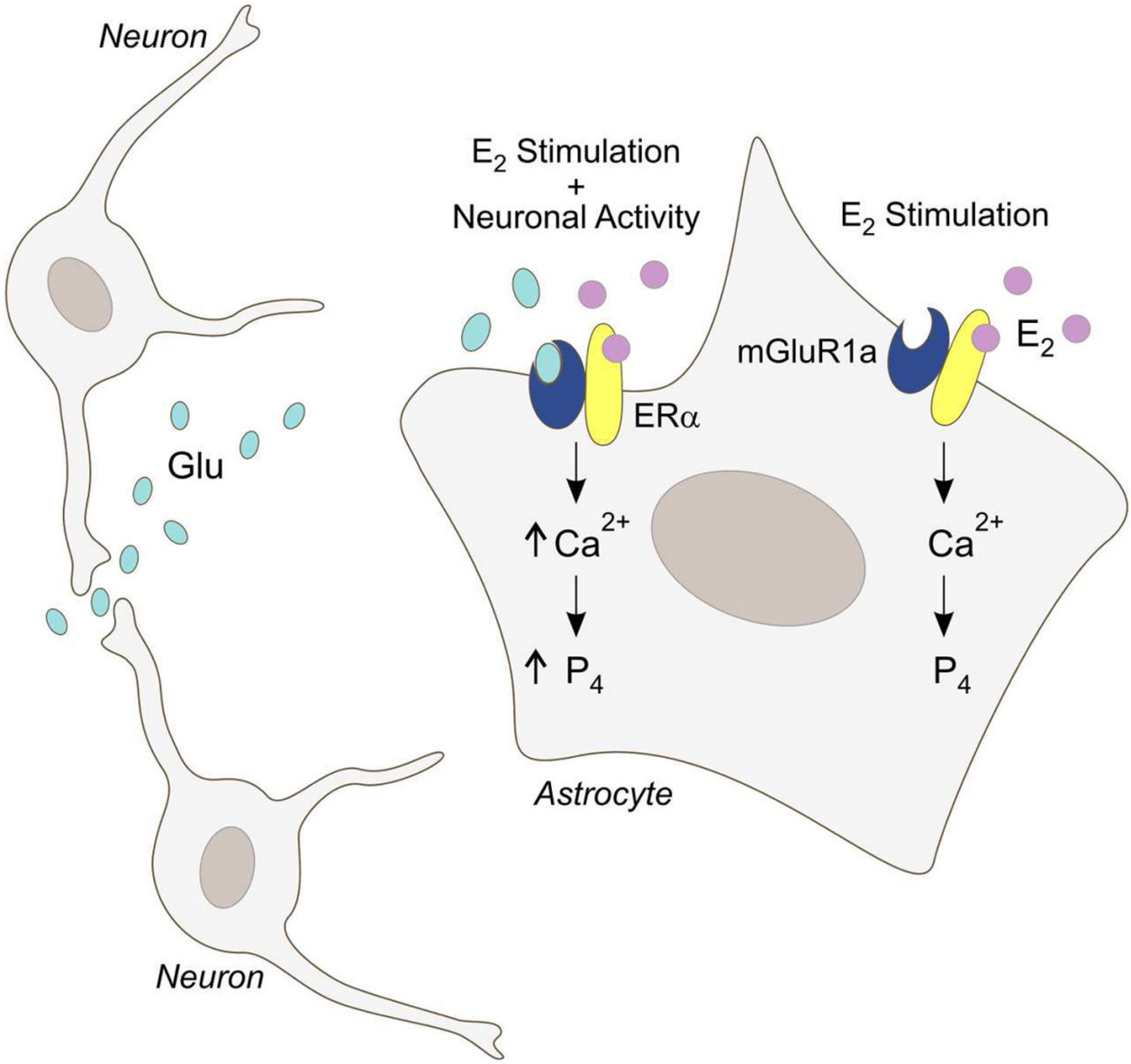

Figure 1.

Figure 1A. ER/mGluR signaling in the arcuate nucleus (ARH) – medial preoptic nucleus (MPN) circuit regulates female sexual receptivity. Low estradiol levels do not activate the circuit and the rat is not sexually receptive (first row). As circulating levels of estradiol increase, they stimulate ERα in the ARH, which induce the release of β-endorphin in the MPN activating and internalizing MORs, producing lordosis (second row). If mGluR1a are activated in the presence of low circulating estradiol levels, the ARH-MPN projection is activated: MOR is activated/internalized leading to lordosis behavior (third row). Conversely, antagonizing mGluR1a prevents the estradiol activation of the ARH-MPN circuit: MOR is not activated/internalized and lordosis is attenuated (fourth row). (From (12)).

Figure 1B. A model of estradiol signaling in astrocytes and the mechanism regulating 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 $[Ca^{2+}]_i$ are needed for neuroprogesterone (P4) synthesis in astrocytes. When both mGluR1a and ERα are simultaneously stimulated, the resulting $[Ca^{2+}]_i$ flux is significantly increased. This suggests that, in *vivo*, neural activity modulates the astrocytic response to E2: when local neural activity releases glutamate (Glu), the synthesis of progesterone is augmented. (Modified from (12)).