Rapid Estrogen Signaling in the Brain

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Abstract

Estrogen has multiple actions in the brain to modulate homeostasis, synaptic plasticity/cognition and neuroprotection. While many of these actions undoubtedly involve mediation via the classical genomic mechanism of regulation of transcription of genes via estrogen nuclear receptors, there has been growing interest in the rapid nongenomic effects of estrogen and the role they may play in the neural actions of estrogen. In this review, we will focus on these rapid nongenomic actions of estrogen in the brain and discuss the potential physiological significance of these actions. The evidence for rapid estrogen regulation of cell signaling pathways, including calcium, ion channel and kinase signaling pathways in the brain will be reviewed, as will evidence derived from plasma-membrane impermeable estrogen-peptide conjugates in the regulation of these cell signaling pathways. Evidence supporting classical and nonclassical estrogen receptor localization to the plasma membrane of neurons will also be reviewed, including the putative new membrane estrogen G-protein-coupled receptor, GPR30. Precisely how membrane estrogen receptors couple to kinase signaling pathways is unclear, but we will discuss the latest findings on estrogen receptor-interacting scaffold proteins, such as MNAR/PELP1, striatin and p130Cas, which are capable of linking estrogen receptors and kinases such as Src and PI3K, to potentially mediate estrogen-induced kinase signaling. Finally, we will review the growing evidence that rapid membrane-mediated effects of estrogen play an important physiological role in the neural actions of estrogen in the brain, including estrogen feedback control and modulation of homeostasis, regulation of synaptic plasticity/cognition, and estrogen-mediated neuroprotection.

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Introduction

Estrogen (17β-estradiol) exerts regulatory effects in a variety of tissues in the body, including the brain, breast, cardiovascular system, uterus and bone [1–7]. Its effects in the brain are of considerable importance, as estrogen can modulate homeostasis through its neuroendocrine feedback effects, as well as exert neuroprotective and cognitive-enhancing effects [1–11]. The brain regulatory effects of estrogen have been of significant interest due to evidence that estrogen may delay onset or ameliorate the severity of several neurological disorders, such as Alzheimer's disease, Parkinson's disease, and stroke [1, 8–13], although controversies do exist in this area as exemplified by the controversial WHI results [for review, see 1]. Understanding how estrogen signals in the brain is critical to understanding how it exerts its purported beneficial effects. This review will focus on the rapid, nongenomic signaling effects of estrogen, which is a fast-emerging area.

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Classically, estrogen has been thought to exert its effects via a genomic mechanism of action involving binding of estrogen to nuclear estrogen receptors and subsequent regulation of transcription. The genomic mechanism of action typically takes several hours for the effect to be manifested due to the time needed for transcription and translation of estrogen-regulated genes [14]. However, a growing body of evidence has emerged, which describes rapid estrogen effects that occur within minutes and thus cannot be attributed to a genomic mechanism of action. These rapid effects have thus been designated as 'nongenomic' effects of estrogen [3, 15–19]. A search in PubMed with the keywords 'estrogen and nongenomic' yields over 334 published papers, with 94% of these papers being published within the last 10 years. Thus, this area has seen an explosion of interest in the past decade, and represents one of the fastest emerging areas in the field of estrogen research. In particular, in the past few years new information has emerged on the types of estrogen receptors at the plasma membrane, targeting of estrogen receptors to the membrane, and scaffold proteins that may link estrogen receptors to kinase signaling pathways in the brain. These areas will be reviewed with interpretations and future directions for the field discussed. The evidence for functional significance of rapid estrogen signaling in the brain will also be reviewed and discussed, as new data has also emerged on this important topic. It should be mentioned that there is also evidence of ligand-independent activation of estrogen receptors which involves crosstalk with peptide growth factors. This, however, is outside the focus of this review, and the reader is referred to previous excellent reviews on this subject for further elaboration and discussion [20, 21]. It is hoped that by gaining a more thorough understanding of estrogen's effects on the brain and a better grasp of the signaling cascade involved, we will gain new insights that lead to better diagnostic tools and improved therapeutics in humans.

Evidence of Rapid Estrogen Signaling in the Brain

Electrophysiological Activity

Reports of rapid estrogen (17β-estradiol) effects in the brain first began to appear in the literature over 40 years ago, when it was observed that estrogen rapidly modulated hypothalamic electrical activity [22–26]. The latency of the estrogen effects was within minutes, and typically two types of effects were observed on hypothalamic neurons – some units had a decreased firing rate that lasted up to 150 min, whereas other units responded with a transitory increase in firing rates. The remaining units were unresponsive to estradiol [24]. Kelly et al. [26–29] showed that neurons from several hypothalamic regions displayed rapid electrophysiological responses to estrogen, including preoptic, arcuate and parvicellular ventromedial hypothalamic neurons. Further studies extended the rapid estrogen effects on electrophysiological responses to other regions of the brain, including the amygdala, cerebellum, and hippocampus [28, 30–33]. These early observations set the stage for subsequent studies on the mechanisms underlying the rapid effects of estrogen in the brain. Along these lines, subsequent work revealed that estrogen rapidly modulated several cell signaling pathways in neurons (and non-neuronal cells), including kinases, ion channels and second messengers. The evidence supporting the regulation of these signaling pathways by estrogen is discussed below.

Kinases
ERKs
There are now many studies in the literature demonstrating that estrogen rapidly activates multiple kinases in various types of brain cells within minutes, including extracellular signal-regulated kinases (ERKs), Akt and cAMP response element binding protein (CREB). The first report of rapid estrogen regulation of ERK1/2 in neuronal cells came from the study of Watters et al. [34] in 1997, which showed that estrogen enhanced ERK1/2 phosphorylation in human neuroblastoma cells within 5–10 min. Subsequent in vitro studies by many laboratories confirmed and extended the rapid estrogen regulatory ERK effect to other species and other neuronal populations such as rat cerebral cortical explants [35, 36], rat cortical neurons [37, 38], and rat and mouse hippocampal neurons [39, 40]. In vivo confirmation of the rapid estrogen effect on ERK phosphorylation has also been demonstrated. Kuroki et al. [41] showed that estrogen administered intracerebroventrically into the lateral ventricle of rats enhanced ERK phosphorylation within 5 min in the hippocampal CA1 region and dentate gyrus, while Bryant et al. [42] demonstrated that subcutaneous injection of estrogen in rats increased ERK phosphorylation in many regions of the brain at 20 min after estrogen administration.

Akt/CREB
In addition to regulation of ERK activation, estrogen has been shown to rapidly enhance phosphorylation of Akt and CREB in cortical neurons and explants [37, 38, 43–45] and hippocampal neurons [40]. Furthermore,

Mannella and Brinton [37] have reported that estrogen induces phosphorylation of ERK and Akt in the same population of cortical neurons in vitro, suggesting a possible unified mechanism of action for estrogen. Further work supported this possibility as it was shown that estrogen receptors interact with the PI3K P85 regulatory subunit in cortical neurons, and that pretreatment with a PI3K inhibitor blocked estrogen-induced activation of pAkt and pERK [37].

Calcium and Ion Channels

In addition to regulation of kinase activation, estrogen has been shown to rapidly regulate intracellular calcium levels in neurons. For instance, patch clamp experiments show that estrogen can reduce Ca^{2+} currents following estrogen influx in rat neostriatal neurons via a nongenomic mechanism [46]. Additionally, Huang et al. [47] used fluorescent Ca^{2+} probes to show that estrogen rapidly attenuates glutamate-induced Ca^{2+} overload in rat primary hippocampal neurons. In contrast, under basal conditions, estrogen acts to increase intracellular calcium concentrations in midbrain neurons [48] and in the cytoplasm, dendrites and nucleus of rat hippocampal neurons, which seems important for changes in hippocampal dendritic spine protein regulation by estrogen [49]. The calcium changes also may be important for estrogen-induced kinase changes. Along these lines, Wu et al. [40] showed that estrogen induced a rapid rise of intracellular Ca^{2+} concentration within minutes of exposure in rat hippocampal neurons in vitro, which was blocked by an L-type Ca^{2+} channel antagonist. If the L-type Ca^{2+} channels are inhibited by an inhibitor, it results in a loss of estrogen activation of Src/ERK and CREB signaling pathways in hippocampal neurons. Estrogen can also rapidly modulate K^+ channels in hypothalamic (POMC, dopamine, GABA, GnRH) neurons that are involved in many homeostatic functions [50]. Kelly et al. [51] have proposed that the estrogen regulation of K^+ channels in the brain involves G-coupled protein receptors and cAMP-dependent phosphorylation. Thus, estrogen can exert rapid regulation of calcium and potassium channels in neurons from various regions of the brain, and these effects may help mediate estrogen actions in the brain. The evidence for a physiologically important role of nongenomic signaling in estrogen actions in the brain is discussed below.

Functional Significance of Rapid Estrogen Signaling

There is now increasing evidence that the rapid effects of estrogen may be important for manifestation of several of its key biological actions in the brain, such as neuroprotection, plasticity/cognition, and homeostasis. Along these lines, in vitro studies by several laboratories have shown that the rapid activation of ERKs by estrogen is critical for its neuroprotective effects, as administration of a MEK inhibitor blocks the neuroprotective effects of estrogen in neurons [40, 52–54]. Furthermore, in vivo studies by Jover-Mengual et al. [55] suggest that estrogen-induced ERK activation in the CA1 region after global cerebral ischemia is critical for its neuroprotective effects, as treatment with the MEK inhibitor (PD98059) blocked estrogen-induced ERK activation and estrogen neuroprotection in the hippocampus. Likewise, a role for the serine kinase Akt in estrogen neuroprotection has been implicated, as estrogen rapidly upregulated Akt activation in cortical neurons in vitro [43], and in the hippocampus in vivo following cerebral ischemia [56], while treatment with a PI3K inhibitor attenuated the neuroprotective effects of estrogen both in vitro and in vivo. Estrogen activation of kinase signaling may also be important for the synaptic plasticity effects of estrogen, as administration of kinase inhibitors (ERK or PKA pathway inhibitors) has been shown to block estrogen-induced enhancement of neurite outgrowth [57], dendritic spinogenesis [58] and synapse formation [59].

There is also growing evidence that rapid nongenomic effects of estrogen play a role in homeostatic neuroendocrine feedback control exerted by estrogen. For instance, Gidewell-Kenney et al. [60] developed a 'knockin' mouse expressing a mutant form of ERα with ablated ERE-dependent but intact ERE-independent signaling. They then bred the knockin mouse onto the ERα-null background and studied the effects on female neuroendocrine feedback control and reproductive physiology. They found that estrogen negative feedback signaling on luteinizing hormone signaling was preserved in the knockin mice, but that estrous cyclicity, spontaneous ovulation and positive feedback signaling were lost. These results suggest that nongenomic estrogen signaling may mediate negative feedback of estrogen, but is not required or sufficient to mediate positive feedback control and maintain estrous cyclicity and ovulation. A similar result was demonstrated in male animals (e.g. that nongenomic signaling was important for the negative feedback effects of estrogen on gonadotropin secretion, but was not required or sufficient for expression of sexual behavior in males) [61]. These studies using mutant ERE knockin ERα–/– mice provide evidence of a significant physiological role of nongenomic estrogen signaling in the brain. The studies are well suited (in mice) to show a required

role of ERE-independent estrogen signaling. However, they are not well suited to show crosstalk between the nongenomic and genomic actions of estrogen that may contribute to mediation of estrogen functions in the brain. This issue will be discussed in greater detail in subsequent sections below. Thus, while progress has been made, clearly further studies are needed using multiple approaches to continue to enhance our understanding of the physiological significance of nongenomic estrogen signaling in the brain.

Membrane Estrogen Receptor Mediation of Rapid Estrogen Effects in the Brain

Estrogen-BSA and Estrogen-Dendrimer Conjugates
The rapid effects of estrogen on calcium and ion channel signaling, as well as kinase activation, suggest a potential membrane receptor-mediated effect of estrogen. One piece of evidence supporting a membrane receptor-mediated mechanism is the use of membrane impermeable conjugates of estrogen, such as estrogen-BSA and estrogen-dendrimers. Similar to estrogen, estrogen-BSA was shown to rapidly enhance phosphorylation of ERK1/2 in the human neuroblastoma SK-N-SHR cells [34], in rat hypothalamic neurons in vitro [62], and in the rat hippocampus in vivo [41]. Estrogen-BSA conjugates have also been shown to rapidly enhance protein kinase A activation in hippocampal neurons [63], increase intracellular calcium levels within minutes in both astrocytes and neurons [64, 65], and to rapidly modulate potassium channel activation in hypothalamic neurons [66]. Nevertheless, it should be pointed out that there has been some criticism of estrogen-BSA conjugates, in that some estrogen (3–5%) can be found in the free unconjugated form in commercially available estrogen-BSA conjugates [67]. For this reason, it is advisable that the estrogen-BSA be subjected to filtration in order to remove free estrogen, and that studies that fail to perform the filtration should be interpreted cautiously. Stevis et al. [67] reported that estrogen-BSA did not bind ERα or ERβ in binding studies; however, a subsequent study demonstrated that estrogen-BSA indeed binds to purified estrogen receptors in vitro and to membrane estrogen receptors in intact cells, albeit at a slower rate than estrogen [68].

Recently, a new class of estrogen conjugates has been developed – the estrogen dendrimers (EDCs) [69]. EDCs are constructed by the attachment of an estrogen through the 17-position, to an abiological macromolecule, a poly(amido)amine (PAMAM) dendrimer, via a hydrolytically stable linkage. The PAMAM dendrimer's core provide flexibility in macromolecule size and surface charge, and produces conjugates that can be further characterized with fluorophores to trace the cellular distribution of the EDCs. While not yet tested in neurons, EDCs have been shown in MCF-7 breast cancer cells to bind to the membrane and rapidly enhance ERK, Shc and Src phosphorylation, yet are very ineffective in enhancing transcription (10,000-fold less potent than estradiol in genomic actions) [69]. Similarly, estrogen-BSA has been reported not to increase transcription of an ERE-based reporter gene in human neuroblastoma cells, further suggesting that estrogen conjugates do not enter the cell and are unable to activate nuclear estrogen receptors and genomic signaling [70, 71]. Rai et al. [72] also showed in breast cancer cells that transfection of a modified, membrane-targeted estrogen receptor that lacked a nuclear localization sequence, led to membrane localization of the modified estrogen receptor and that the membrane-targeted estrogen receptor failed to regulate estrogen-responsive genes that are upregulated by wild-type nuclear estrogen receptors. However, Pfaff and coworkers [71, 73, 74] have provided evidence that estrogen-BSA may act to potentiate genomic actions of estrogen using a two-pulse paradigm (first pulse = estrogen-BSA, and second pulse = estrogen). In their study, they showed that the first pulse estrogen-BSA can enhance transcription from a consensus ERE-driven luciferase gene in neuroblastoma cells induced by a second pulse of estrogen. The potentiation effect of estrogen-BSA on estrogen genomic actions was blocked by PKA or PKC inhibitors, suggesting that the rapid membrane signaling induced by estrogen-BSA exerts crosstalk with the nucleus that enhances the genomic effects of estrogen. Further work showed that this two pulse estrogen-BSA/estrogen paradigm enhanced lordosis sexual behavior of female rats [74], demonstrating that the nongenomic/genomic crosstalk by estrogen could enhance a functionally relevant endpoint. Taken as a whole, the estrogen conjugate data suggest that rapid signaling effects of estrogen involve mediation via a membrane estrogen receptor. The subsequent sections of this review will examine the evidence supporting membrane localization of estrogen receptors (ERα, ERβ, ER-X, GPR30) in neurons and discuss the role each may play in mediating rapid estrogen effects in the brain.

Evidence of Estrogen Receptor Localization to the Plasma Membrane
Ultrastructural electron-microscopic studies have provided direct evidence of extranuclear localization of
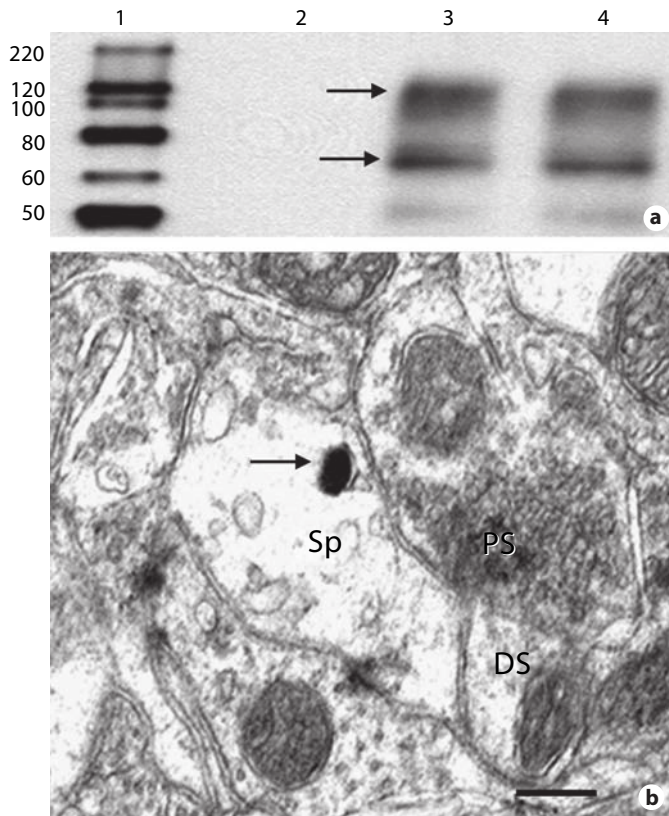
Fig. 1. a Expression of estrogen receptor alpha (ERα) on the membrane surface in 4–14 days in vitro (DIV) cultured rat cortical neurons. Surface labeling of ERα was performed by biotinylation method using a commercially available kit. Lane 1 shows the positions of various markers, lane 2 represents total cell lysate before biotinylation, while lanes 3–4 represent total cell lysates after biotinylation of surface proteins from 4 DIV (lane 3) and 14 DIV cultured neurons (lane 4). Total cell lysate from biotinylated (lanes 3, 4) as well as nonbiotinylated (lane 2) samples were incubated with immobilized 'NeurtrAvidin' agarose gel for 1 h at room temperature and the bound proteins were eluted using SDS-DTT sample buffer. Two ERα bands (arrows) were detected – a dimer (~120 kDa) and a monomer (~65 kDa), demonstrating that ERα is localized in the plasma membrane of rat cortical neurons. **b** Electron micrograph showing ERα-reactive gold particle (arrow) in the dendritic spine from the prefrontal cortex of an adult female rat brain. Sp = Postsynaptic dendritic spine; DS = dendritic shaft; PS = presynaptic terminal. Scale bar = 200 nm.

estrogen receptors in the brain [75–77]. Milner et al. [75] performed ultrastructural studies of ERα localization in the rat hippocampal formation and demonstrated that ERα, in addition to nuclear localization, is found in cytoplasm and plasma membrane of neurons, and in unmyelinated axons and axon terminals. Furthermore, approximately 25% of ERα is localized in dendritic spines,

often associated with spine apparati and/or polyribosomes, suggesting it may act to regulate calcium availability, local protein translation, and synaptic growth. Figure 1 shows a similar observation in the rat cerebral cortex, in that electron microscopy studies by our laboratory demonstrate ERα immunolocalization in dendritic spines in the rat cerebral cortex. Moreover, biotinylation of cultured rat cortical neuron membrane proteins, followed by pull down with avidin bead column and subsequent Western blot analysis for ERα, showed that ERα is present in the plasma membrane of rat cortical neurons (fig. 1). Additional work by Romeo et al. [77] showed that there is a 114% increase in ERα in hippocampal dendritic spines in proestrus female rats as compared to diestrus female rats and male rats. Thus, there appears to be some cycle-related fluctuations in ERα in dendritic spines in the hippocampus. Fluorescent-tagged estrogen-BSA conjugates and transfection of GFP-tagged ERα constructs have also been used to determine membrane or subcellular localization of estrogen receptors in neurons in vitro. Using Western blot and estrogen-horseradish protein-FITC or estrogen-BSA-FITC conjugates, Marin et al. [78, 79] showed plasma membrane localization of ERα in SN56 septal-derived cholinergic neurons, and that the estrogen conjugates protected the cells against A-beta toxicity as effectively as estrogen. Xu et al. [80] transfected GFP-tagged human ERα in rat cortical neurons in vitro and determined its subcellular localization. The studies revealed that there was localization of the GFP-tagged human ERα in neurites of the cortical neurons and that the ERα appears to be directed to the neurites directly from its site of translation and not from nuclear stores. Furthermore, as shown and discussed in figure 1, biotinylated membrane protein studies also support localization of ERα at the plasma membrane of rat cortical neurons. Finally, ultrastructural studies have also demonstrated that the other ER subtype, ERβ is also localized at extranuclear sites such as cytoplasm, plasma membrane, dendritic spines, axons and axon terminals in the rat hippocampus [76]. Thus, ERβ could also have a role in mediating nongenomic actions of estrogen in the brain. We should also mention that a novel plasma-membrane-associated estrogen receptor called ER-X has been proposed as well in neurons of the brain, but attempts to clone it have so far been unsuccessful [81].

Palmitoylation – A Key Factor in Localization of ER at the Plasma Membrane?

It is unclear how estrogen receptors are targeted to the plasma membrane. However, recent work in non-neuro-

nal cells has implicated palmitoylation of the estrogen receptor as an important mechanism for targeting ER to the plasma membrane [82–84]. Palmitoylation is a post-translational modification that consists of the addition of a 16-carbon fatty acid, palmitate, to a cysteine residue through the creation of a thioester link. Work from several laboratories has shown that palmitoylation occurs on residues in the E domain (ligand-binding domain) of ERα and ERβ, and that mutation of these key residues in the E domain significantly reduces ER localization to the plasma membrane and decreases ERK and Akt activation [82–84]. Furthermore, administration of a pamitoyl-acyltransferase inhibitor, 2-bromo-hexadecanoic acid, has been shown to attenuate ER localization to the plasma membrane and ERK activation in HeLa cervix cancer cells [82]. These studies suggest that palmitoylation of ER may help target ER to the plasma membrane. Correlative studies in the brain on the role of palmitoylation in ER subcellular targeting and nongenomic signaling are currently lacking. Studies to address this issue and determine how palmitoylation of the ER might be regulated are needed.

Estrogen Rapid Signaling in Estrogen Receptor Knockout Mice

One approach to determine the role of classical estrogen receptors in mediating rapid signaling effects of estrogen in the brain is to utilize mutant mice with one or both classical ERs knocked out. Using this approach, Chaban and Micevych [85] demonstrated that rapid estrogen inhibition of ATP-induced calcium signaling in dorsal root ganglion neurons is blocked by an ER antagonist, and that the effect of estrogen on calcium signaling is lost in ERα knockout mice. Herbison and colleagues [86] have demonstrated that estrogen-induced phospho-CREB in hypothalamic GnRH neurons is preserved in ERα knockout mice, but lost in ERβ knockout mice. Likewise, other studies also show the loss of rapid estrogen-induced phospho-CREB responses in ERβ knockout mice, particularly in areas where ERβ is densely localized [87]. With regard to ERK regulation, there are contradicting reports on the estrogen regulation of phospho-ERKs in ER-knockout mice. Singh et al. [88] reported that estrogen-induced phosphorylation of ERKs was not lost in cortical neurons from ERα knockout mice, suggesting that ERα may not mediate the rapid phospho-ERK-enhancing effects of estrogen. In contrast, Abraham et al. [87] reported that rapid estrogen induction of phosphorylation of ERKs in the medial preoptic nucleus was lost in both ERα and ERβ knockout mice, suggesting that

both ERα and ERβ may mediate the ERK regulatory effect of estrogen. Finally, estrogen can also rapidly induce c-Fos protein in the brain, an effect which has been shown to be lost in double ERα/β knockout mice in the ventral medial nucleus, preoptic area, granule accessory layer of the olfactory bulb, and pyramidal layer of the CA3 hippocampus [89]. Interestingly, two regions did not show a loss of estrogen induction of c-Fos in the double ERα/β mice: the olivary pretectal nucleus and cingulate cortex. As a whole, the ER knockout mice results shed some light on involvement of ERα or ERβ in rapid signaling effects of estrogen in the brain. Additionally, they suggest that the role of ERα or ERβ in rapid estrogen signaling effects may differ from one brain region to another, and results in one region cannot be immediately extrapolated to another region or another kinase.

GPR30 – A Novel Membrane Estrogen Receptor?

Recent interest in the field of rapid estrogen signaling has focused on a novel seven transmembrane domain G-protein-coupled receptor called GPR30, which has recently been proposed to function as a membrane estrogen receptor [90–94]. GPR30 was first identified and cloned by Carmechi et al. [90] in 1997. Using a differential cDNA library screening technique, the investigators identified GPR30 as a differentially expressed gene in a screen of genes from an ER-positive breast cancer cell line (MCF-7) versus an ER-negative breast cancer cell line (MDA-MB-361). Most studies show that GPR30 is localized in the plasma membrane of cells, but an endoplasmic reticulum localization for GPR30 has also been reported [91–94]. Additionally, electron microscopy by our laboratory and others has confirmed that GPR30 is localized in the plasma membrane [95, 96] and endoplasmic reticulum [95] of neurons in the brain. It should be mentioned that seven-transmembrane domain receptors are known to be retained in the endoplasmic reticulum as a consequence of carbohydrate processing, disulfide bond exchange, and proteolytic editing, which may explain the observation of GPR30 localization in the endoplasmic reticulum. Alternatively, GPR30 may have a functional role in the endoplasmic reticulum, which remains to be determined.

Radioreceptor assays have shown that membrane expression of GPR30 in human ER-negative breast SKBR3 cancer cells is associated with high-affinity, saturable, low-capacity binding for estrogen [93, 94]. Treatment of the SKBR3 cells with GPR30 siRNAs significantly reduced GPR30 mRNA and protein and resulted in an 80%

decrease in estrogen binding [93, 94]. Further work has implicated GPR30 as having a role in mediating rapid estrogen modulation of calcium, cAMP, and ERK signaling in human breast cancer and liver cell lines [91–94]. However, there are a number of controversies surrounding the putative role of GPR30 as an estrogen receptor involved in physiological estrogen signaling. For instance, even though ER-negative SKBR3 breast cancer cells have abundant GPR30 expression, they have an extremely low estrogen binding interaction, with only modest generation of cAMP observed in cells transfected with GPR30 [94]. Additionally, Pedram et al. [97] demonstrated that GPR30 siRNA knockdown in MCF-7 breast cancer cells did not affect estrogen rapid signaling to cAMP, ERK, PI3K, calcium or cell survival, while ERα siRNA knockdown prevented the rapid signaling and survival effects of estrogen in MCF-7 cells. Furthermore, the preponderance of work in the field has been performed in cell lines, and studies in vivo are lacking. Finally, few studies assessed functional endpoints, so the functional significance of the purported GPR30-mediated signaling effects remains unclear.

With respect to the brain, information has now begun to appear concerning GPR30 distribution in various brain regions in rodents. Work from several laboratories has shown that GPR30 is expressed in many regions of the brain with high expression noted in the hypothalamic-pituitary axis, hippocampus, brainstem autonomic nuclei, cortex and striatum [95, 96, 98]. Additionally, a putative GPR30 selective agonist, G-1, has been shown to increase intracellular calcium concentrations in cultured hypothalamic neurons [98], but unfortunately no functionally relevant endpoints were assessed in the study to determine the physiological significance of the observation. To date, there are no studies establishing a physiological role for GPR30 in estrogen signaling in the brain. Studies using GPR30 knockout mice may help address this issue and clarify the role of GPR30 in estrogen actions and signaling in the brain, as well as in other estrogen-target tissues in the body.

Finally, one must consider the possibility that classical ERα or ERβ may couple to G-protein signaling via an unknown mechanism to mediate rapid estrogen signaling. Navarro et al. [99] have demonstrated by coimmunoprecipitation studies that ERα interacts with Galphai3 in immortalized GnRH neurons, and that estrogen-induced inhibition of cAMP can be blocked by pertussis toxin, a G-protein signaling inhibitor. Additionally, Kelly and coworkers [3, 66, 100] have provided evidence of a Galphaq-coupled estrogen receptor that regulates the PLC-PKC-

PKA signaling pathway in hypothalamic neurons and is involved in hypothalamic control of energy homeostasis. Taken as a whole, the above studies provide evidence in specific brain regions that G-protein signaling may be involved in the rapid estrogen effects in the brain, but considerable work is needed to fully understand the mechanisms whereby estrogen couples to and regulate G-protein signaling. Considering the current controversies surrounding the role of GPR30 in estrogen signaling in the brain and other parts of the body, this area should be an important focal point for future research.

Do Scaffold Proteins Help Mediate Rapid Estrogen Signaling?

It is currently unclear as to how membrane estrogen receptors regulate kinase signaling in the brain. However, work in the last few years has identified estrogen receptor (ER)-interacting scaffold proteins that may serve to link membrane estrogen receptors to kinase signaling pathways via the formation of a signaling complex that includes the ER, ER-interacting protein and various kinases. We will discuss three such ER-interacting proteins – MNAR/PELP1 (modulator of nongenomic action of estrogen receptor/proline-, glutamic acid-, and leucine-rich protein-1), striatin and p130Cas [101–105]. It should be mentioned that the majority of the work on these proteins has been performed in non-neuronal cells such as breast cancer and endothelial cells; however, where available, we will discuss correlative findings on these scaffold ER-interacting proteins in the brain.

MNAR/PELP1
Of all the ER-interacting proteins, we know the most about MNAR/PELP1. Human MNAR/PELP1 was initially cloned and characterized as a novel human coregulator of ERα, with high expression levels present in the brain, testes, and mammary glands [101, 102]. Subsequent work by Brann and coworkers [103, 106] cloned the rat and monkey MNAR/PELP1 gene. Figure 2 shows the primary amino acid sequence for rat MNAR/PELP1 (fig. 2a), domain structure deduced from the amino acid sequence (fig. 2b), and RT-PCR expression results for MNAR/PELP1 in various regions of female rat brain (fig. 2c) [103]. The primary structure of rat MNAR/PELP1 protein was shown to consist of 1,130 amino acids organized into various functionally conserved domains, similar to other species [101–103, 106]. These conserved domains are comprised of various LXXLL, PXXP motifs

Fig. 2. Primary amino acid sequence of rat MNAR/PELP1 deduced from cDNA cloned into pT7-blue vector (**a**), and domain/binding motifs organization in the primary structure of the rat MNAR/PELP1 (**b**). **c** RT-PCR analysis of MNAR/PELP1 mRNA expression in various tissues from the proestrous female rat. –RT = Negative control where reverse transcriptase was omitted; +control = cloned rat MNAR/PELP1 plasmid. From Vadlamudi et al. [101], with permission.

and glutamic acid clusters. The LXXLL motifs have been shown to interact with the AF2 domains of classical steroid receptors, whereas the PXXP domains serve as SH3-binding sites for Src, PI3K and potentially other SH3 domain proteins, thereby functioning as a scaffold protein to link the estrogen receptor with kinase pathways [101, 102].

MNAR/PELP1 has been shown to be expressed in many regions of the brain that are targets for estrogen action, including the hypothalamus, cerebral cortex, hippocampus, pituitary, and cerebellum (fig. 2) [103, 106]. Furthermore, colocalization studies revealed that MNAR/PELP1 is colocalized with ERα in many regions of the brain [103]. Light- and electron-microscopic studies have demonstrated that MNAR/PELP1 is located at the plasma membrane and in the nucleus of non-neuronal and neuronal cells, supporting its putative nongenomic and genomic roles [101, 103, 106]. Evidence in support of its genomic role in estrogen action has come from studies demonstrating that siRNA to MNAR/PELP1 significantly attenuated estrogen-induced transcription of genes in cells [101, 102, 107]. Likewise, support for a role

of MNAR/PELP1 in nongenomic estrogen effects has come from studies demonstrating that estrogen activation of Src-ERK can be blocked by MNAR/PELP1 antisense or siRNA in breast cancer cells [101, 102]. Corollary studies confirming the genomic and nongenomic roles of MNAR/PELP1 in estrogen actions in the brain are underway in our laboratory. Our preliminary work has confirmed that ER-α and MNAR/PELP1 do interact in the brain and that estrogen may enhance this interaction, similar to what has been observed in breast cancer cells [Zhang and Brann, unpubl. obs]. Recent work also suggests that MNAR/PELP1 is phosphorylated and that this phosphorylation promotes estrogen activation of nongenomic signaling pathways, such as PI3K in breast cancer cells [108]. Overall, the above studies support a role for MNAR/PELP1 as an estrogen receptor coactivator and ER-interacting scaffold protein which appears critical for the genomic and nongenomic effects of estrogen. Clearly, while a lot of work has been performed in non-neuronal cells; additional work is needed to understand the role of MNAR/PELP1 in estrogen actions in the brain.

Striatin

A second ER protein identified is striatin, a 110-kDa protein which is highly conserved [104, 109, 110]. The protein structure of striatin includes a putative caveolin-binding motif, a coiled-coil structure [111], a Ca^{2+}-calmodulin binding site and a large WD-repeat domain [109, 112–114]. Work in endothelial cells led to evidence that striatin interacts with ERα, implicating its importance in nongenomic signaling [104]. Along these lines, co-immunoprecipitation experiments revealed an ERα-striatin complex formation, with striatin promoting localization of ERα to the membrane [104]. In vascular endothelial cells, striatin has been shown to directly bind to amino acids 183–253 of ERα and to serve as a scaffold for the formation of an ERα–G-protein (Gαi) complex [104]. Overexpression of a peptide consisting of the striatin-binding domain within ERα disrupts complex formation between ERα and striatin, and blocks estrogen-induced rapid activation of ERKs, Akt, and eNOS, but has no effect on ER-dependent regulation of an estrogen response element-driven reporter plasmid [104]. Little work has been done in any other cell or tissue type on the role of striatin in estrogen actions, including the brain. Interestingly, however, studies have shown abundant localization of striatin to the dendritic spines and somatodendritic regions of the brain [111, 115], and thus it appears to act at the postsynaptic level of information processing [109, 116]. Intense immunostaining of striatin has been reported in the dorsal striatum, nucleus accumbens, olfactory tubercle, red nucleus and subthalamic nucleus, while lower levels were detected in the cerebral cortex, amygdala, hippocampus, midbrain and cerebellum [112]. Preliminary data acquired by our lab from the female rat brain verified these results [117]. Moreover, the presence of striatin in a large proportion of efferent neurons has led to the suggestion that it is involved in executive output function [112]. Interestingly, in vitro data from a variety of non-neuronal cells shows that striatin directly binds caveolin-1, a membrane microdomain responsible for the docking of scaffold proteins and the center of signal transduction, thus providing additional evidence of striatin's membrane association [111, 118]. This is intriguing as several studies have suggested that estrogen receptors may associate with caveoli [118–121]. Obviously, future studies are needed to determine if striatin is colocalized with estrogen receptors in the brain and whether it is modulated by estrogen, as well as to determine what role, if any, it has in mediating estrogen signaling and physiological actions in the brain.

P130Cas

The third ER-interacting protein, a 130-kDa, tyrosine-phosphorylated p130 protein designated p130Cas (Crk-associated substrate), was identified in 1994 and has been shown to localize both to the membrane and to the nucleus in cells [105, 117, 122]. A full characterization of the localization and distribution of p130Cas in the brain is lacking, but preliminary studies by our laboratory revealed intense immunolocalization of p130Cas in the amygdala, cerebral cortex and striatum of the female rat brain [117]. Analysis of the protein structure of p130Cas revealed a unique SH3 region in the N-terminal domain [123] as well as a proline-rich domain clustered with tyrosines that functions as SH2-binding motifs [124]. Work in breast cancer cells has shown that ERα interacts with p130Cas in a multi-molecular complex with Src and PI3K following estrogen administration [105]. Gene knockdown studies suggested an important role for p130Cas in estrogen nongenomic signaling in human breast cancer cells, as siRNA knockdown of p130Cas significantly reduced the ability of estrogen to increase ERK activation [105]. Unfortunately, parallel work in the brain on the role of p130Cas in estrogen signaling has not been conducted to date. However, there is evidence suggesting a role for p130Cas in neurite formation [125] and commissural axon guidance possibly via interactions with GTPase molecules such as Rac and Cdc42 [126–129]. Further research is necessary for the characterization of the functional and physiological roles of p130Cas in the brain and its precise involvement in estrogen signaling.

Conclusions

In conclusion, there is abundant evidence that estrogen exerts rapid nongenomic effects in the brain to regulate various ion channel, second-messenger and kinase signaling pathways. Figure 3 illustrates the various mechanisms for nongenomic signaling by estrogen that are postulated to occur in neurons. The genomic signaling mechanism is also included in the diagram to illustrate that estrogen can utilize both types of signaling mechanisms to regulate neuronal function. As discussed in the review, an increasing body of work shows that the rapid nongenomic effects of estrogen have a physiologically important role in estrogen-induced neuroprotection, regulation of homeostasis and modulation of synaptic plasticity in the brain. The rapid effects of estrogen in the brain appear to be mediated by a membrane estrogen receptor, although it is not yet entirely clear whether it is ERα, ERβ,
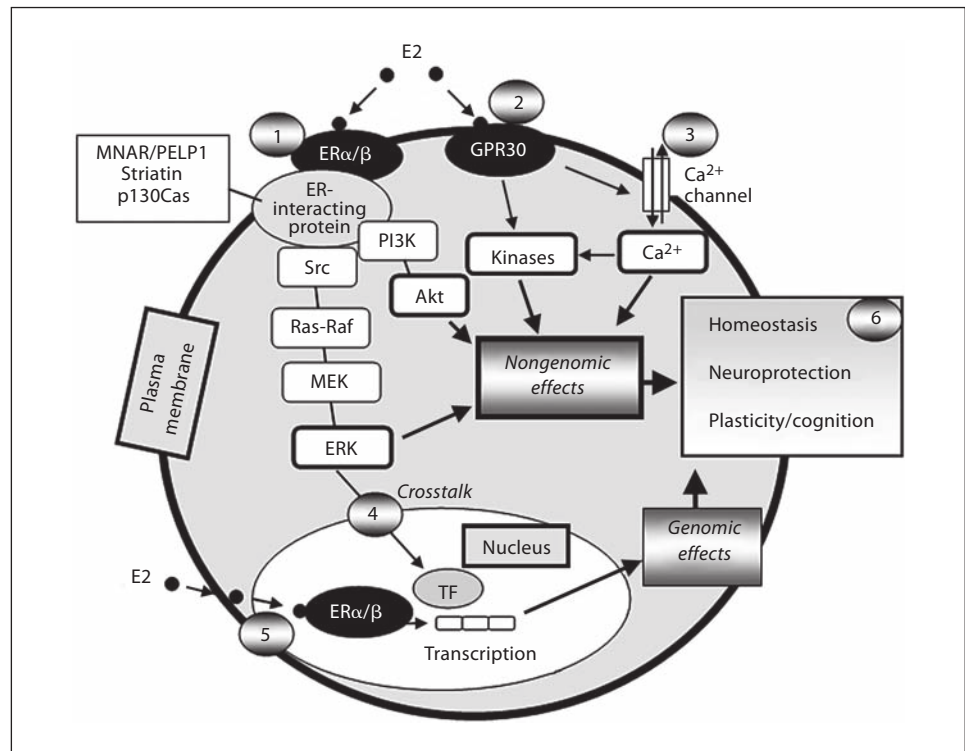
Fig. 3. Summary diagram of potential nongenomic and genomic signaling pathways of estrogen in the brain. (1) Estrogen interaction with classical ERα/β in plasma membrane that is in a complex with ER-interacting protein, which facilitates estrogen activation of kinases and subsequent downstream regulation of various cellular proteins and functions. (2) Estrogen interaction with GPR30 in the plasma membrane that leads to activation of kinase and second-messenger signal pathways such as calcium, which exerts multiple downstream effects in the cell. (3) Estrogen, via membrane ERα/β or GPR30-mediated mechanisms, modulates calcium channels to influence intracellular calcium levels, which subsequently regulates activation state of kinases and various other cellular proteins and functions. Estrogen can also modulate calcium release from intracellular calcium stores via kinase regulation, as well as regulate other ion channels – such as potassium channels as discussed in the review. (4) Activated phospho-ERK translocates to the nucleus where it can regulate transcription factors such as CREB and Elk1 – and thereby modulate transcription of genes. The actions of phospho-ERK in the nucleus to regulate transcription thus provide a mechanism for crosstalk between the nongenomic and genomic pathways. (5) Classical estrogen genomic signaling via ERα/β in nucleus regulates transcription of genes that influence cell functions. (6) Nongenomic signaling effects of estrogen postulated to contribute to homeostasis, neuroprotection and plasticity/cognition actions of estrogen as discussed in the review. (Contributions by genomic effects of estrogen may contribute to these functions as well.)

GPR30, or an as yet unidentified membrane estrogen receptor. Results from ERα and ERβ knockout models provide some evidence supporting classical estrogen receptors in the mediation of rapid estrogen signaling in the brain. However, these studies show that the role of classical ERα or ERβ in mediating rapid estrogen signaling in the brain can vary depending on the specific kinase and precise brain region examined, making it difficult to make clear-cut global conclusions and extrapolations. The discovery of GPR30 and its proposal as a putative membrane estrogen receptor has generated much excitement. However, a role for GPR30 in nongenomic estrogen signaling remains controversial, and detailed studies in the brain are lacking. Thus, additional studies are urgently needed to determine the precise role, if any, of GPR30 in estrogen rapid signaling in the brain. Finally, there is growing interest in ER-interacting proteins such as MNAR/PELP1, striatin and p130Cas and the role they may play in mediating estrogen activation of kinase signaling in the brain. These ER-interacting proteins have been shown to couple ER to kinases such as Src and PI3K and to mediate rapid estrogen activation of ERKs and Akt in non-neuronal cells. While the role of these ER-interacting proteins in rapid estrogen signaling in the brain

has not been examined, MNAR/PELP1 has been shown to be localized in key brain regions that are targets of estrogen action, and it has been shown to be colocalized with ERα in the brain. Striatin and p130Cas are also localized in the brain, but there colocalization with ERα has not been established. For the field to advance, studies using gene knockdown or knockout animal models are needed to establish whether these ER-interacting proteins have a physiologically important role in the signaling and functional effects of estrogen in the brain. In conclusion, while much has been learned in the past decade on nongenomic estrogen signaling in the brain, considerable work remains ahead before we can fully understand the mechanisms that underlie these rapid signaling effects of estrogen, as well as their full physiological significance.

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