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Zhen Zhao[a], Cheryl Park[a], Melissa A. McDevitt[a], Christine Glidewell-Kenney[b], Pierre Chambon[c], Jeffrey Weiss[b], J. Larry Jameson[b], and Jon E. Levine[a,1]

[a]Department of Neurobiology and Physiology, Northwestern University, Evanston, IL 60208-3113; [b]Department of Endocrinology, Metabolism, and Molecular Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; and [c]Institut de Génétique et de Biologie Moléculaire et Cellulaire, 1 Rue Laurent Fries, 67404 Illkirch, France

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Nonclassical estrogen receptor α (ERα) signaling can mediate E_2 negative feedback actions in the reproductive axis; however, downstream pathways conveying these effects remain unclear. These studies tested the hypothesis that p21-activated kinase 1 (PAK1), a serine/threonine kinase rapidly activated by E_2 in nonneural cells, functions as a downstream node for E_2 signaling pathways in cells of the preoptic area, and it may thereby mediate E_2 negative feedback effects. Treatment of ovariectomized (OVX) rats with estradiol benzoate (EB) caused rapid and transient induction of phosphorylated PAK1 immunoreactivity in the medial preoptic nucleus (MPN) but not the arcuate nucleus. To determine whether rapid induction of PAK phosphorylation by E_2 is mediated by nonclassical [estrogen response element (ERE)-independent] ERα signaling, we used female ERα null (ER$\alpha^{-/-}$) mice possessing an ER knock-in mutation (E207A/G208A; AA), in which the mutant ERα is incapable of binding DNA and can signal only through membrane-initiated or ERE-independent genotropic pathways (ER$\alpha^{-/AA}$ mice). After 1-h EB treatment, the number of pPAK1-immunoreactive cells in the MPN was increased in both wild-type (ER$\alpha^{+/+}$) and ER$\alpha^{-/AA}$ mice but was unchanged in ER$\alpha^{-/-}$ mice. Serum luteinizing hormone (LH) was likewise suppressed within 1 h after EB treatment in ER$\alpha^{+/+}$ and ER$\alpha^{-/AA}$ but not ER$\alpha^{-/-}$ mice. In OVX rats, 5-min intracerebroventricular infusion of a PAK inhibitor peptide but not control peptide blocked rapid EB suppression of LH secretion. Taken together, our findings implicate PAK1 activation subsequent to nonclassical ERα signaling as an important component of the negative feedback actions of E_2 in the brain.

GnRH | LH | estrogen receptor α

Ovarian estradiol-17β (E_2) conveys negative feedback actions within the reproductive axis that include inhibition of gonadotropin-releasing hormone (GnRH) neurosecretion and suppression of gonadotrope responsiveness to GnRH stimulation. Both actions can be sustained by E_2 treatment regimens that maintain serum E_2 levels in low physiological ranges (1) and they can also be manifested rapidly, within minutes after E_2 injection (2). Studies of estrogen receptor α (ERα), ERβ, and ERα/β null mutant mice have clearly implicated ERα as the isoform essential for E_2 negative feedback regulation in vivo (3, 4).

Cell signaling pathways that transduce ERα-mediated negative feedback are not well understood. In classical ERα signaling mechanisms, E_2 binds nuclear ERs and recruits coactivators to consensus palindromic estrogen response elements (EREs). Direct binding of ERs to EREs thereby mediates alterations in transcription of target genes. Nonclassical ERα signaling mechanisms operate independently of ERα binding directly to EREs and include protein–protein interactions with transcription factors, such as AP1, SP1, and NF-κB (5), which in turn mediate transcriptional regulation at their cognate response elements. Nonclassical ERα signaling also includes membrane-associated receptor activation coupled to stimulation of cytoplasmic signaling pathways. To distinguish relative contributions of classical versus nonclassical ERα signaling to E_2 actions in vivo, Jakacka et al. (6) developed a gene knockin mouse that expresses a mutant (E207A/G208A; AA) form of ERα with disrupted classical (ERE-dependent) but intact nonclassical (ERE-independent) ERα-signaling capacities. By using animals in which the AA mutant allele was introduced onto the ERα-null (ER$\alpha^{-/-}$) mutant background (ER$\alpha^{-/AA}$ mice), we determined that nonclassical ERα signaling can rescue the majority of E_2 negative feedback effects that are present in wild-type (ER$\alpha^{+/+}$) mice and completely absent in ER$\alpha^{-/-}$ mice (7).

Here, we attempt to identify a downstream mediator of nonclassical ERα signaling mechanisms conveying E_2 negative feedback in the brain. E_2 can modulate dendrite morphogenesis and induce synaptogenesis in hypothalamic (8–11) and extrahypothalamic neuronal populations (12, 13), and such structural plasticity may mediate E_2 feedback effects (14). That the ERα isoform appears to mediate many of these effects rapidly (15) is consistent with the idea that membrane-initiated, nonclassical ERα signaling can rapidly induce the actin-cytoskeletal reorganization required for synaptic remodeling (16). In nonneural cells, ERα signaling produces rapid alterations in cell shape, polarity, and motility by activating p21-activated kinase 1 (PAK1) (17), the best-characterized member of a family of conserved mammalian serine/threonine kinases that function as downstream effectors of activated Rho GTPases, Rac1 and Cdc42, as well as phosphatidylinositol 3-kinase (PI3K) (18). Because nonclassical ERα signaling and activated PAK1 share common effects on neuronal morphology (19), and because E_2 can activate PAK1 through nonclassical mechanisms (20), we tested the hypothesis that the nonclassical negative feedback actions of E_2 are conveyed in part via ERα-mediated activation of PAK1.

Results

E_2 Rapidly Induces PAK1 Phosphorylation In Vivo. The effects of EB on the expression of pPAK1 were assessed by peroxidase immunohistochemical analyses of the lateral and medial subdivisions of medial preoptic nucleus (MPNl and MPNm, respectively) and the arcuate nucleus (AN). Treatment with EB but not oil vehicle produced a rapid and transient increase in the number

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[1]To whom correspondence should be addressed. E-mail: jlevine@northwestern.edu.

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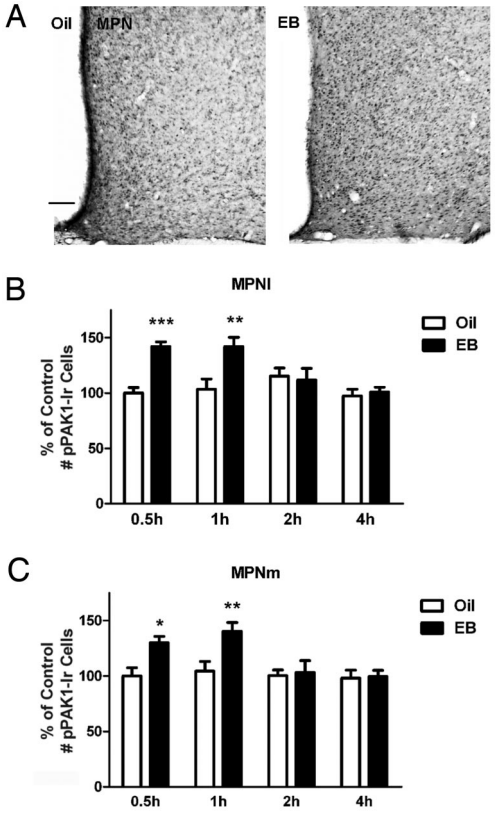

Fig. 1. Time dependence of E_2-induced pPAK1-Ir in the MPN of female rats. OVX females rats treated with oil or EB were killed at 0.5, 1, 2, and 4 h after injection. (*A*) Representative photomicrographs of pPAK1-Ir in the MPN and adjacent regions of OVX rats 0.5 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir cells in the MPNl (*B*) and MPNm (*C*) was significantly greater after 0.5 and 1 h of EB treatment compared with corresponding oil treatment ($n = 6$–9). Data are represented as the mean ± SEM (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

of phosphorylated PAK1 immunoreactivity (pPAK1-Ir) cells in both subdivisions of the MPN. Micrographs of representative sections from 1 oil-treated and 1 EB-treated ovariectomized (OVX) rat 0.5 h after injections are given in Fig. 1*A*, demonstrating a greater number of pPAK1-Ir cells in the MPNl and MPNm of an EB-treated versus an oil-treated rat. Fig. 1 *B* and *C* summarizes pPAK1-Ir cell counts in the MPNl and MPNm for the 2 treatment groups at all time points. EB significantly increased the number of pPAK1-Ir cells as early as 0.5 h in the MPNl ($P < 0.001$; Fig. 1*B*) and MPNm ($P < 0.05$; Fig. 1*C*). This increase was maintained at 1 h after EB treatment ($P < 0.01$; Fig. 1 *B* and *C*). The number of pPAK1-Ir cells at 2 and 4 h was not significantly different from corresponding values in oil-treated controls. In the same OVX animals, the oil and EB treatments were without effect on the number of pPAK1-Ir cells in the AN. Representative tissue sections containing the AN from an oil-treated and an EB-treated rat are provided in Fig. 2*A*, along with the summary data of pPAK1-Ir cell counts for the 2 treatments at each time point (Fig. 2*B*). No significant differences between the treatment groups were observed for the pPAK1-Ir cell number in the AN at any time point.

Nonclassical ERα Signaling Mediates Rapid E_2 Induction of pPAK1-Ir. ERα can mediate negative feedback actions of E_2 by nonclassical signaling mechanisms, and rapid, nonclassical ERα-mediated activation of PAK1 has been shown to occur in breast cancer cells (17). We therefore sought to determine whether nonclassical

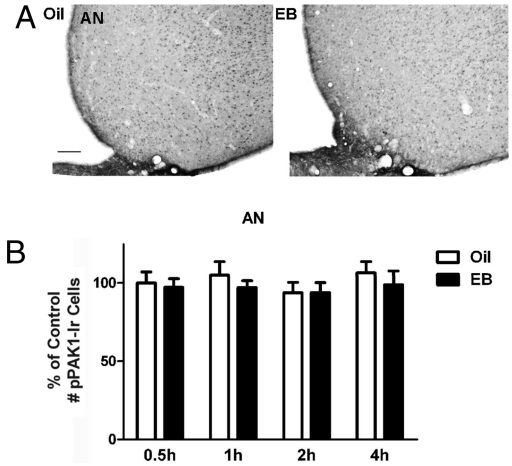

Fig. 2. E_2-independent pPAK1-Ir in the AN of female rats. Animals were treated as described in Fig. 1. (*A*) Representative photomicrographs of pPAK1-Ir in the AN and adjacent regions of OVX rats 1 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir neurons in the AN was not significantly different after EB treatment compared with corresponding oil treatment (*B*). Data are represented as the mean ± SEM ($n = 6$–9).

ERα signaling mediates PAK1 activation in preoptic cells. To assess the role of nonclassical ERα signaling, we compared the ability of 1 μg of EB to induce pPAK1-Ir in the preoptic areas of ER$\alpha^{+/+}$, ER$\alpha^{-/-}$, and ER$\alpha^{-/AA}$ mice within 1 h of treatment. Consistent with our findings in OVX rats, acute E_2 treatment induced a significant increase in the number of pPAK1-Ir cells within 1 h in the MPNl and MPNm in OVX wild-type ER$\alpha^{+/+}$ mice ($P < 0.01$; Fig. 3 *B* and *C*). This effect was completely absent in the ER$\alpha^{-/-}$ mice (Fig. 3 *B* and *C*), confirming the obligatory involvement of ERα in E_2-mediated action. In the OVX ER$\alpha^{-/AA}$ mice, the ability of E_2 to induce PAK1 was restored to levels observed in the ER$\alpha^{+/+}$ mice ($P < 0.05$; Fig. 3 *B* and *C*), indicating that nonclassical ERα signaling is sufficient to mediate E_2 effects on pPAK1 in the MPN. Representative photomicrographs of pPAK1-Ir in the MPN of the 3 genotypes are provided in Fig. 3*A*. The summary values for pPAK1-Ir in the 3 genotypes are depicted in Fig. 3 *B* and *C*. In contrast to the effects of E_2 in the preoptic area, E_2 was without any effect on pPAK1-Ir in the AN in any of the groups (Fig. 4).

Nonclassical ERα Signaling Mediates Rapid Negative Feedback Actions of E_2. If E_2 can rapidly activate pPAK1 through nonclassical ERα signaling, and this mechanism is integral to E_2 negative feedback, then it should be true that E_2 can engage this mechanism to effect a rapid suppression of LH secretion. We therefore tested the ability of acute EB injections to rapidly suppress LH by nonclassical ERα signaling. To examine the rapid feedback actions of E_2 on LH release, ER$\alpha^{+/+}$, ER$\alpha^{-/-}$, and ER$\alpha^{-/AA}$ female mice were ovariectomized, and 7 days later (1,000–1,200 h) they received s.c. injections of 1 μg of EB or oil vehicle. Animals were killed 1 h after the injection. A total of 3 blood samples were collected from each mouse—one at the time of OVX, one just before the EB injection, and one at sacrifice 1 h later. LH RIA of the plasma samples revealed that LH levels before OVX were low in ER$\alpha^{+/+}$, slightly elevated in ER$\alpha^{-/AA}$, and greatly increased in ER$\alpha^{-/-}$ mice, as reported previously (7). The serum LH levels were significantly elevated in both ER$\alpha^{+/+}$ ($P < 0.001$; Fig. 5, a) and ER$\alpha^{-/AA}$ ($P < 0.05$; Fig. 5, b) mice at 7 days after OVX compared with pre-OVX levels. In contrast, LH levels in ER$\alpha^{-/-}$ mice were elevated before OVX and remained at these levels at 7 days after OVX. Treatment of OVX ER$\alpha^{+/+}$ mice with EB resulted in a suppression of LH

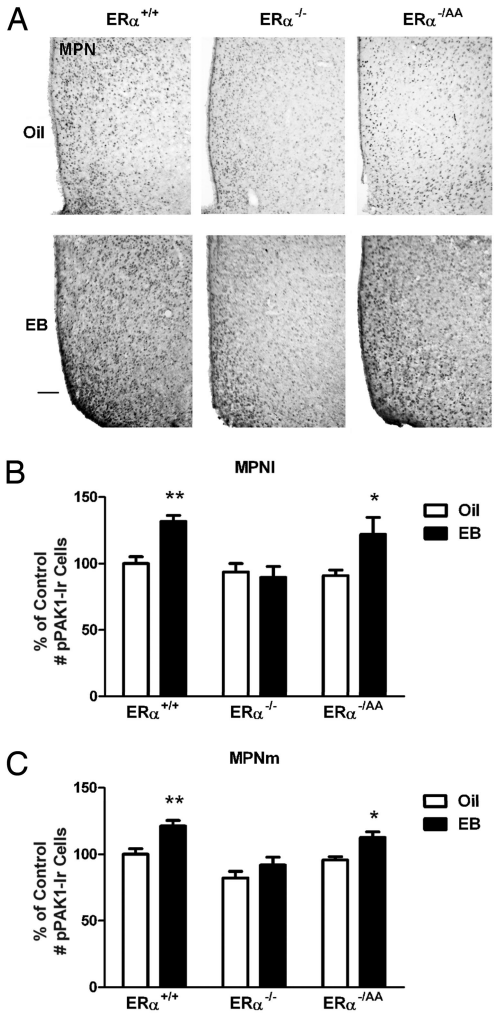

Fig. 3. E_2-induced pPAK1-Ir in the MPN of female ER$\alpha^{+/+}$, ER$\alpha^{-/-}$, and ER$\alpha^{-/AA}$ mice. OVX female mice treated with oil or EB were killed at 1 h after injection. (*A*) Representative photomicrographs of pPAK1-Ir in the MPN and adjacent regions of OVX mice 1 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir cells was significantly increased in the MPNl (*B*) and MPNm (*C*) in ER$\alpha^{+/+}$ and ER$\alpha^{-/AA}$ but not ER$\alpha^{-/-}$ mice (n = 5–9). Data are represented as the mean ± SEM (**, $P < 0.01$; *, $P < 0.05$).

within 1 h, whereas the same treatment was without effect in the OVX ER$\alpha^{-/-}$ mice. In the OVX ER$\alpha^{-/AA}$ mice, EB suppressed LH to the pre-OVX levels ($P < 0.05$; Fig. 5, d). The inhibitory action of EB in OVX ER$\alpha^{-/AA}$ mice constituted ≈70% of the suppression seen in the OVX ER$\alpha^{+/+}$ mice ($P < 0.001$; Fig. 5, c).

Inhibition of PAK Phosphorylation Blocks Acute EB Suppression of LH Secretions. Within the PAK kinases, a conserved, proline-rich sequence of 18 aa called PAK18 binds tightly to the SH3 domain of PAK-interacting exchange factor (PIX). The PIX–PAK interaction was shown to be essential for PAK activation (21). The PAK18 peptide has been used to interfere selectively with the activation of PAKs 1–3 in cell cultures (22–26) and in vivo in the rat forebrain (22). PAK18 was conjugated to a cell-permeant HIV-1 TAT peptide sequence, which has been used to deliver functional biomolecules into cells (27). The TAT-facilitated cellular translocation has been shown to happen very rapidly (within 1–2 h) and results in neurofunctional effects in vitro and in vivo (28, 29). The PAK18 peptide but not the inactive peptide PAK18 R192A has been shown to reduce pPAK levels in the

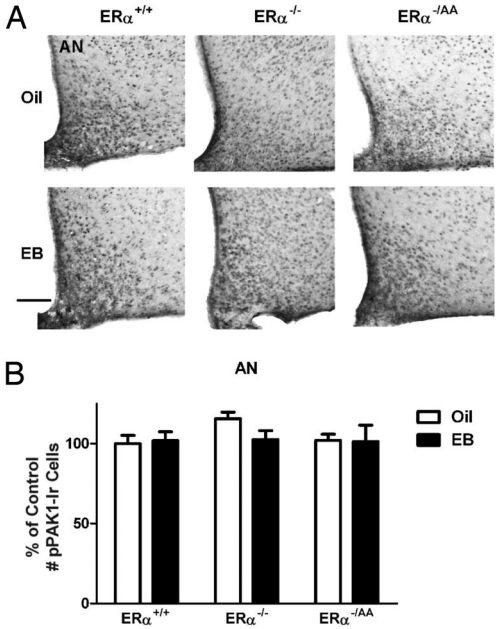

Fig. 4. E_2-independent pPAK1-Ir in the AN of female ER$\alpha^{+/+}$, ER$\alpha^{-/-}$, and ER$\alpha^{-/AA}$ mice. Animals were treated as described in Fig. 3. (*A*) Representative photomicrographs of pPAK1-Ir in the AN and adjacent regions of OVX mice 1 h after oil or EB injection. (Scale bar: 100 μm.) The number of pPAK1-Ir cells in the AN was not significantly different after EB treatment compared with values in animals receiving corresponding oil treatment (*B*). Data are represented as the mean ± SEM (n = 5–8).

hippocampus by 80% after intracerebroventricular (icv) treatments, an effect that was accompanied by drebrin loss, cofilin pathology, and memory deficits (22). This suggests that PAK18 and PAK18 R192A are valid tools for inhibiting PAKs. We first verified the ability of PAK18 to block PAK phosphorylation by using hypothalamic GT1-7 cells in vitro. Western blot analysis of pPAK demonstrated that inhibition of PAK phosphorylation is rapid and significant when these cells are incubated with the peptide PAK18 (10 μM) for 1 h compared with R192A ($P < 0.05$; Fig. 6*A*). Subsequently, we infused peptide PAK18 (6 μg/μL, 1 μL/min in 5 min) or R192A in the lateral ventricle of OVX rats. The cerebrospinal fluid (CSF) volume was 250 μL per rat, with a physiological flow rate of 2.9 μL min^{-1} (30); therefore, the concentration of PAK18 and R192A in the CSF was ≈10 μM 1 h after the infusion, a dose found effective in vitro. Immediately after the peptide infusion, OVX animals were given an s.c. injection of EB (30 μg per rat). At 1 h after EB injection, animals were killed, and brains were removed rapidly to assess the ability of PAK18 to reduce pPAK1-Ir by immuno-

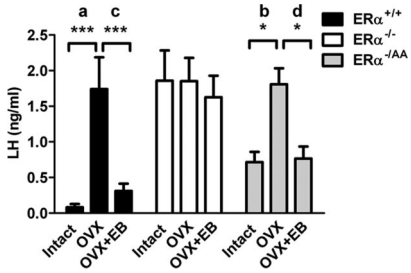

Fig. 5. The ERE-independent ERα signaling pathway is sufficient to convey rapid E_2 negative feedback actions. Serum LH from intact, OVX, and OVX/EB-injected (1 h) females in the morning (n = 8–14). Data are represented as the mean ± SEM (***, $P < 0.001$; *, $P < 0.05$).

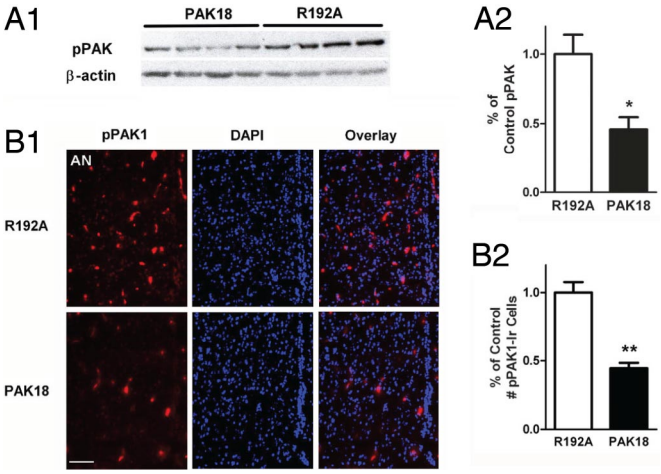

Fig. 6. Inhibition of PAK phosphorylation. Inhibition of PAK phosphorylation by treatment with PAK18 inhibitory peptide to GT1-7 cells (*A*) and in the lateral ventricle of OVX rats (*B*) for 1 h ($n = 4–6$). (Scale bar: 100 μm.) Data are represented as the mean ± SEM (**, $P < 0.01$; *, $P < 0.05$).

histofluorescence, indicating a suppression of PAK1 phosphorylation. As depicted in Fig. 6*B*, infusion of PAK18 peptide for 1 h resulted in a significant reduction in the number of pPAK1-Ir cells in the preoptic area compared with the control treatment ($P < 0.01$; Fig. 6*B*). To determine the effects of PAK18 infusions on responsiveness to the negative feedback actions of E_2, blood samples were obtained just before infusions and at autopsy 1 h after the EB treatment. Analysis of serum LH levels by RIA revealed that in OVX rats, 5-min icv infusion of PAK18 but not control peptide R192A blocked rapid suppression of serum LH 1 h after EB administration (Fig. 7).

Discussion

Our laboratories recently demonstrated that the majority of E_2 negative feedback actions in the mouse can be exerted by a nonclassical ER α signaling mechanism that proceeds in the absence of direct binding of ERα to EREs in the promoters of target genes (7). In the present study, we have further determined that these inhibitory effects can occur within 1 h of E_2 administration, and thus are likely exerted via nongenotropic signaling mechanisms. Our findings specifically implicate the PAKs as components of the nongenotropic ERα signaling pathways leading to the suppression of GnRH and LH, because they show that (*i*) E_2 rapidly induces PAK1 phosphorylation in the MPN, (*ii*) nonclassical ERα signaling is sufficient to rescue rapid phosphorylation of PAK1 in preoptic cells, as well as suppression of LH secretion in ERα-null mutants, and (*iii*) acute

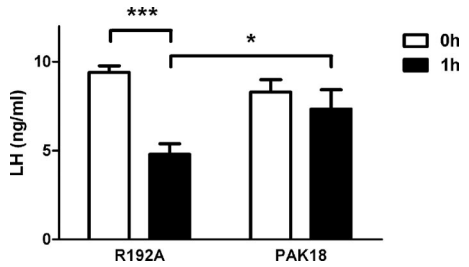

Fig. 7. Inhibition of PAK phosphorylation blocks acute E_2 suppression of LH secretions. The LH level was significantly decreased at 1 h after R192A icv infusion and EB s.c. injection. In the contrast, the LH level was not significantly altered by treatment with PAK18 and EB ($n = 4–6$). Data are represented as the mean ± SEM (***, $P < 0.001$; *, $P < 0.05$).

inhibition of PAK phosphorylation in preoptic-hypothalamic areas blocks the acute negative feedback actions of E_2.

The involvement of a nongenotropic mechanism in E_2 negative feedback has long been suspected, given the ability of acute E_2 treatments to suppress LH secretion in as little as 20 min (31). Although rapid steroid hormone effects on gene transcription are known, the rapid (<60 min) modulation of LH by E_2 occurs within a temporal window that is generally held to be too short to additionally include RNA processing, translation, posttranslational enzymatic processing, intracellular transport, and neurosecretion. A variety of membrane-integrated or membrane-associated receptors and cell signaling mechanisms have instead been suggested to mediate at least some of the E_2 effects on GnRH and LH. Classic work by Kelly et al. (62) revealed rapid effects of E_2 on neuronal firing in preoptic neurons that were best explained by activation of membrane receptors for E_2. Further work demonstrated that GnRH neurons themselves are rapidly hyperpolarized by E_2, even during tetrodotoxin-induced blockade of synaptic inputs (32), suggesting direct electrophysiological suppression through membrane-associated receptors in GnRH neurons. The absence of ERα in GnRH neurons has led some to suggest that rapid, direct effects of E_2 on GnRH neurons may be mediated by ERβ (33) and/or an unidentified G protein-coupled receptor (34). It has remained unclear, however, to what degree the rapid electrophysiological effects of E_2 on GnRH neuronal activity in vitro may reflect the operation of negative feedback loop governing GnRH neurosecretion in vivo. Our studies clearly implicate the ERα isoform, which is not expressed in GnRH neurons, in the rapid feedback actions of E_2 in vivo, and thus alternatively suggest an indirect mechanism conveyed via ERα-expressing afferents to GnRH neurons. The direct ERα-independent effects of E_2 on GnRH neurons may provide additional components of negative feedback control of GnRH and LH release, or they may function to regulate other aspects of GnRH neuronal function. We also cannot exclude the possibility that ERα-dependent mechanisms may up-regulate ERα-independent E_2 signaling through other receptors, which may in turn mediate E_2 feedback effects.

Rapid inhibition of LH secretion can also occur via direct suppression of gonadotrope responsiveness to GnRH (35). It does not appear to be the case, however, that the nonclassical ERα signaling mechanisms described in the present studies are mediated by any such direct actions on the gonadotrope; pituitary responsiveness to GnRH is not enhanced in ERα-null mutants (3), nor is it reduced by E_2 in ER$\alpha^{-/AA}$ mice (7). We have also determined that nonclassical ERα signaling fails to rescue E_2 effects on LH mRNA and pituitary LH content (36), even as it does effectively restore the majority of E_2 suppression of LH secretion (7). Taken together, the weight of the foregoing evidence suggests that the majority of the rapid, nonclassical ERα-mediated suppression of LH secretion occurs through inhibitory actions on afferents to GnRH neurons. These effects are also complemented by classical, ERE-mediated transcriptional effects, because a residual 30% of E_2 negative feedback actions are not rescued by nonclassical ERα signaling. As reported by Christian et al. (63), the portion of preopticohypothalamic negative feedback that is conveyed by classical ERα signaling may be mediated by suppression of GnRH neuronal firing.

We have observed that E_2 induces rapid phosphorylation of PAK1 in MPN cells through a nonclassical ERα signaling mechanism, and that the inhibition of PAK1 in the basal forebrain blocks the inhibition of LH secretion, presumably by preventing E_2 suppression of GnRH neurosecretion. Our findings are therefore consistent with the hypothesis that PAK activation in preoptic neurons mediates the negative feedback actions of E_2 on GnRH release. Although these results do not reveal the cellular signaling pathways that mediate E_2 activation of PAK1, the rapidity of this

process effectively limits the possibilities to those that are initiated at the plasma membrane or within the cytoplasm and that are independent of transcriptional modulation. The actions of E_2 in MPN cells parallel those observed previously in breast cancer cells, where E_2 was found to activate PAK1 through a rapid, nontranscriptional mechanism.

A number of kinases have been reported to phosphorylate PAK1 and regulate its activity (37), including the cyclin B-bound Cdc2, which phosphorylates PAK1 at Thr-212, a site also targeted by the p35-bound form of Cdk5, a neuron-specific protein kinase (38, 39). Moreover, extracellular signal-regulated kinase 2 (ERK2) mediates phosphorylation of PAK1 at Thr-212 (40). PAK1 is also known to function as an effector protein of PI3K (18, 41). In the present study, ERα signaling was found to induce rapid phosphorylation of PAK1 at Thr-212 in MPN cells. Because E_2 can rapidly influence MAPK/ERK and PI3K/Akt signaling pathways in a variety of cell types (42, 43) and in a variety of brain regions, including the MPN (44), it is possible that ERα signaling induces phosphorylation of PAK1 by ERα-mediated activation of either or both intermediate signaling kinases.

Consistent with the hypothesis that PAK1 mediates E_2 feedback effects, recent studies have revealed that many of the cellular actions of E_2 in the CNS are shared by those of activated PAK1. Both activated PAK1 (23) and ERα signaling (15, 45, 46) can induce rapid alterations in the dendritic cytoskeleton that are integral to morphological plasticity in central neurons. In cortical neurons, PAK1 activity promotes formation and maintenance of dendritic spines (47). A recent study showed that E_2 also rapidly increases the number of nascent dendritic spines in cortical neurons (46), and thereby acutely increases neuronal connectivity, although these effects appear to be exerted in an ERα-independent manner. Estradiol also stimulates dendritic spinogenesis in hippocampal CA1 neurons, an effect that may occur rapidly via activation of ERα and MAPK signaling (45). Previous studies have also documented that E_2 induces synaptic remodeling in the ventromedial nucleus, the anteroventral periventricular nucleus, the MPN, and the AN of the hypothalamus (10, 14).

Because ERα signaling and PAKs can both mediate rapid actin cytoskeletal organization and morphological plasticity (11, 23, 48–51), it is thus possible that these downstream cellular events comprise a major route by which E_2-activated PAKs may mediate negative feedback control over GnRH and, hence, LH secretion. It remains to be determined whether such a rapid, PAK-mediated alteration in cell connectivity functions in this manner and, if so, which of the many known PAK substrates (e.g., actin-related protein 2/3 complex, filamin, and/or cofilin) (19) may mediate these actions. Further studies will also be necessary to identify the ERα-expressing cell populations in which PAKs mediate these E_2 effects. The absence of ERα expression in GnRH neurons (52) makes it unlikely that the ERα-mediated negative feedback mechanism that we have characterized in these studies operates within GnRH neurons themselves. Expression of ERα does occur in GABAergic neurons, including those that appear to be afferents to GnRH neurons (53), and GABA release is modulated by E_2 in a variety of brain regions. Moreover, ERα has been shown recently to be localized to axon terminals in hippocampal GABAergic interneurons (54, 55), suggesting their involvement in nongenotropic modulation of synaptic function. The localization of ERs to dendrites and axon terminals has been shown previously in hypothalamic neurons as well (56). Herbison and colleagues (57) have also recently characterized a nonclassical ERα signaling mechanism that mediates E_2 modulation of GABAergic transmission on GnRH neurons.

Taken together, our findings support a model for the homeostatic negative feedback actions of E_2 wherein the activation of extranuclear ERα is coupled via one or more cytoplasmic kinase signaling cascades to the activation of PAKs. Activated PAKs, which would presumably include the brain-enriched PAK1, thereafter mediate alterations in cell function and/or connectivity in neural circuitries that govern GnRH neurosecretion, and thereby suppress the release of the GnRH decapeptide into the hypophysial portal vasculature. Although several of the specific features of this model await testing, the present results provide initial evidence in a physiological context that nonclassical ERα signaling, leading to the activation of one or more of the PAKs, is a signaling pathway that is integral to the manifestation of physiological E_2 negative feedback control in the reproductive axis.

Materials and Methods

Animals. The ER$\alpha^{-/-}$ and ER$\alpha^{-/AA}$ mutant mice were generated as described (6, 58, 59). Further details appear in *SI Materials and Methods*.

Effects of EB Treatment on PAK1 Phosphorylation. Rats and mice were anesthetized and bilaterally OVX. On the morning of day 7 after OVX (0800–1000 hours), animals were given an s.c. injection of sesame oil vehicle or EB (10 μg per rat; 1 μg per mouse). Animals were anesthetized with 75 mg/kg i.p. ketamine (Fort Dodge Laboratories) and 5 mg/kg i.p. xylazine (Burns Veterinary Supply Inc.) and transcardially perfused with 4% paraformaldehyde (Sigma), pH 7.4, at the following time points after injection: 0.5, 1, 2, or 4 h for rats; 1 h for mice (60). Further details appear in *SI Materials and Methods*.

Effects of OVX and Acute EB Treatment on LH Release. Female mice 2–4 months of age were anesthetized (0800–1000 hours), and blood samples were collected immediately before OVX. At 7 days after OVX, blood samples were obtained, and either 1 μg of EB or sesame oil vehicle injections was administered s.c. At 1 h after injections, blood samples were obtained by exsanguination following cardiac puncture. Further details appear in *SI Materials and Methods*.

Effects of PAK18 Inhibitory Peptide on EB Suppression of LH Release. Details of stereotaxic surgery, PAK18 inhibitory peptide infusion, EB treatment, and blood sample collection can be found in *SI Materials and Methods*.

Cell Culture and Western Blot Analysis. The effectiveness of the PAK18 peptide in suppressing PAK phosphorylation was tested in GT1-7 cells, an immortalized GnRH-producing cell line (61). Further details appear in *SI Materials and Methods*.

Immunohistochemistry and Analysis. Brain sections were processed for immunohistochemistry following standard procedures. Details can be found in *SI Materials and Methods*.

Additional experimental procedures are presented in *SI Materials and Methods*.

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1. Leipheimer RE, Bona-Gallo A, Gallo RV (1986) Influence of estradiol and progesterone on pulsatile LH secretion in 8-day ovariectomized rats. *Neuroendocrinology* 43:300–307.
2. Johnston CA, Tesone M, Negro-Vilar A (1984) Cellular mechanisms of acute estrogen negative feedback on LH secretion: Norepinephrine, dopamine and 5-hydroxytryptamine metabolism in discrete regions of the rat brain. *Brain Res Bull* 13:363–369.
3. Couse JF, Yates MM, Walker VR, Korach KS (2003) Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. *Mol Endocrinol* 17:1039–1053.
4. Wintermantel TM, et al. (2006) Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron* 52:271–280.
5. Coleman KM, Smith CL (2001) Intracellular signaling pathways: Nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci* 6:D1379–D1391.
6. Jakacka M, et al. (2002) An estrogen receptor (ER)alpha deoxyribonucleic acid-binding domain knock-in mutation provides evidence for nonclassical ER pathway signaling in vivo. *Mol Endocrinol* 16:2188–2201.

7. Glidewell-Kenney C, et al. (2007) Nonclassical estrogen receptor alpha signaling mediates negative feedback in the female mouse reproductive axis. *Proc Natl Acad Sci USA* 104:8173–8177.
8. Olmos G, et al. (1989) Synaptic remodeling in the rat arcuate nucleus during the estrous cycle. *Neuroscience* 32:663–667.
9. Langub MC, Maley BE, Watson RE (1994) Estrous cycle-associated axosomatic synaptic plasticity upon estrogen receptive neurons in the rat preoptic area. *Brain Res* 641:303–310.
10. Parducz A, et al. (2006) Synaptic remodeling induced by gonadal hormones: Neuronal plasticity as a mediator of neuroendocrine and behavioral responses to steroids. *Neuroscience* 138:977–985.
11. Cohen RS, Pfaff DW (1981) Ultrastructure of neurons in the ventromedial nucleus or the hypothalamus in ovariectomized rats with or without estrogen treatment. *Cell Tissue Res* 217:451–470.
12. Gould E, Woolley CS, Frankfurt M, McEwen BS (1990) Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J Neurosci* 10:1286–1291.
13. Woolley CS, Gould E, Frankfurt M, McEwen BS (1990) Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *J Neurosci* 10:4035–4039.
14. Naftolin F, et al. (2007) Estrogen-induced hypothalamic synaptic plasticity and pituitary sensitization in the control of the estrogen-induced gonadotrophin surge. *Reprod Sci* 14:101–116.
15. Mukai H, et al. (2007) Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons. *J Neurochem* 100:950–967.
16. Carlisle HJ, Kennedy MB (2005) Spine architecture and synaptic plasticity. *Trends Neurosci* 28:182–187.
17. Mazumdar A, Kumar R (2003) Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells. *FEBS Lett* 535:6–10.
18. Tsakiridis T, Taha C, Grinstein S, Klip A (1996) Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. *J Biol Chem* 271:19664–19667.
19. Nikolic M (2008) The pak1 kinase: An important regulator of neuronal morphology and function in the developing forebrain. *Mol Neurobiol* 37:187–202.
20. Rayala SK, Kumar R (2007) Sliding p21-activated kinase 1 to nucleus impacts tamoxifen sensitivity. *Biomed Pharmacother* 61:408–411.
21. Manser E, et al. (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell* 1:183–192.
22. Zhao L, et al. (2006) Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease. *Nat Neurosci* 9:234–242.
23. Penzes P, et al. (2003) Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. *Neuron* 37:263–274.
24. Maruta H, He H, Tikoo A, Nur-e-Kamal M (1999) Cytoskeletal tumor suppressors that block oncogenic RAS signaling. *Ann NY Acad Sci* 886:48–57.
25. Obermeier A, et al. (1998) PAK promotes morphological changes by acting upstream of Rac. *EMBO J* 17:4328–4339.
26. Nheu T, et al. (2004) PAK is essential for RAS-induced upregulation of cyclin D1 during the G1 to S transition. *Cell Cycle* 3:71–74.
27. Wender PA, et al. (2000) The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters. *Proc Natl Acad Sci USA* 97:13003–13008.
28. Aarts M, et al. (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science* 298:846–850.
29. Cao G et al. (2002) In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J Neurosci* 22:5423–5431.
30. Kusuhara H, Sugiyama Y (2001) Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (Part 2). *Drug Discov Today* 6:206–212.
31. Arreguin-Arevalo JA, Nett TM (2006) A nongenomic action of estradiol as the mechanism underlying the acute suppression of secretion of luteinizing hormone in ovariectomized ewes. *Biol Reprod* 74:202–208.
32. Kelly MJ, Ronnekleiv OK, Eskay RL (1984) Identification of estrogen-responsive LHRH neurons in the guinea pig hypothalamus. *Brain Res Bull* 12:399–407.
33. Abraham IM, et al. (2003) Estrogen receptor beta mediates rapid estrogen actions on gonadotropin-releasing hormone neurons in vivo. *J Neurosci* 23:5771–5777.
34. Qiu J, et al. (2003) Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C. *J Neurosci* 23:9529–9540.
35. Strobl FJ, Gilmore CA, Levine JE (1989) Castration induces luteinizing hormone (LH) secretion in hypophysectomized pituitary-grafted rats receiving pulsatile LH-releasing hormone infusions. *Endocrinology* 124:1140–1144.
36. Glidewell-Kenney C, et al. (2008) Estrogen receptor alpha signaling pathways differentially regulate gonadotropin subunit gene expression and serum follicle-stimulating hormone in the female mouse. *Endocrinology* 149:4168–4176.
37. Bokoch GM (2003) Biology of the p21-activated kinases. *Annu Rev Biochem* 72:743–781.
38. Nikolic M, et al. (1998) The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* 395:194–198.
39. Thiel DA, et al. (2002) Cell cycle-regulated phosphorylation of p21-activated kinase 1. *Curr Biol* 12:1227–1232.
40. Sundberg-Smith LJ, Doherty JT, Mack CP, Taylor JM (2005) Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation. *J Biol Chem* 280:2055–2064.
41. Adam L, et al. (1998) Heregulin regulates cytoskeletal reorganization and cell migration through the p21-activated kinase-1 via phosphatidylinositol-3 kinase. *J Biol Chem* 273:28238–28246.
42. Kato S, et al. (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270:1491–1494.
43. Sun M, et al. (2001) Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. *Cancer Res* 61:5985–5991.
44. Abraham IM, Todman MG, Korach KS, Herbison AE (2004) Critical in vivo roles for classical estrogen receptors in rapid estrogen actions on intracellular signaling in mouse brain. *Endocrinology* 145:3055–3061.
45. Murakami G, et al. (2006) Comparison between basal and apical dendritic spines in estrogen-induced rapid spinogenesis of CA1 principal neurons in the adult hippocampus. *Biochem Biophys Res Commun* 351:553–558.
46. Srivastava DP, et al. (2008) Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity. *Proc Natl Acad Sci USA* 105:14650–14655.
47. Hayashi K, Ohshima T, Hashimoto M, Mikoshiba K (2007) Pak1 regulates dendritic branching and spine formation. *Dev Neurobiol* 67:655–669.
48. Meng Y, et al. (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* 35:121–133.
49. Boda B, et al. (2004) The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. *J Neurosci* 24:10816–10825.
50. Tolias KF, et al. (2005) The Rac1-GEF Tiam1 couples the NMDA receptor to the activity-dependent development of dendritic arbors and spines. *Neuron* 45:525–538.
51. Zhao L, Brinton RD (2007) Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. *Brain Res* 1172:48–59.
52. Hrabovszky E, et al. (2001) Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology* 142:3261–3264.
53. Christian CA, Moenter SM (2007) Estradiol induces diurnal shifts in GABA transmission to gonadotropin-releasing hormone neurons to provide a neural signal for ovulation. *J Neurosci* 27:1913–1921.
54. Milner TA, et al. (2001) Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J Comp Neurol* 429:355–371.
55. Hart SA, Snyder MA, Smejkalova T, Woolley CS (2007) Estrogen mobilizes a subset of estrogen receptor-alpha-immunoreactive vesicles in inhibitory presynaptic boutons in hippocampal CA1. *J Neurosci* 27:2102–2111.
56. Blaustein JD, Lehman MN, Turcotte JC, Greene G (1992) Estrogen receptors in dendrites and axon terminals in the guinea pig hypothalamus. *Endocrinology* 131:281–290.
57. Romano N, et al. (2008) Nonclassical estrogen modulation of presynaptic GABA terminals modulates calcium dynamics in gonadotropin-releasing hormone neurons. *Endocrinology* 149:5335–5344.
58. McDevitt MA, et al. (2007) Estrogen response element-independent estrogen receptor (ER)-alpha signaling does not rescue sexual behavior but restores normal testosterone secretion in male ERalpha knockout mice. *Endocrinology* 148:5288–5294.
59. Dupont S, et al. (2000) Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* 127:4277–4291.
60. Gu G, et al. (1996) Hormonal regulation of CREB phosphorylation in the anteroventral periventricular nucleus. *J Neurosci* 16:3035–3044.
61. Mellon PL, et al. (1990) Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 5:1–10.
62. Kelly MJ, Moss RL, Dudley CA (1976) Differential sensitivity of preoptic-septal neurons to microelectrophoresed estrogen during the estrous cycle. *Brain Res* 114:152–157.
63. Christian CA, Glidewell-Kenney C, Jameson JL, Moenter SM (2008) Classical estrogen receptor alpha signaling mediates negative and positive feedback on gonadotropin-releasing hormone neuron firing. *Endocrinology* 149:5328–5334.