BIOLOGY OF REPRODUCTION **69,** 1771–1778 (2003)
Published online before print 30 July 2003.
DOI 10.1095/biolreprod.103.019745

Minireview

Direct and Indirect Regulation of Gonadotropin-Releasing Hormone Neurons by Estradiol[1]

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ABSTRACT

Estrogen signaling to GnRH neurons is critical for coordinating the preovulatory surge release of LH with follicular maturation. Until recently it was thought that estrogen signaled GnRH neurons only indirectly through numerous afferent systems. This minireview presents new evidence indicating that GnRH neurons are directly regulated by estradiol (E_2), primarily through estrogen receptor (ER)-β, and indirectly through E_2-sensitive neurons in the anteroventral periventricular (AVPV) region. The data described suggest that E_2 generally represses GnRH gene expression but that this repression is transiently overcome by indirect E_2-dependent signals relayed by AVPV neurons. We also present evidence that the AVPV neurons responsible for relaying E_2 signals to GnRH neurons are multifunctional gamma aminobutyric acid-ergic/glutamatergic/neuropeptidergic neurons.

estradiol, glutamate, gonadotropin-releasing hormone, luteinizing hormone, neuroendocrinology

INTRODUCTION

Tightly regulated communication between the ovary and brain ensures that the neural signal for ovulation occurs when ovarian follicles are mature. A critical component of this communication system is the estrogen-dependent regulation of GnRH synthesis and hypersecretion that precedes a preovulatory surge in LH release. Understanding the neural mechanisms through which estradiol (E_2) regulates GnRH neurons has been the goal of numerous studies over the past three decades. Early evidence indicated that few GnRH neurons had estrogen receptors (ER), thus research efforts have been focused on identifying afferent neurons that communicate E_2 signals to GnRH neurons. As a result, researchers have found numerous brain regions and neurotransmitters that are regulated by E_2 and are important for GnRH and LH surge release [1–7]. These findings led to the hypothesis that E_2 regulates GnRH neurons indirectly through many afferent neurotransmitter and neuromodulatory systems. In this minireview, we present recent evidence indicating that it is now appropriate to refine this long-standing concept.

IN VITRO EVIDENCE THAT GnRH-EXPRESSING CELL LINES CONTAIN ER

A fundamental question regarding steroid effects on GnRH neurons is whether these effects are exerted directly or indirectly. In one of the first efforts to address this issue, Shivers et al. [8] found only rare immunoreactive GnRH (irGnRH) neurons that concentrated tritiated E_2 in ovariectomized (OVX) rats. Subsequent work using double-label immunocytochemistry (ICC) [9–16] verified that few, if any, GnRH neurons in females of a variety of species contain ER. The results of these early studies led to the logical and generally accepted conclusion that GnRH neurons are not regulated directly by E_2.

Until recently, the main challenge to this dogma came from investigators using immortalized GnRH neurons of the GT1 and GN lines [17, 18]. These cells overcome the problem that GnRH neurons in vivo are few in number and scattered throughout the preopticohypothalamus, making biochemical analyses difficult. Results of initial studies showed that GT1 cells have specific binding sites for E_2, albeit at significantly lower concentrations than in ER-containing neurons in the preoptic area [POA] [19]. Studies in GN cells derived from an olfactory bulb tumor, and possibly representative of less mature GnRH neurons, also contain high-affinity ER binding sites in cell nuclei [20]. These findings were not widely accepted, largely because the weight of evidence argued against the presence of ER in GnRH neurons in vivo. Moreover, it was suggested that tumor-derived GT1 cells may behave as embryonic rather than adult GnRH neurons [21]. Therefore, the conclusion that GnRH neurons are regulated only indirectly by E_2 generally persisted and directed much of the research in the field.

Recent findings resulting from the discovery of a second ER isoform (ERβ) [22] and from the introduction of more sensitive techniques indicate that it may have been premature to conclude that GnRH neurons are not regulated directly by E_2 (see also [23]). Using reverse transcription polymerase chain reaction (RT-PCR) and Southern blots, researchers found mRNA encoding ERα [24, 25] and ERβ [25] in GT1 cells. Others confirmed the presence of ERα mRNA in a subset of GT1-7 cells [26] and found both ERα and ERβ proteins in these cells [25, 27]. Using ICC, investigators found that although only 7%–11% of GT1-7 cells contain irERα [27], nearly all have irERβ [28]. At least one of these receptors appears to be functional because

[1]This work was supported by NIH grant HD27305 to S.L.P.
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Received: 28 May 2003.
First decision: 16 June 2003.
Accepted: 15 July 2003.

ISSN: 0006-3363. http://www.biolreprod.org

E_2 regulates the activity of a minimal promoter construct containing tandem estrogen response element (ERE) sequences when the promoter is transiently transfected into GT1-7 cells [24]. These findings provide support for the idea that initial binding studies in GT1 cells identified bona fide ER and suggest that ERβ predominates in these cells.

IN VITRO EVIDENCE THAT GnRH NEURONS CONTAIN FUNCTIONAL ER

Similar to results of studies in GT1 cells, accumulating in vivo evidence indicates that GnRH neurons also express ERβ preferentially. Earlier studies that failed to show ER in GnRH neurons generally used antibodies specific for ERα, suggesting that this isoform was not present. However, Coen and coworkers [27] subsequently used fixation procedures that maximize detectability of irERα and found a small percentage of ERα-positive GnRH neurons. To date no other researchers have replicated these findings nor have recent investigations using sensitive techniques detected ERα mRNA in GnRH neurons in mice [23] or rats [29]. (One group of investigators suggested that GnRH neurons contain ERα mRNA [30] but subsequently reported this finding to be in error [23].) GnRH neurons may express ERα, but at very low levels. An alternative explanation for discrepancies among results is that under some fixation conditions antibodies to ERα may recognize proteins with similar structural features. For example, the gene encoding ER-related receptor α (ERRα), one of a family of proteins structurally similar to ER [31], is detected in GnRH neurons [23, 30]. In particular, the DNA binding domain and hinge regions of ERRs are similar to those of ERα. Therefore, it is interesting that these domains [32] were targeted for production of the ERα antibodies used to detect ERα in GnRH neurons [27]. It remains to be determined whether different fixatives alter structures such that these antibodies could recognize ERRs.

Consistent with evidence that ERβ predominates in GT1 cells, a consensus is emerging that native GnRH neurons express ERβ preferentially. In rats, a sensitive dual-label in situ hybridization histochemical (ISHH) study revealed that as many as 73.8% of GnRH neurons contain ERβ mRNA [33]. Based on evidence that the proportion of GnRH neurons with ERβ immunoreactivity ranges from approximately 64% [28] to 88% [29] in OVX rats, it appears that this mRNA is translated into protein. There is also evidence that GnRH neurons in mice express the ERβ gene; however, this work relied on morphological identification of native GnRH neurons in situ and analyzed them with single-cell RT-PCR approach [30]. As a result of the practical constraints of this innovative approach, <50 GnRH neurons were examined from a total of 22 adult animals, and neurons were sampled only if they had a clear bipolar morphology. Therefore, as suggested by Herbison and Pape [23], results of this study probably underestimate the proportion of GnRH neurons with ERβ mRNA. One previous study using combined ISHH and ICC failed to colocalize ER-β mRNA with irGnRH [34]. However, we [35] and others [36, 37] have found that fixation procedures similar to those used in that study increase background and dramatically decrease ISHH signal. Therefore, although combined ISHH and ICC is a useful approach for determining colocalization when both targets are abundant, it may not be sufficiently sensitive to detect ERβ mRNA in GnRH neurons.

Very few studies have investigated the regulation of ERβ expression in GnRH neurons. Work from Coen and coworkers [28] indicates that administration of 1.0 μg E_2 3.5 h before death reduces the proportion of GnRH neurons with detectable irERβ by less than 10%, whereas administration of a 50-fold higher dose of E_2 16 h before death reduces this proportion by half. Although we found that the percentage of GnRH neurons with ERβ mRNA did not change as a result of E_2 administration 48 h before the animals were killed [33], we did not measure changes in cellular levels of ERβ in GnRH neurons. This is an important caveat because our ISHH method is quite sensitive; therefore, we have yet to detect a significant difference in the number of cells expressing a particular mRNA, even when we observe dramatic changes in cellular levels of that mRNA. Therefore, it remains to be determined whether E_2 reduces ERβ gene expression in individual GnRH neurons in rats. Skynner et al. [30] found no significant differences in the percentage of GnRH neurons containing ERβ mRNA among mice examined at diestrus, proestrus, and estrus. However, the small numbers of observations in that study may have been insufficient to provide the statistical power necessary to detect differences among groups.

Despite the convincing evidence that GnRH neurons primarily synthesize the ERβ isoform, the functional significance of this finding is unclear. The first question is whether ERβ binds E_2 in native GnRH neurons. In vivo autoradiographic studies using 17α-iodovinyl-11β-methoxyestradiol as an ER ligand [33] detected more E_2-concentrating GnRH neurons than did the original studies using a tritiated ligand [8]. However, despite the use of the higher specific activity ligand, ER binding was detected in <10% of GnRH neurons [33]. One interpretation of these data is that although ER-β mRNA appears to be translated into protein in a majority of GnRH neurons, the protein is functional in only a very small subpopulation of these neurons. Another possibility is that ER binding in GnRH neurons is underestimated by the technique of in vivo autoradiography.

A second question is whether activation of ERβ in GnRH neurons alters the functioning of these cells. A very recent report shows that E_2 rapidly increases the phosphorylation of cAMP response element-binding protein (CREB) in GnRH neurons in wild-type and ERα knockout but not ERβ knockout mice [38]. Using slice preparations and tetrodotoxin to electrically isolate neurons, the authors verified that CREB phosphorylation by E_2 was at least partially mediated by a direct action on GnRH neurons. This finding is consistent with previous evidence that E_2 directly and rapidly alters GnRH electrical excitability [39] and suggests that activation of ERβ in GnRH neurons may alter the responsiveness of these cells to other stimuli.

IN VITRO EVIDENCE THAT E_2 DIRECTLY REGULATES GnRH GENE TRANSCRIPTION

Several possible functions for ER in GnRH neurons have been reviewed recently [23]. In this minireview, we limit our focus to effects of E_2 on the regulation of GnRH gene expression, a key step in the regulation of GnRH biosynthesis. Although another gene encoding GnRH II has also been found in human brain [40], there is little information available on the regulation of this gene in neuronal cells [41]. Therefore, we concentrate here on studies investigating E_2 regulation of GnRH-1 (hereinafter referred to as GnRH).

Early work showed that E_2 stimulates human GnRH promoter activity through ER binding to a putative ERE [42].

Subsequently, it was found that transcription of the human gene can be initiated at two different start sites and that an upstream site is used primarily in reproductive tissues and a downstream site is used in hypothalamic neurons [43, 44]. In nonneural cell lines, researchers detected both stimulatory and inhibitory EREs in the upstream promoter [45]. In contrast, the rather low level of basal reporter activity of the neuron-specific downstream site is not significantly affected by E_2 [46]. Unlike human [43, 44] and monkey [47] GnRH genes, specific upstream transcriptional start sites do not appear to be used in the regulation of GnRH gene expression in nonneural tissue of rats [47]. Nevertheless, several regions of the rat GnRH promoter region confer negative regulatory responsiveness to E_2 [48]. Although ERα is required for this inhibitory effect, the receptor does not bind directly to the putative ERE sequence in the GnRH promoter [46] nor does ERα bind ERE in the upstream promoter of human GnRH [43]. Thus, the promoter regions of GnRH genes in several species appear to be negatively regulated by E_2 through ER, but some regulatory responses may involve indirect effects of liganded ERα at putative ERE sites.

The studies described above relied on introduction of ERα expression vectors and specific reporter constructs into cells to determine the effects of E_2 on GnRH promoter activity. No studies have determined whether ERβ mediates E_2 effects on the promoter. Since the discovery that GT1 cells contain endogenous ERα and ERβ, researchers have begun to investigate the role of each isomer in the regulation of GnRH gene expression. In agreement with results of transient transfection studies described above, E_2 represses mouse GnRH gene expression in GT1-7 cells [25]. This effect is blocked by ICI 182,780, an ER antagonist that inhibits both ERα and ERβ activities, and is mimicked by 2,2-bis-(*p*)-hydroxyphenyl-1,1,1-trichloroethan [25], a putative ERα agonist and ERβ antagonist, in several cell lines [49]. These data suggest that ERα may be responsible for repressive effects of E_2 on GnRH gene expression. This idea is supported by recent findings that E_2 suppression of GnRH mRNA levels in GT1-7 cells is not antagonized by *R,R*-diethyltetrahydrochrysene (*R,R*-THC) [50], an ERβ antagonist [51, 52] with partial [51, 52] or no [53] ERα agonist activity. However, the phytoestrogen coumestrol also suppresses GnRH gene expression and *R,R*-THC blocks that suppression, suggesting that ERβ mediates the effect. In summary, the preponderance of in vitro evidence indicates that E_2 negatively regulates neural relevant GnRH promoter activity and that both ERα and ERβ are active in GT1 cell lines.

E_2 REGULATION OF GnRH GENE EXPRESSION IN VIVO

It is difficult to demonstrate that E_2 directly represses GnRH transcription in vivo, but results of studies in mammals, including humans, are consistent with this idea. In one of the first studies to address this issue, E_2 suppressed GnRH mRNA in OVX rats [54], a finding that has been verified in adult OVX rats [55, 56] and in explant cultures prepared from Postnatal Day 4 rats [57]. More recent work demonstrated that E_2 from ovarian and extraovarian sources depresses GnRH gene expression in rats [58]. E_2 also decreases GnRH mRNA levels in neurons of sheep POA [59, 60]; however, this decrease is transient and occurs at the onset of and during LH surge release. Work on human brain identified region-specific GnRH neuronal subtypes and provided evidence that GnRH mRNA levels are suppressed by steroids only in type I neurons found in the medial basal hypothalamus [61]. E_2 also suppresses GnRH gene expression in nonhuman primates [62] and, similar to humans, does so specifically in type 1 GnRH neurons [63].

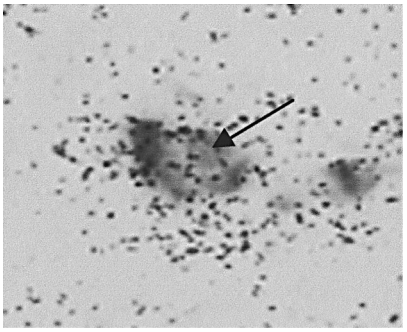

FIG. 1. Results of dual-label ISHH analysis using digoxigenin-labeled cRNA probes for GnRH mRNA (indicated by arrow) and ^{33}P-labeled cRNA probes for the mRNA encoding the R1 subunit of the $GABA_B$ receptor (black grains).

Despite this convincing evidence that E_2 decreases GnRH gene expression in vitro and in several in vivo models, not all investigators found such effects. As a result, the issue was debated in the literature for several years. Work showing that E_2 has time- and region-specific effects on GnRH gene expression in OVX rats largely reconciles these data as described previously [5, 56]. Specifically, E_2 treatment decreases GnRH mRNA levels during the periods prior to and following LH surge release in adult OVX rats [54]. In contrast, levels in E_2-treated OVX animals are similar to or above levels in OVX animals several hours before and during the surge [56, 64]. Consequently, investigators examining animals during the early morning hours on the day of the surge found E_2-dependent suppression of GnRH gene expression [54–56], whereas researchers who examined levels at mid-morning or late afternoon found no differences [56, 65–67]. When levels were examined during early to mid-afternoon, a stimulatory effect of E_2 was often seen [56, 65, 68, 69]. The stimulatory effect of E_2 occurs preferentially in rostral populations of GnRH neurons [56] and may be masked if tissue samples including the entire POA are assayed. These data emphasize the importance of considering time of day, animal model, and brain region when evaluating the effects of steroids on GnRH neurons.

INDIRECT REGULATION OF GnRH GENE EXPRESSION BY E_2—ROLE OF THE ANTEROVENTRAL PERIVENTRICULAR REGION

The findings described above indicate that E_2 may repress GnRH gene expression in vivo as it represses GnRH promoter activity in vitro. On the day of LH surge release, this repression is overcome by an afferent signal that is indirectly triggered by E_2 and necessary for the surge [70]. Many brain regions and neural systems have been implicated as regulators of GnRH neurons in different animal models [1–7, 71, 72]. Here, we focus primarily on recent work that examined the role of the anteroventral periventricular (AVPV) region, the medial region of the POA lying just caudal to the organum vasculosum of the lamina terminalis (OVLT) and rostral to the suprachiasmatic nucleus (SCN) [73, 74] (previously referred to as the medial pre-

optic nucleus [MPN] [70, 75–78]). In rats, the AVPV region is a sexually dimorphic nucleus [79] with abundant ERα [80, 81] and ERβ [81] mRNAs and a high density of E_2-concentrating cells [82]. This nucleus receives inputs from other brain regions that are critical for the daily LH surge, and therefore it is thought to be a key site for integrating and transducing environmental and hormonal signals to GnRH neurons [79].

The importance of the AVPV region in LH surge release in rats was first demonstrated by findings that electrolytic lesions of the region blocked the surge [76, 77], probably by destroying neurons required for the appropriate regulation of GnRH neurons [78]. Microimplants of antiestrogen placed into the AVPV region also blocked both the E_2-induced LH surge release and the phasic increase in GnRH gene expression seen several hours before the surge [56]. In addition, neurons of the AVPV region project to the rostral POA [83], where the most dramatic effects of E_2 on GnRH gene expression have been observed [56]. A group of neurons in a medial subdivision of the AVPV region, designated the periventricular POA and found only in females (G. Hoffman, personal communication), is coactivated with GnRH neurons at the time of the LH surge [84]. Thus, it seems likely that ER-containing neurons in the AVPV region of rats may be responsible for overcoming direct repressive effects of E_2 on GnRH gene expression.

The E_2-dependent LH surge release is seen in females but not in males, and the AVPV region has a number of sexually dimorphic E_2-sensitive neurotransmitter/neuropeptide systems known to regulate LH release [5, 79]. In addition, consistent with evidence that a daily circadian signal is required for the afternoon LH surge, neurons from the SCN project to the AVPV region, where they synapse on ER-containing neurons [85, 86]. Therefore, it is intriguing that neurons in the AVPV region show a rise in cAMP concentrations in OVX animals and on all days of the estrous cycle [87]. Together, these data suggest that a daily circadian signal is communicated to AVPV neurons, where it is reflected in an increase in cAMP; however, only in females and only in the presence of E_2 is this signal communicated to GnRH neurons as a trigger for hypersecretion and LH surge release.

The identity of the AVPV neurons that communicate E_2 signals to GnRH neurons has yet to be established, but several lines of evidence suggest that gamma aminobutyric acid (GABA) neurons are likely candidates. First, GABA is abundant in the POA and plays a key role in the regulation of GnRH release and gene expression [88–91]. We recently found that virtually all GABA neurons in the AVPV region have ERα and/or ERβ mRNA (unpublished results). Considering that less than half of the GABA neurons in the POA of rats concentrate E_2 [92], AVPV GABA neurons appear to be a distinctive E_2-sensitive subpopulation. This sensitivity may explain why GABA neurons in the AVPV region, but not in the rat POA, show E_2-induced changes in glutamic acid decarboxylase 67 mRNA [93] that parallel changes in GABA release linked to the LH surge [94–96]. Another effect of E_2 in the AVPV region is to induce progestin receptor (PR) gene receptor expression [97], and this induction is required for E_2-dependent LH surge release [98–100]. Therefore, it is significant that we recently showed that PR mRNA levels are increased by E_2 specifically in GABA neurons of the AVPV region (unpublished results). Finally, although it is not clear that AVPV GABA neurons project to GnRH neurons, GnRH neurons have $GABA_A$ receptors [35, 101–104] and contain mRNA encoding the $GABA_B$ receptor (see Fig. 1). These findings provide convincing evidence that GABA neurons in the AVPV region are important for transmitting E_2-dependent signals to GnRH neurons.

Like GABA, glutamate is abundant in the POA [105], and its release is regulated by E_2 [95, 106]. Activation of glutamate receptors stimulates both GnRH gene expression and LH release [107–109]. This activation is probably direct because GnRH neurons express *N*-methyl-D-aspartate R1 mRNA [110] and protein [108, 111, 112] and metabotropic glutamate receptors [113, 114]. Until recently, there was no reliable marker of glutamatergic neurons, making it difficult to localize and characterize these neurons. Identification of vesicular glutamate transporters (VGLUT-1, -2, and -3) and characterization of these molecules as Na^+/inorganic P cotransporters responsible for loading glutamate into synaptic vesicles [115–123] established them as specific markers of glutamatergic neurons. Researchers showed recently that VGLUT-2 is the predominant isoform in the preopticohypothalamus [122–127] and that VGLUT-2 mRNA-containing cells are found in the AVPV region [127–129].

Researchers have generally assumed that GABA and glutamate are released from separate neurons in the AVPV region, but until now it was not possible to critically test this hypothesis. We recently made the surprising discovery that all VGLUT-2 mRNA in the AVPV region occurs in GABA neurons and that numerous terminals in the rat POA contain both VGLUT-2 and vesicular GABA transporter proteins (unpublished results). Others have detected terminals containing VGLUT-2 protein on GnRH cell bodies [129, 130] and shown that some of these terminals also contain synaptophysin [129]. These findings suggest the intriguing possibility that dual-function GABA/glutamate neurons in the AVPV region contain ER and directly regulate GnRH neurons.

It is also possible that these AVPV GABAergic/glutamatergic neurons are also neuropeptidergic. This idea is based on our findings that most ERα and ERβ expression is found in GABA neurons and that a high percentage of some neuropeptidergic neurons in the AVPV also contain ER [5, 79]. One of these peptides, neurotensin (NT), is regulated by E_2, shows sexually dimorphic expression, and augments LH surge release [131–136]. Our preliminary studies show that virtually all NT mRNA is found in GABA neurons in the AVPV region and adjoining MPN. Studies in progress will determine whether other sexually dimorphic neuropeptides are also expressed in GABAergic/glutamatergic neurons of the AVPV region.

These findings suggest that GABAergic/glutamatergic/neuropeptidergic neurons of the AVPV region are targets of E_2 and are likely candidates for regulating phasic increases in GnRH synthesis prior to the induction of LH surge release. More research is necessary to determine how release of neurotransmitters from these multifunctional AVPV neurons is coordinated by E_2 and by afferent signals.

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