Estrogen and Thyroid Hormone Receptor Interactions: Physiological Flexibility by Molecular Specificity

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I. Introduction

Cross-talk between members of the nuclear receptor superfamily theoretically can multiply the possible modes of gene regulation, leading to a greater and more flexible array of transcriptional responses to environmental changes. In the central nervous system (CNS), such gene regulation conceivably can help to coordinate behavioral responses of the organism to climatic and social stimuli (217). Such cross-talk can also underlie metastatic processes. The activation of the estrogen-dependent growth responses by a nonestrogen such as the growth factor, insulin growth factor (IGF), may promote the growth of various cell types. Cross-talk between IGF and estrogens, for example, can lead to cell proliferation in breast carcinoma (41 and references therein) and hence is of considerable interest as a target for adjunct therapy.

Estrogen (ER) and thyroid hormone receptors (TR) are members of the nuclear receptor superfamily that bind the low-molecular-weight ligands, estrogens and thyroid hormones, respectively. They transduce these signals into gene regulation events. These receptors have a modular protein structure with high homology in the central DNA binding domain. They are ligand-activated transcription factors that influence transcription from target genes (43, 105). Nuclear receptors bind enhancer elements on DNA called hormone response elements to regulate transcription from genes (43, 105).

How do nuclear receptors regulate gene transcription? Gene activation events require the recruitment of
specific coactivators by ligand-bound nuclear receptors (33, 116, 122). This leads to the remodeling of chromatin, since many coactivators possess histone acetyltransferase (HAT) activity (27, 178). The remodeling and “opening” of chromatin leads to gene activation (27). Unlike the ER, the TR can regulate transcription even in the absence of ligand (213). Two well-characterized corepressors termed nuclear corepressor (NCoR) and SMRT exist for the TRs and the retinoic acid receptors (RAR)α (71, 93). In the absence of ligands, TR and RARs recruit corepressors, which have histone deacetylase activity and antagonize HAT coactivators. This, in turn, represses gene transcription (68).

Estrogens are critical in the control of reproduction in both male and female mammals (88). The deletion of the ERα isoform causes infertility in both male and female mice (104, 128). The nonreproductive functions of estrogens include maintenance of bone mass (64 and references therein) and cardioprotective effects (170). The importance of the expression of this gene is underscored by the ability of antisense oligonucleotides against PPE in the hypothalamus to reduce lordosis in the estrous cycle (12). When antisense oligonucleotides are infused into the VMH at proestrus, showing a correlation with the estrogen surge that occurs during this part of the estrous cycle (92); they also bind the consensus the estrogen response element (ERE) with significantly higher rejection behavior and lower lordosis, thus demonstrating the need for an intact OT-OTR system for reproductive success (107).

In a similar manner, estrogen treatment also upregulates preproenkephalin (PPE) mRNA in the rodent VMH (97, 168, 169). The importance of the expression of this gene is underscored by the ability of antisense oligonucleotides against PPE in the hypothalamus to reduce lordosis behavior (121).

II. HORMONAL INDUCTION OF GENES IN THE CENTRAL NERVOUS SYSTEM AND IN CELL LINES

A. Estrogen Induction of Genes in the Brain

Estrogens are necessary for the induction of the primary female-typical sexual behavior lordosis (91) in several species. Estrogens regulate the expression of several genes in the brain, some of which are responsible for the facilitation of lordosis (reviewed in Ref. 145). For example, the nonapeptide oxytocin (OT) and its receptor, the oxytocin receptor (OTR), are expressed in the uterus during pregnancy (96) and are thought to be important for gestation, parturition, and lactation. Mice that do not have OT (OT knock-out mice) cannot lactate and therefore cannot nurse pups (123). The expression of OT and OTR in the CNS is believed to be important for the facilitation of affiliative (24, 44) and sexual behaviors (7, 8, 22) that are, in turn, required for optimal reproduction.

Estrogen administration to ovariectomized female rats increases OTR mRNA in the ventromedial hypothalamus (VMH), a brain region critical for lordosis (153). Estrogens also upregulate OTR mRNA in the medial amygdala, hippocampus, and anterior pituitary but do not change the concentation of OTR mRNA in the caudate putamen or arcuate nucleus of the rat (153). Concomitantly, estrogen treatment increases oxytocin binding in the bed nucleus of the stria terminalis, lateral ventral septum, amygdala, and VMH (20, 59, 188). In situ hybridization studies show that OTR mRNA is highest in the rat VMH at proestrus, showing a correlation with the estrogen surge that occurs during this part of the estrous cycle (12). When antisense oligonucleotides are infused into the VMH of estrogen-primed female rats, the rats show significantly higher rejection behavior and lower lordosis, thus demonstrating the need for an intact OT-OTR system for reproductive success (107).

In a similar manner, estrogen treatment also upregulates preproenkephalin (PPE) mRNA in the rodent VMH (97, 168, 169). The importance of the expression of this gene is underscored by the ability of antisense oligonucleotides against PPE in the hypothalamus to reduce lordosis behavior (121).

B. Isoforms From Genes for ER and TR:
Distinct and Overlapping Functions

1. Two ER genes: α and β

Among vertebrates, the ER exists in two isoforms, α and β, which are products of different genes (186). The newly discovered ERβ isoform was cloned from rat prostate and the ovary. The isoforms exhibit considerable homology in the DNA binding domain and COOH-terminal AF2 domain but high divergence in the NH2-terminal transactivation AF-1 domain (65). Both isoforms can bind several ligands with similar affinities (92); they also bind the consensus the estrogen response element (ERE) with similar affinities (31). The dissociation of ERα and ERβ from such a consensus ERE is similarly affected in the presence and absence of ligand at elevated temperature (136). They also bind many EREs (72) derived from estrogen-regulated genes that have deviations from the consensus ERE sequence. Both receptors contain a functionally conserved AF-2 domain, which can be stimulated by binding the steroid receptor coactivator (SRC1) (31, 186). Despite limited homology in the NH2-terminal AF-1 do-

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main, both ERα and ERβ contain a mitogen-activated protein kinase (MAPK) phosphorylation site that results in enhanced transcription (186). Hall and McDonnell (66) show that despite similar binding affinities for several ligands, activation of transcription from simple target promoters containing EREs by ERβ is dependent on pure agonists. On the other hand, ERα can activate transcription when bound to agonists and partial agonists. If a chimeric ERβ receptor containing the A/B domain of the ERα is tested for transcriptional activation, antiestrogens such as tamoxifen, which showed no transcriptional activation with ERβ, could now show some degree of transactivation (110). Jones et al. (76) investigated the ability of ERα and ERβ to activate transcription from a number of different promoters that are estrogen responsive but lack classical EREs in human breast, bone, and uterine cell lines. These included a collagenase promoter containing an AP-1 element important in estrogen induction, a nonconsensusERE containing complement C3 promoter, and a transforming growth factor (TGF)-α promoter containing both ERE and Sp1 elements. All antiestrogens studied were agonistic on the collagenase reporter in the uterine cell lines when ERβ was transfected, but tamoxifen alone was agonistic when ERα was transfected (76). Also, the ability of ERβ to repress transactivation of NFκB in osteoblasts occurs only in the presence of 17β-estradiol, whereas ERα can repress NFκB transactivation in the absence or presence of ligand (152). This suggests important mechanistic differences, possibly arising from differences in amino acid sequence in the AP-1 domain (66, 195).

2. Differences between ERα and ERβ

At AP-1 sites, classical estrogens such as diethylstilbestrol and 17β-estradiol activated transcription when bound to ERα but were antiestrogens when bound to ERβ (137). On the human RARα-1 promoter, ERα activates transcription in response to estrogens through nonclassical ERE and not by direct DNA-receptor binding. However, in response to estrogens, ERβ does not activate this promoter; it activates it in response to tamoxifen, raloxifene, and ICI-164,384 (220). Therefore, the ER isoforms show considerable promoter site specificity. In vivo and in vitro, heterodimerization between ERα and ERβ has been shown (129, 143), and tissues that coexpress both isoforms are thought, therefore, to respond differently to various ER ligands compared with tissues that express predominantly one isoform (65, 66). Therefore, there is likely a considerable contribution of ERβ to the pharmacology of estrogens and antiestrogens.

3. TRs: two genes and four isoforms

The four TR isoforms are protooncogene products derived by differential splicing of two different genes: TRα1 and TRα2 are from the TRα gene, while TRβ1 and TRβ2 are from a separate TRβ gene. In chicken, a shorter TRα1 transcript lacking the NH2-terminal A/B domain is also present. Also, two TRβ1 transcripts, which possess very short A/B domains, are also present in the chicken. *Xenopus laevis* has several transcripts with homology to TRβ1 but none similar to TRβ2 (98). Again, although there is considerable homology in the central DNA binding domain among the isoforms, significant dissimilarity exists in the NH2-terminal A/B domain. Not all TR isoforms can bind ligand; the TRα2 isoform lacks the ability to bind ligand due to a loss of 40 amino acids in the COOH-terminal hormone-binding domain. The role of TRα2 in physiology is unclear; ex vivo studies in cell culture implicate it as a dominant negative inhibitor of TR action. However, the potency of dominant negative action is lower than the unliganded TR isoforms, possibly due to deficient interactions with corepressors (182).

4. Transcriptional properties of TR isoforms

The transcriptional properties of different TR isoforms have been poorly studied, but there do exist some differences. The differences in the TR isoforms could allow for differential interactions with other proteins, thereby regulating transcription. For example, unliganded TRβ2 can bind SRC-1, unlike TRα1 and TRβ1 (125). The TRβ2 is a more potent mediator of ligand-independent activation than TRα1 or TRβ1 of TRβ target genes such as the thyroid stimulating hormone (TSH) subunit gene and the TRH gene (70, 94, 171). This ability is independent of NCoR and may be due to differential binding of corepressors. The TRβ2 isoform also is unable to mediate ligand-independent repression on the growth hormone promoter, unlike the TRβ1 and TRα1 isoforms, due to lack of NCoR binding ability (70). Zhu et al. (215) have noted an increased ability of TRβ1 to transactivate from a F2 thyroid hormone response element (TRE) compared with TRα1 (215). Differential interaction with other proteins, including other nuclear receptors, may therefore play a role in thyroid hormone physiology.

Since the consensus DNA sequences bound by ER and TR share a common half site, it is possible that competition between the two receptors may lead to antagonism of the other’s effect. Indeed, this was first demonstrated for the vitellogenin ER by Glass et al. (60); the thyroid hormone receptor could decrease ERα-mediated transactivation.

Steroid hormone receptors have been shown to decrease ligand-dependent TR transactivation from a TRE (210). Estrogens were also shown to suppress the T3 effect on the α-glycoprotein hormone subunit promoter (207). In both pituitary-derived GH4 cells and JEG-3 choriocarcinoma cells, T3 mediates suppression of the α-glycoprotein hormone subunit promoter. 17β-Estradiol sup-
pressed this inhibition. In vitro synthesized ERα could bind to the TRE present in this promoter, thereby suggesting competition between these two nuclear receptor systems as a distinct possibility (207). However, in both these studies, pure TR and ER isoforms were not used. Hence, the investigation of possible differential interactions between distinct ER and TR isoforms is of interest.

C. Molecular Interactions Between the ER and TR Isoforms: Cell Culture Studies

Two different promoters were initially used to examine the interactions between the ER and the TR. The consensus ERE derived from the vitellogenin gene promoters has long been used as a model system to explain molecular mechanisms by which estrogen regulates genes. In CV-1 kidney fibroblast cells, the consensus ERE linked to a CAT reporter has been shown previously to be transcriptionally upregulated by estrogen-ligated ERα (218). Transiently transfected TRα1 was able to inhibit this ERα-mediated induction, but TRβ1 and TRβ2 had no effect (218). Contrasting to another promoter, when three tandem copies of the estrogen response EREs from the progesterone receptor promoter are used in the CV-1 cell line, no TR isoform could inhibit the ERα-mediated induction (173). This suggested that the interactions between TR and ER isoform were different on different promoters.

1. Modulation of ERα transcriptional activity by the ligand-binding TR isoforms

The interaction of ERα, the classical ER isoform, and the ligand-binding TR isoforms has been observed on a consensus vitellogenin ERE linked to a minimal thymidine kinase promoter in kidney fibroblast, CV-1, cells (190, 218). CV-1 cells were chosen since they have low endogenous ER and TR isoforms (215). On cotransfection of ERα and TRα1 expression vectors, the TRα1-ligated TRα1 isoform could interfere with the ERα induction of this simple promoter (Fig. 1A) (190, 218). However, the TRβ1 or TRβ2 isoforms did not have any effect on the ERα induction of this promoter (190, 218) (Fig. 1B and C). This demonstrates that on a simple consensus ERE, there is considerable difference in the modulation of transcriptional activity of ERα by the ligand-binding TR isoforms.

2. Interactions of ERβ with the ligand binding TR isoforms on the consensus ERE

ERβ could also induce this promoter in CV-1 cells, albeit at a lower level than the ERα isoform (Fig. 2). When the ligand binding TR isoforms were cotransfected with ERβ in CV-1 cells, the TR isoforms showed differential effects on ERβ-mediated induction from the consensus ERE. In contrast to the inhibitory effect of the TRα1 isoform on the consensus ERE, the TRα1 isoform stimulated ERβ-mediated transcription (Fig. 2A). The TRβ1 isoform also stimulated ERα-mediated transcription (Fig. 2B), while the TRβ2 isoform inhibited ERβ-mediated transcription (Fig. 2C). However, neither TRβ1 nor TRβ2 had any effect on ERα-mediated transcription. This shows that a single TR isoform can lead to differential transcriptional outcomes depending on the ER isoform present in the cell.

Are the interactions between the various TR and ER isoforms different on a physiologically relevant promoter? To address this question, the estrogen-responsive, behaviorally relevant PPE and OTR promoters cloned upstream of reporter genes were transfected into CV-1 cell lines and neuronal (SK-N-BE2C) cell lines. The PPE promoter has two EREs located within 450 bp of the transcription start site (77), whereas the OTR promoter has a distal ERE located ~4 kb from the transcription start site (11).

3. ERα versus ERβ: TRα1 modulation of induction of the PPE promoter in CV-1 cells

Similar to the inhibitory effect by the TRα1 on ERα-mediated transcription from the consensus ERE, the TRα1 inhibited ERα induction from the PPE promoter (Fig. 3A). In contrast, the TRα1 isoform stimulated ERβ-mediated transcription (Fig. 3B), demonstrating differences in the interaction of a given TR isoform with an ER isoform. In the CV-1 cell line, the TRβ isoforms had no effect on ERα or ERβ-mediated induction of the PPE promoter (192). Again, similar to the consensus ERE, the ability of the TRα1 isoform to inhibit or stimulate ERα-mediated transcription of the PPE depends on the ER isoform present in the cell (192).

4. TR isoform modulation of ERα-mediated induction of the OTR promoter

To test the interactions between the TR and ER isoforms in a cell line with neuronal properties and to compare these interactions with those occurring in a nonneuronal cell line, the OTR promoter containing the distal ERE was investigated in both CV-1 and SK-N-BE2C cell lines (189). Again, the TRα1 isoform was capable of inhibiting the ERα-mediated induction from the OTR promoter in both CV-1 and SK-N-BE2C cell lines. However, a complex pattern of interactions emerged when the TRβ isoforms were expressed in conjunction with the ERα isoform. Although the TRβ2 isoforms were inhibitory to ERα-mediated induction of this OTR promoter in either cell line, the TRβ1 effect on ERα induction depends on the cell line. In the CV-1 cell line, this isoform stimulated the ERα induction while inhibiting it in the neuronal cell line (189). A neuronal specific cofactor, NIX1, which can
bind liganded TRβ1 and downregulate transcription, could be responsible for this phenomenon (63). The expression of the cofactor appears to be confined to dentate gyrus, the amygdala, as well as thalamic and hypothalamic regions and may contribute to the differences in transcriptional activation observed with the TRβ1 isoform in these cell lines. Therefore, cell-specific effects are also important in the interactions between nuclear receptor isoforms. These data underscore the need to test physiological promoters in different cell lines.

Table 1 summarizes the transcriptional pattern obtained using different promoters and various combinations of ER and TR isoforms. The different outcomes show that the interaction between the ER and TR isoforms demonstrates considerable promoter specificity. It is easy to visualize that different levels of TR and ER isoforms in cells may, therefore, allow for flexible regulation of EREs depending on stimuli.

5. Mechanisms of TRα1-mediated inhibition

A) COMPETITIVE DNA BINDING. What are the possible mechanisms by which the TR isoforms interact with the ER isoforms? Since the consensus DNA sequences, the hormone response element (HRE), bound by both ER and TR, are very similar, TR can interfere with ER-mediated transcription by competing with the ER for binding to the ERE on DNA (60). A TRα1 mutant called the TR P-box mutant has a mutation in the DNA binding region; this disallows binding by this isoform to DNA (209). If DNA binding were important in the inhibitory interaction between TRα1 and the ERα isoform, then such a mutant
should not be able to inhibit the ERα induction. With the consensus ERE in CV-1 cells, inhibition by the TRα1 isoform was lost when the TR P-box mutant is used (190, 218), implicating DNA binding and hence competition for the ERE as important in this inhibition. In addition, the TRβ1 and TRα1 isoforms can bind to the consensus ERE, thus making inhibition by competitive DNA binding possible (218). However, differences arise when “physiological” promoters such as the OTR and PPE promoters are used. Despite lack of DNA binding ability, the TR P-box mutant could nonetheless inhibit the ERα-mediated induction of both PPE (Fig. 4A) and OTR promoters, suggesting that DNA competition may not be a universal mechanism in inhibition. Also, to check if the levels of ER isoforms expressed in the CV-1 cell lines significantly differ, binding of [3H]estradiol to extracts of cells transfected with ERα, ERβ, and TRα1, TRβ1, TRα2, and TRβ2 expression plasmids was done using ANOVA followed by Student-Newman-Keuls post hoc tests. A (TRα1): *P < 0.01 compared with the vehicle-treated group. #P < 0.001 compared with the vehicle-treated group. *P < 0.001 compared with the estrogen-treated group. B (TRβ1): *P < 0.001 compared with the vehicle-treated group. #P < 0.001 compared with the estrogen-treated group. C (TRβ2): *P < 0.01 compared with the vehicle-treated group. #P < 0.001 compared with the estrogen-treated group. [Modified from Vasudevan et al. (190).]
TABLE 1. ERα compared with ERβ in the specificities of interactions with different TR isoforms: promoter and cell type dependence

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Summary of the interaction between the ligand-binding thyroid hormone receptor (TR) isoforms and the estrogen receptor (ER)α and ERβ isoforms in CV-1 (kidney fibroblast) and SK-N-BE2C (neuroblastoma) cells. All effects of thyroid hormone-liganded TR isoforms are tested only if there is estrogen (E) induction via either ERα or ERβ of the promoter. The promoters used were as follows: ppe-cat, physiological rat preproenkephalin (PPE) gene promoter (−457 to +53) linked to the chloramphenicol acetyltransferase reporter gene (77); otr-luc, physiological rat oxytocin (OT) receptor gene promoter linked to the luciferase reporter gene (11); erectcat, a single copy of the consensus (ERE) derived from the vitellogenin gene linked to the chloramphenicol acetyltransferase reporter gene (218). ↑, T4 through TR isoform increases E-induced transcription; ↓, T4 through TR isoform decreases E-induced transcription; ~, T4 through TR isoform has no effect on E-induced transcription. I, data from Vasudevan et al. (192); II, data from Vasudevan et al. (189); III, data from Vasudevan et al. (190).

fected with either ERα or ERβ was done. There was no difference in the levels of ERα or ERβ; this also correlates well with the similar level of transcriptional activation promoted by either isoform in response to 17β-estradiol. The nuclear corepressor NCoR mediates basal repression by unliganded TR isoforms (30). However, because the inhibition detailed in Table 1 is ligand dependent, this must represent a novel, NCoR-independent mechanism.

B) RESCUE OF TRα INHIBITION BY THE EXPRESSION OF A COACTIVATOR. The p160 group of coactivators, of which

FIG 4. Mechanisms of TR-mediated interference of ER-driven transcription. A: a TRα1 mutant unable to bind DNA, the TR P-box mutant, can still cause inhibition of the ERα-mediated induction from the rat PPE promoter in CV-1 cells. B: SRC-1 overexpression rescues TRα1 inhibition of ERα-mediated induction from the rat OTR in CV-1 cells. A: CV-1 cells were cotransfected with PPE reporter plasmids and the expression plasmids for ERα and the TR P-box mutant (209) as detailed in the legend to Figure 1. [Modified from Vasudevan et al. (192).] B: SRC-1, a general steroid receptor coactivator, was overexpressed in CV-1 along with the TRα1 and ERα isoforms as detailed in the legend to Figure 1. The samples that were transfected with SRC-1 expression vector (4th, 5th, and 6th bar from left) were treated with 17β-estradiol (E) (10⁻⁷ M) or both 17β-estradiol and triiodothyronine (E + T). Corresponding hormone treatment was given to a set of samples that received the empty control SRC-1 expression plasmid (first 3 bars from left). [Modified from Vasudevan et al. (180).] Forty-eight hours after hormone treatment, cells were lysed, and each sample was assayed for both β-gal and CAT activity. The reporter gene activity was normalized to the β-gal activity for every sample. Results (fold over vehicle control) represent means ± SE. Statistical comparisons between treatment groups were done using ANOVA followed by Student-Newman-Keuls post hoc tests. A (n = 8 treatment group): *P < 0.01 compared with vehicle-treated group, #P < 0.01 compared with estrogen treatment. B (n = 5 treatment group): *P < 0.05 compared with the vehicle-treated group. #P < 0.05 compared with the vehicle-treated −SRC-1 group. ∇P < 0.001 compared with the estrogen-treated −SRC-1 group.
SRC-1 is a member, has been shown to bind both the ER ligand binding domain and the TR (103). Therefore, SRC-1 appeared to be an attractive candidate that could be tested for its ability to restore transcriptional activation by ERα on the rat OTR and PPE promoters in the presence of the inhibitory TRα1 isomorph. To explore the idea that coactivators are sequestered by the TR isoforms, we overexpressed a general steroid coactivator, the SRC-1, along with TRα1 and ERα. On both PPE and OTR (Fig. 4B), promoters as well as the minimal promoter containing the consensus ERE, SRC-1 overexpression could rescue TRα1 inhibition, suggesting that sequealing of common coactivators is an important mechanism of inhibition by the TR isoforms. Recently, Auger et al. (10) have shown that reduction in brain SRC-1 levels by antisense oligonucleotide injection reduces the ability of the ER to defeminize the brain during postnatal sexual differentiation in Sprague-Dawley rats. Inhibition of T3-dependent transcriptional activation by other nuclear receptors such as the glucocorticoid and estrogen receptor has been reported to be due to titration of essential coactivators (209). The ligand-bound TRα and TRβ proteins could interfere with progesterone receptor (PR)-mediated transactivation from a progesterone responsive reporter in the CV-1 cell line. Deletion of the DNA binding region did not affect the inhibitory properties; however, deletion of the six amino acids in the ligand binding domain needed for binding coactivators abolished the interference (214). Therefore, sequealing of proteins has important consequences for gene regulation. For example, the coactivator proteins RIP140 and TIF2 compete for a common binding site on the glucocorticoid receptor (GR), allowing TIF2 to relieve the inhibitory effect of RIP140 on GR action (180). Although it is not clear if ER-containing neurons also coexpress SRC-1, the widespread distribution of SRC-1 in the brain makes this likely (10). Also, many ER containing VMH neurons coexpress SRC-1, thus making such alleviation of inhibition possible in vivo (10). Therefore, the ability of the TRα1 isomorph to bind DNA as well to sequeal coactivators such as SRC-1 provides a rationale for the inhibition observed with this isoform.

6. PPE and OTR, “downstream genes,” providing routes from estrogens/ERs to behavior

The PPE gene plays a role in analgesic responses which can help the female to put up with somatosensory stimuli during mating which otherwise would be treated as noxious (17). Therefore, PPE gene induction represents a causal route which allows us to link a hormone’s genomic effects with a specific behavior, lordosis. In the rat VMH, PPE mRNA in the afternoon of proestrous was significantly higher than diestrous (56). In the female ewe, PPE mRNA increased in the VMH both during lactation and with estrogen treatment (21). On a single dose of 17-estradiol-3-benzoate given to female ovariectomized mice, PPE mRNA was upregulated in the VMH, medial amygdala (MeAmyg), and arcuate nucleus (ARC) at 24 and 48 h (154). However, PPE is not regulated in the caudate putamen or in the cortical amygdala by estrogens (154). A single dose of estradiol benzoate to ovariectomized female rats resulted in a biphasic increase in PPE mRNA producing cells both in the ARC and VMH with a peak at 48 h (151). This biphasic response of PPE consists of a primary peak at 1 h and a second peak between 24 and 48 h postinjection. The rapid first peak was stress induced and could be blocked by adrenalectomy or constant low levels of corticosterone. A peak of plasma corticosterone also coincided with this peak. In the medial amygdala, the antiestrogen tamoxifen blocked the second peak of PPE mRNA expression. These data indicate that both steroids and noxious mild somatosensory stimuli interact to give increases in PPE expression (177). This is consistent with a role for PPE in female reproductive behavior. Acute, mild stress in the form of male approach behavior may activate limbic and hypothalamic circuits known to be important for the full display of reproductive behavior (177).

The OTR gene is also critical for reproductive success. Infusion of oxytocin into the VMH increases sexual behavior and maternal behavior. How does it do so? It is thought that most laboratory tests for sexual behavior and maternal behavior involve unfamiliar, novel and potentially threatening surroundings for rodents. Exposure to such apparatuses or to an unfamiliar animal could trigger inhibitory stress responses. In Swiss Webster mice pre-treated with estrogens, peripherally administered OT increased entries into open arms in the elevated plus maze (108). In mice given intracerebroventricular injections of OT, entries into open arms were increased compared with mice given arginine vasopressin (108). Therefore, estrogen upregulation of OT could be a vital component in anxiolytic actions, decreasing stress and facilitating social interactions (109).

Therefore, both PPE and OTR are downstream genes with proven behavioral roles and are upregulated by a primary reproductive effector, estrogen. These downstream gene products are expressed in behaviorally relevant neurons that possess ERs. Indeed, they can be visualized as systems that link a small hormonal signal with an identifiable behavior.

III. PHYSIOLOGICAL DATA AND THEIR IMPLICATIONS

A. Lordosis Behavior

Neuronal and genetic mechanisms for lordosis behavior have been worked out in such detail (reviewed in
Ref. 145) that the behavior virtually stands as an expression system for ER transcriptional activation. In turn, interactions between liganded TRs and ER function can be charted thereby.

The neuroanatomy of estrogens liganded to ERα or ERβ has been charted in considerable detail (144, 146, 175). VMH neurons binding estrogens sit on top the lordosis behavior neuronal circuit (148). Hormone implant experiments establish that it is the estrogenic sensitivity of these neurons that accounts for hormonal facilitation of lordosis. New RNA and protein synthesis are required for the behavioral facilitation. Specific, hypothalaminically expressed genes have the following properties: they are turned on by estrogens, and their products facilitate lordosis (150). Therefore, in the manner of a logical syllogism, their induction comprises part of the mechanisms by which estrogenic hormones turn on the behavior.

These particular genes in no way exhaust the possibilities of hormone-stimulated messages, which are behaviorally relevant. New DNA-microarray experiments have revealed hitherto unimagined genes that are hormone sensitive (113) which indicate linked glial/neuronal and possible leptomeningeal/neuronal cooperation in neuroendocrine function.

Normal gene expression for ERα is required for normal lordosis behavior (128, 132). In dramatic contrast, active gene expression for ERβ actually suppresses lordosis; ERβ knock-out female mice show the behavior during a larger portion of their estrous cycles than wild-type female littermate controls (126).

Therefore, estrogen-dependent lordosis behavior is well suited to look for thyroid/estrogenic interactions. Thyroid hormone administration, in fact, reduces lordosis behavior both in female rats (38) and in female mice (114). The molecular mechanisms involved are not necessarily simple and require further investigation; that is, against all predictions, the contribution of the TRα gene to the regulation of female reproductive behavior is diametrically opposite to that of the TRβ gene (36).

B. Differences in Isoforms From Nuclear Receptor Genes: Use of Knock-out Models

1. **TR gene knock-out mice**

Differences in the physiological roles of the different isoforms have been explored using knock-out mice models. Despite similar ligand binding characteristics, the differential distribution of the TR isoforms as well as data obtained from knockout mice suggest a unique role for the various TR isoforms. The TRα1 and the TRβ isoforms have both common and specific roles in vivo. Predicated on the high concentration of TRβ2 in the anterior pituitary (69), the TRβ2 isoform plays a major role in the negative-feedback regulation of TSH by thyroid hormone. Lack of TRβ2, therefore, causes hyperthyroidism in mice (2). On the other hand, lack of the TRα1 isoform results in a mild hypothyroidism in mice (75). The distribution of the TRβ2 isoform in the developing retina of the mouse is also indicative of an important developmental role for this isoform. In rodents, cones contain different opsins sensitive to different wavelengths. The TRβ2 isoform is responsible for a commitment to M-cone (M, middle or green wavelengths) identity. Deletion of this isoform results in a lack of M cones and a concomitant increase in S-opsin (S, short wavelength) immunoreactive cones (119). The inability for one isoform to substitute for another is also exemplified by T3-controlled type 1 deiodinase expression (6). Although both TRα1 and TRβ are present in both liver and kidney, expression of the deiodinase was highly dependent on TRβ in the liver and completely dependent on TRβ in the kidney (6). Another example, the TRβ1 isoform, is implicated in hearing loss, whereas the TRα knock-out mice remain unaffected (2).

The TRα and TRβ isoforms also play distinct roles in the facilitation of lordosis in female mice. Deletion of the TRα1 isoform resulted in decreased lordosis behavior in female mice, whereas loss of the TRβ isoforms resulted in increased lordosis (36). OT immunoreactivity in the paraventricular nucleus (PVN) was elevated in TRβ knock-out female mice treated with estradiol compared with wild-type mice given the same treatment, implicating OT increase in the PVN as important in increased lordosis (36). Both behavioral and molecular data on the cross-talk between the ERα and TR isoforms on the PPE and OTR promoters point to opposing effects of the TRα and TRβ isoforms.

2. **ERα knock-out mice versus ERβ knock-out mice:**

Reproductive and affiliative behaviors are differentially affected according to which of the ERs is deleted in mice. ERα knock-out (ERKO) female mice show virtually no lordosis (128, 132), whereas ERβ knock-out mice (BERKO) not only show normal lordosis behavior but express this behavior during a larger portion of the estrous cycle than wild-type littermate controls (126). ERKO females have striking deficits in maternal behavior. Dramatically, aggressive behaviors in young adult BERKO males are heightened (124), whereas they are markedly suppressed in ERKO males (131). Note that there appears to be genotype/age interactions in aggressive behavior by BERKO mice, in that the relatively inexperienced young BERKO mice are more aggressive in resident-intruder tests. Finally, the increase in locomotor activity in both genetic females and genetic males, following estrogen administration, depends absolutely on the potency of the ERα gene but not the ERβ gene (127).
3. Cross-talk between the ERα and ERβ isoforms

It has been suggested that the inability of ERKO mice to induce OTR in response to estradiol benzoate treatment (212) is a factor in their failure to promote social interactions. Antagonistic effects of two ER isoforms expressed in the same cell have also been reported. On a consensus ERE promoter in HeLa cells, Hall and McDonnell (66) have noted that coexpression of ERβ along with ERα reduces the transactivation seen with ERα. We were interested in investigating if there is a similar effect on a physiological promoter in both the cell lines. However, although ERβ did not affect the transactivation observed with the ERα isoform on the OTR promoter in response to 17β-estradiol in the CV-1 cell line (Fig. 5), it decreased the transcriptional activation observed on this promoter in the SK-N-BE2C cell line (Fig. 5). Again, cell-specific effects such as the expression of tissue-specific cofactors may play a role in this phenomenon. The inability of estrogen-ligated ERβ to activate transcription from a physiological OTR promoter may also help explain a result obtained from ERKO mice. The OTR gene promoter is upregulated by estrogens in several brain regions. Unlike in the rat, estrogens do not induce OTR expression in the mouse hippocampus, which does not have ERα but has ERβ, lacks estrogen induction of OTR in many brain regions, such as the cortex, as monitored by OT binding (212). However, the ERKO mouse, which does not have ERα but has ERβ, reduces estrogen induction of OTR in many brain regions, such as the cortex, as monitored by OT binding (212). This may be explained by the differential distribution of ER isoforms as well as the inability of the ERβ to induce the OTR. In the mouse hippocampus, ERα expression is sparse, although ERβ expression is intense (174).

4. Mechanisms for ERα cross-talk with ERβ

Do relative amounts of ERα and ERβ contribute to this result? As monitored by [3H]estradiol binding, ERα and ERβ appeared to be expressed equivalently to each.

**Fig. 5.** Effect of the coexpression of ERα and ERβ on oxytocin receptor (OTR) gene transcription in CV-1 and SK-N-BE2C cells. Both ERα and ERβ expression plasmids were cotransfected into CV-1 and SK-N-BE2C cells at equal concentrations along with the OTR-luciferase construct. A corresponding set of samples received the ERα or the ERβ expression construct alone along with the OTR-luciferase reporter construct. After treatment of each set with 10⁻⁷ M 17β-estradiol or ethanol for 48 h (n = 6/treatment group), cells are lysed and assayed for β-galactosidase and luciferase activity. The results are analyzed using ANOVA followed by Student-Newman-Keuls post hoc test to compare between treatment groups. CV-1 cells: *P < 0.01 compared with vehicle treatment of the ERα group. #P < 0.01 compared with vehicle treatment of the ERβ group. SK-N-BE2C cells: ΔP < 0.01 when compared with vehicle treatment of the same group. \( \nabla P < 0.01 \) when compared with estrogen treatment of the ERα group. [Modified from Vasudevan et al. (189).]
other in both CV-1 cells (190) and SK-N-BE2C (189) cell lines. Although not statistically significant, ERβ binding to [3H]estradiol appeared to be slightly lower and may have contributed to the noninducibility of the rat OTR promoter by ERβ. In contrast, in CV-1 kidney fibroblast cells (Fig. 3B), ERβ was capable of mediating the 17β-estradiol induction of the natural PPE gene promoter fragment. Also, ERβ is capable of promoting neurite elongation in SK-N-BE2C human neuroblastoma cells in response to added 17β-estradiol, thus proving that both cell lines are responsive to estrogens via both ERα and ERβ (140). Therefore, the nonresponsiveness of the OTR gene promoter on transfection of the ERβ isoform in both the cell lines tested is promoter and ER isoform specific. The lower transcriptional efficiency of ERβ has been noted using the consensus CRE construct, CRE-tk-luc, in COS-1 and HepG2 cells. With the use of Gal4 DNA binding fusion proteins fused to the AF-1 domains of either ERα or ERβ, it was determined that the AF-1 activity of ERβ was negligible compared with ERα (32). On promoters and in cell lines which require both AF-1 and AF-2 activity, ERβ appears to be a poorer transcriptional activator than ERα (32).

C. Patterns of Behavior

The above data suggest that isoforms deriving from closely related genes for nuclear receptors play unique roles that are clearly not equivalent in whole animal studies. The ERKO mice do not exhibit the same behavioral phenotype as the BERKO mice in several behavior tests, especially those designed to elicit socially motivated responses. Similarly, the αTRKO females treated with estrogens do not display the same levels of sexual receptivity as the βTRKO females (36). Hence, the αKO are not equivalent to the βKO mice in either ER or TR knock-out models. Table 2 highlights theoretical scenarios for functional relations between ERKO and BERKO gene products. On the right side of Table 2 are summarized some of the data gathered from genetic females and genetic male mice on a number of behavioral and histochemical assays. The variety of relations between ERα and ERβ are reminiscent of the differences seen between ERα and ERβ in their molecular interactions, reviewed above, with a given TR isoform in the cell culture model systems. For both the molecular and the behavioral studies, the specificities of interactions among ER gene products and TR gene product isoforms additionally provide us with internal controls.

In the early 1940s, Beadle and Tatum (13), in their famous genetic and biochemical studies on the fungus Neurospora crassa, found that the loss of an enzyme led to a specific biochemical defect. The underlying cause of lack of enzyme activity was discovered to be a loss of a gene, thus leading to the well-known “one gene-one enzyme” hypothesis (13, 158, 183). The data presented above show us the combinatorial possibilities of nuclear receptor gene product interactions. These force us to redirect our thinking into a new, more organismal framework whereby patterns of gene expressions and interactions in the central nervous system underlie patterns of behavior.

D. Physiological Implications of Thyroid Hormone Modulation of Estrogen Action

1. Neuroendocrine data

The involvement of thyroid hormone in the neuroendocrine control of reproduction has been documented especially well in starlings and sheep. In these species inhabiting temperate latitudes, seasonal reproduction ensures the birth of young in conditions that maximize survival. Therefore, the termination of the breeding season in sheep and the initiation of anestrus occur during the long-day period (spring and summer). An exogenous rhythm, which is entrained by such changes in day length,
controls the timing of seasonal reproduction (78, 166, 167). In starlings, thyroidectomy of starlings prevented the start of photofractoriness and allowed for continuation of the breeding season (201). Thyroxine rise during long days in European starlings is permissive for the neuroendocrine shift to the nonbreeding season, which is primarily dictated by day length (15). In the thyroidectomized male American tree sparrow, administration of T₄ given intracerebroventricularly could restore all components of seasonality. Therefore, T₄ was capable of acting centrally to program already photostimulated male American male sparrows (203).

However, in mammals, both retinal photoreceptors and the pineal gland are required for reproductive responses to photoperiod (120). Long days induced a drop in lutenizing hormone (LH) in thyroid-intact ewes, though thyroidectomy blocked this effect (112). However, thyroidectomy had no effect on the circadian pattern of circulating melatonin or prolactin and the change that occurs with the photoperiod (34). Thyroid hormones may play a permissive role to photoperiod; they need to be present at the end of the breeding season for anestrous to commence. This critical period of neuroendocrine responses to thyroid hormone is late in the breeding season. The minimal effective duration of exposure to circulating levels of thyroid hormone was 60–90 days beginning in late December such that anestrous could develop in spring (184). Do thyroid hormones also play a role in the maintenance of anestrous once it develops? In ewes thyroidectomized (THX) just as they entered anestrous, the timing of the LH rise late in anestrous, indicative of the next breeding season, was the same as non-THX controls. Therefore, although thyroid hormones play a role in initiating anestrus, they do not have any role in the maintenance of the anestrous and the timing of the subsequent breeding season (185) in sheep. However, in the male American tree sparrow, T₃, T₂, and reverse T₃ given intracerebroventricularly could allow for thyroid hormone-dependent photoperiodic testicular growth. The order of potency was T₄ > T₃ > reverse T₃ (203). These data demonstrate that thyroid hormone may play slightly different roles in the maintenance of nonreproductive conditions in mammals and birds.

2. Mechanisms of thyroid hormone-induced anestrous in sheep

The mechanisms of thyroid hormone-induced anestrous in sheep have centered mainly on the gonadal hypothalamic-pituitary axis. There is no effect of THX in female ewes on the ability of a rise in estrogens to elicit the LH surge or in the ability of progesterone to suppress LH secretion (199). However, there is intensified estrogen-mediated negative feedback in control ewes compared with THX ewes (199). High-frequency pulses of both GnRH and LH are observed in THX ewes that did not make the transition to anestrous (198). Central infusion of T₄ to THX and ovariectomized ewes given Silastic implants of estradiol benzoate restored anestrous to these ewes. This demonstrated that thyroxine acts centrally in the brain in ewes to promote changes in GnRH and LH that signal anestrous (194). Also, TRα has been colocalized in 46% of GnRH neurons in sheep (74). In rats, hyperthyroid rats had 25% less LH on proestrous, showing depression of the LH surges. Also, the amount of estrogens required to initiate a LH surge was greater in hyperthyroid animals (53). Hyperthyroid animals could, however, respond to GnRH, suggesting that the pituitary was not a site of action for thyroid hormone. The hypothalamus is more plausible, since stimulation of the arcuate nuclei-median eminence area (ARC-ME) resulted in hyperthyroid rats secreting less LH than control rats (53). In rats devoid of the thyroid gland, the synthesis and metabolism of LH was not affected, but the secretion of LH was higher (52). Propylthiouracil (PTU), a goitrogen, given transiently to neonatal rats dramatically increases sperm production and testis size in the adult rat. However, it leads to a significant drop in GnRH-stimulated LH production. Gonadal feedback is enhanced in PTU-treated males resulting in chronically reduced circulating levels of LH and follicle stimulating hormone (86).

3. Thyroid hormone effects on reproductive behavior in rodents

Thyroid hormone elevation has also been shown to have an adverse effect of reproduction in rodents (38, 114). Concomitant administration of T₄ to ovariectomized rats (38) and mice (114) treated with estrogens has been shown to reduce lordosis, compared with ovariectomized rodents that received estrogens alone. TR knock-out female ovariectomized and estrogen-treated mice deleted for TRβ isoforms showed higher lordosis than the βTRWT, suggesting that TRβ may exert an inhibitory influence on ER-controlled reproduction (36).

Because reproductive behavior is controlled by estrogens via the ER, it is possible that a reduction in ER target genes such as OT, OTR, and PPE could be responsible for TR-mediated inhibition (149, 217). Indeed, injections of thyroid hormone to estrogen-treated female rodents lead to a decrease of OT mRNA in the PVN (39). Ex vivo studies indicate that thyroid hormone upregulates the human OT promoter fivefold through the composite element containing an imperfect ERE located at −148/−172 bp upstream of the transcriptional start site (4). TRα1 protein can bind to this composite element and interfere with the transcriptional induction by estrogens (4). Thyroid hormone elevation also reduces the expression of another estrogen-induced gene in the VMH, the PPE gene, which facilitates lordosis behavior (37).
4. Modulation of estrogen action by thyroid hormone in other species

In other species, thyroid hormone has also been shown to modulate estrogen action. In the fish tilapia, *Oreochromis neoloticus*, three distinct populations of GnRH exist: the terminal nerve neurons in the forebrain, the preoptic neurons, and the midbrain neurons. In castrated male tilapia, terminal nerve neurons express GnRH, which is lowered by exogenous T3 treatment (138). Interestingly, in the tilapia, the ontogeny of terminal nerve neuron GnRH is concomitant with a decrease in T4 levels. Sexually mature tilapia have low levels of thyroid hormones but high levels of terminal nerve GnRH (197). In oviparous species such as the clawed toad, *Xenopus laevis*, metamorphosis is dependent on thyroid hormone while vitellogenesis is strongly dependent on estrogens (155). T3 could enhance ERα production and autoinduction and thereby enhance the estrogenic activation of vitellogenin genes (156).

5. Estrogen and thyroid hormone influence each other’s nonreproductive functions

One of the most prominent effects of estrogens is to promote mitosis in the uterine luminal epithelium, stroma, and myometrium. The ability of hypothyroid rats to increase the mitotic index in these uterine regions is reduced compared with the euthyroid controls (87). This diminished uterine response is not due to a shift in the dose-response curve of the estrogen; rather, it is possible that thyroid hormones have a direct effect on the uterus such that it lowers its responsiveness to estrogens (57). In pregnancy, despite lower levels of free T4 and free T3, there is no rise in serum TSH (50). A similar absence of TSH rise is seen in postmenopausal women receiving estrogen replacement therapy (1). Although estrogen treatment did not augment the serum concentrations of TSH in euthyroid or untreated hypothyroid rats, it in-treatment did not augment the serum concentrations of estrogen replacement therapy (1). Although estrogen production and autoinduction and thereby enhance the estrogenic activation of vitellogenin genes (156).

IV. ROLE OF PROMOTER AND CELL SPECIFICITY IN DISTINCT TRANSCRIPTIONAL RESULTS

How do differences in sequences bound by the ER and TR isoforms explain their transcriptional differences in the context of cell lines and promoters? Different hormone response elements are allosteric mediators of receptor conformation. Therefore, hormone response elements not only position receptors close to basal transcription complexes but also serve to direct the mode of regulation of the target gene (100). Studies with the consensus vitellogenin A2 ERE, or the imperfect pS2, vitellogenin B1 or oxytocin (OT) ERE show that the A2 was the most potent activator of transcription followed by the OT ERE (205). DNase footprinting revealed that MCF-7 proteins protected the OT and A2 EREs to a greater extent than the pS2 or B1 EREs. Although the receptor-interacting domains of the glucocorticoid receptor interacting protein 1 (GRIP) and SRC-1 bound effectively to ERα, TIF2 was bound less by B1-bound ERα than A2-bound ERα, suggesting that allosteric modulation of ERα conformation by different EREs influences coactivator recruitment (205).

Different isoform conformations within the cell could also have an effect in the recruitment of coactivators. The TRβ2 isoform, for example, can bind p160 class
of coactivators in the absence of the hormone and, therefore, mediate ligand-independent activation of target genes. This is mediated by contacts in the unique NH$_2$ terminus of TR$\beta$2 and an internal interaction domain of SRC-1 and GRIP-1 coactivators. These contacts are different from the LXXLL motifs that mediate hormone-dependent coactivator contacts and hence hormone-dependent transcriptional activation (206). The NH$_2$-terminal region in the human PR (hPR) also modulates differential coactivator and repressor binding. The hPR exists as two different isoforms: hPRA, which is a strong ligand-dependent repressor of transcription, and the hPRB, which is a transcriptional activator in most cell and promoter contexts. An inhibitory domain (ID) present in the hPRA and B is active only in the hPRA isoform, facilitating the interaction between this isoform and the transrepressor SMRT. This and the inability of the hPRA isoform to engage coactivators could provide an explanation of the differential activities of the isoforms (58). Also, the specificity of NCoR interactions with TR$\beta$1 could be because of the ability of this TR isoform to interact with a novel NCoR interacting domain, called N3 which is specific for TR and interacts very poorly with other nuclear receptors, such as the RAR$\alpha$ (29). Possible differences in isoform conformation may allow for tissue-specific expression. For example, although a ERE oligonucleotide derived from the rat 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase promoter confers estrogen responsiveness to a heterologous promoter in several cell lines, the “natural” HMG CoA promoter containing the ERE shows estrogen induction in MCF-7 breast carcinoma cells but not in hepatic cell lines (40).

Within the mammalian CNS, the physiological results of hormones liganding to specific nuclear receptor gene products, as well as the consequences of their interactions, may often depend on the details of neuronal or glial localization; that is, the neuroanatomical pattern of expression of ER$\alpha$ is much different from that for ER$\beta$ (175), and the TR$\alpha$ and TR$\beta$ gene products likewise are distributed differently both inside and outside the CNS (19, 90, 101, 111). Most importantly, differential distributions that depended on promoter sequences active during certain developmental stages may well underlie functional differences between nuclear receptor isoforms. In turn, what about the requirement that for direct TR/ER interactions they must be expressed in the same neuron? Kiar and colleagues (83–85), using a sensitive double in situ hybridization methodology, showed that a high percentage of forebrain neurons expressing ER also expressed TRs.

Why are isoforms of the nuclear receptors such as ER and TR maintained in organisms? The differential codistribution of the isoforms is a clue that they have distinct nonoverlapping functions. The phenotypes of the isoform-specific knock-outs also appear to indicate that a subset of functions is indeed unique to a certain isoform and cannot be substituted by the other isoforms. The experiments suggest that a variety of transcriptional outcomes are possible depending on the combination of isoforms present in the cell. These represent different flexible outputs, presumably to ensure homeostasis, in response to the same external stimulus, i.e., presence of ligand. We suggest that the isoforms have evolved to allow for different cell responses to the same signal, possibly by differential cross-talk between isoforms, to achieve neuroendocrine integration.

V. QUESTIONS UNANSWERED

A. Gene Duplication and Splice Variants

Gene duplication events and splice variations comprise likely origins for a number of hormone receptor isoforms. Those isoforms, which are gene duplication products, provide fascinating opportunities to compare their physiological roles. Some current examples from molecular endocrinology and neuronal biophysics suggest that diametrically opposite functions represent an interesting possibility.

Gustafsson and colleagues’ (122) have made the case that in peripheral organs ER$\beta$ can oppose the actions of ER$\alpha$. Neurobiological results both with behavioral end points (126, 131–133) and during transfection studies (189, 190, 192) support this point of view. With respect to the CNS, both transcriptional end points in cell lines (189, 192) and behavioral results (36) offer the possibility of markedly different TR$\alpha$ and TR$\beta$ actions.

Among nuclear hormone receptors, PRs show the wide diversity of actions of PR splice variants; that is, under circumstances where PR$\beta$ can act as a transcriptional activator, the smaller splice variant PR$\alpha$ can have the opposite transcriptional effect (58, 193, 200). The same can be illustrated for neuuropeptides. OT and vasopressin are extremely similar nonapeptides, which almost certainly represent gene duplication products. OT is well known to promote affiliative behaviors (23, 142). In contrast, vasopressin promotes aggressive and territory-defensive behaviors (5, 46, 47). The electrophysiological actions of neurotransmitter receptors make the same case. For the transmitter norepinephrine ($\alpha$1 vs. $\alpha$2 receptors), dopamine (D1 vs. D2 receptors), serotonin (5-HT$_1$ vs. 5-HT$_2$ receptors) and acetylcholine (M1 vs M2) receptors, markedly different and even opposing actions of likely gene duplication products are the rule rather than the exception. All of these examples constitute new opportunities for the fine-grained analysis of gene domains governing specific neuroendocrine functions.
B. Rapid Versus Slow Effects of Estrogen: Two Separate Opportunities For Thyroid Hormone Modulation?

Although the classical mode of estrogen action is via the nuclear ER and leads to the modulation of transcription rate of estrogen-target genes, nonnuclear ER-mediated effects exist and are certainly prominent in neurophysiological literature (14, 80–82). These nonnuclear ER effects are typically rapid and are mediated by a putative membrane receptor (mER) and involve several signal transduction pathways (79 and references therein). Membrane-limited estradiol conjugates are used to investigate the membrane effects of estrogens. The MAPK has been implicated as a downstream effector molecule in estrogen’s rapid action at the membrane in a neuroblastoma cell line, the SK-N-SH cell line (196). In a cell line with endogenous ERα (the breast carcinoma MCF-7 line), non-genomic mechanisms have been shown to activate MAPK (73). Protein kinase C activation in growth plate chondrocytes is seen on administration of both 17β-estradiol and E-BSA (181). The rapid rise in intracellular free Ca²⁺ has been thought to be mediated by mERs (9, 115, 179). The identity of the putative mER has been explored in a transient transfection assay gave rise to a single transcript and functional protein expression in both nuclear and membrane fractions (159). Microscopy on fetal rat hippocampal neurons reacted with affinity-purified anti-ERα revealed abundant staining at the neurites. Also, incubation with antisense oligonucleotides directed against ERα reduced the immunostaining (28).

Although work on the nuclear ER and membrane ER has been ongoing for several years, they have usually been presented as alternate modes of estrogen action. However, this laboratory has recently shown that these two modes of estrogen action can in fact synergize. Therefore, it is possible that an early, preliminary administration of estrogen leads to rapid activation of signal transduction cascades at the membrane, and this facilitates transcription by a later administration of 17β-estradiol (191).

The study utilized a two-pulse hormonal schedule in an ex vivo neuroblastoma cell culture model system. Such a pulse regimen was shown to be previously effective in investigating estrogen’s effects on cell division (67). The first pulse (of either 20-min or 2-h duration) utilized E-BSA, a membrane-limited estrogen conjugate designed to limit estrogen actions to the membrane and the second 2-h pulse utilized 17β-estradiol to stimulate transcription. Transcription from a transiently transfected reporter gene, consisting of three tandem consensus EREs linked to luciferase, was then measured. A 20-min first pulse using E-BSA and a second 2-h pulse utilizing 17β-estradiol, separated by a hormone free interval of 4 h, showed greater transcription than either pulse alone (Fig. 6). Reversal of the order of addition, 17β-estradiol in the first pulse and E-BSA in the second pulse, did not have any effect on transcription, showing that primary membrane effects of estrogens are important to the nuclear effects of estrogens. Addition of inhibitors to the signal transduction cascade in the first 2-h pulse along with E-BSA could inhibit this synergism, demonstrating that activation of protein kinases A and C plays a role in facilitation of transcription by the nuclear 17β-estradiol-bound ER. In contrast, addition of these inhibitors in the second 2-h pulse along with 17β-estradiol did not reduce synergism. This synergistic transcription could be blocked by concomitant addition of the nuclear ER antagonist ICI in either the 20-min first or 2-h second pulse, demonstrating...
that in this model system the mER is similar to the nuclear ER (191).

Could thyroid hormone modulation of ER gene transcription affect either or both of these modes of estrogen action? Discrete targets of modulation impart specificity to thyroid hormone action. Also, the mechanisms of stimulatory cross-talk between the ER and TR remain currently unknown and would be an avenue of future research.

C. Thyroid Hormone Elevation: Does It Signal Cold Temperatures?

Impetus to the examination of TR and ER cross-talk was provided by a series of studies investigating the nature of the mammalian response to cold temperatures and its dependency on thyroid hormone. Cold temperatures are also correlated with seasonal shifts in photoperiod (short day length), which is in turn signal anestrous in several mammalian species. Cold exposure increases the blood levels of thyroid hormones and TSH. Despite elevated inhibitory levels of thyroid hormones, it also causes an increase of TRH mRNA in the PVN, demonstrating the ability of cold to override inhibition by T₃ (219). Such cold stress also transiently elevated corticotropin releasing hormone (CRH) levels, cortisol, and ACTH levels in rats (55). Acute stressors such as uncontrollable foot shock in rats also elevated brain T₃ levels in both male and female rats (54). The role of brown adipose tissue (BAT) in adaptive nonshivering thermogenesis in mammals has been well studied. Intracellular conversion of T₄ to T₃ is adaptive nonshivering thermogenesis in response to cold also'

"duce" this environmental signal to neuroendocrine systems mediating sexual behavior.

VI. SUMMARY

Transcriptional influences of enhancer complexes on gene promoters can depend on complex interplay among
transcription factors. Here we reviewed evidence that two classes of transcription factors, TRs and ERs, can interact in several different modes, which depend on TR isofrom, ER isofrom, gene promoter, and cell type.

The in vivo data utilizing gene knock-out mice also point to different roles for different isoforms for both TRs and ERs. This is evident from the behavioral data where the deletion of one isoform does not lead to the same phenotype as deletion of the other isoforms. For ERα compared with ERβ, enough behavioral assays have been completed to show that different patterns of ER gene expression are required for different patterns of neuronal function and behavior. We note that nonredundancy of expression are required for different patterns of neuronal completed to show that different patterns of ER gene expression

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