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Estrogen action: a historic perspective on the implications of considering alternative approaches

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Abstract

In the 50 years since the initial reports of a cognate estrogen receptor (ER), much has been learned about the diverse effects and mechanisms of estrogens, such as 17 β -estradiol (E₂). This expert narrative review briefly summarizes perspectives and/or recent work of the authors, who have been addressing different aspects of estrogen action, but take a common approach of using alternative considerations to gain insight into mechanisms with clinical relevance, and inform future studies, regarding estrogen action. Their “Top Ten” favorite alternatives that are discussed herein are as follows. 1- E₂ has actions by binding to a receptor that do not require its enzymatic conversion. 2- Using a different strategy for antibody binding could make the estrogen receptor (ER) more discernible. 3- Blocking ERs, rather than E₂ production, may be a useful strategy for breast cancer therapy. 4- Secretion of α -fetoprotein (AFP), rather than only levels of E₂ and/or progesterone, may influence breast cancer risk. 5- A peptide derived from the active site of AFP can produce the same benefits of the entire endogenous protein in endocrine cancers. 6- Differential distribution of ER subtypes in the body and brain may underlie specific effects of estrogens. 7- ER β may be sufficient for the trophic effects of estrogen in the brain, and ER α may be the primary target of trophic effects in the body. 8- ER β may play a role in the trophic effects of androgens, and may also be relevant in the periphery. 9 - Downstream of E₂'s effects at ER β , there may be consequences for biosynthesis of progestogens and/or androgens. 10- Changes in histones and/or other factors, which may be downstream of ER β , potentially underlie the divergent effects of E₂ in the brain and peripheral tissues.

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Keywords

Estradiol; estrogen receptor; trophic; alpha feto-protein; breast cancer; neurosteroid

1. Introduction to the idea of alternative approaches

Estrogen, such as 17 β -estradiol (E₂), is secreted cyclically in adult women from puberty until menopause, and profoundly influences physiology and behavior of females. The breadth of what E₂ does can be observed during menopause, when cyclical E₂ secretion from the ovaries declines. Menopausal women can experience physical (hot flashes, dryness of eyes and vaginal mucosa, increased risk of stroke and coronary heart disease) and/or psychological (sleeplessness, forgetfulness, anxiety, depression, decreased sex drive) changes. Some preclinical and clinical studies show that hormone therapies (HT) can benefit these sequelae. However, the large, and well-publicized, Women's Health Initiative clinical trials were ended early because some women on the most commonly-prescribed therapies (e.g. conjugated equine estrogen) had increased risk to develop breast cancer, stroke, and/or cardiovascular complications. As such, it is important to more fully understand the mechanisms of how E₂ may contribute to both physical and psychological effects when assessing treatment options. Differences in the favorable effects of HT may depend in part upon estrogens' actions. To this end, the authors of this expert narrative review provide their perspectives and discussion of recent studies about how alternative approaches have contributed to the discovery of estrogens' actions and/or implications, over the last fifty years ago, from when the "estrogen receptor (ER)" was discovered, to now. The authors "Top Ten" favorite alternative approaches to their studies of E₂ follow the background of how an alternative approach to climbing the Matterhorn in the Swiss Alps eventually led to the major discovery of a binding site for E₂. How other alternative approaches/considerations have built upon this fundamental work to extend our understanding of estrogen's effects and mechanisms are then described.

2. Dr. Jensen's perspective of how a natural phenomenon shaped his studies on estrogen

It is hard to believe that it is fifty years since our first report of the ER. The whole field, over that fifty year period, has benefited from the consideration of alternative approaches. Alternative approaches for me originated in the Swiss Alps, and, in particular, the Matterhorn, which stands on the Swiss-Italian border. Although I had no experience at all as a mountain climber, I was in pretty good physical shape. With a guide, and a student from Colorado, who was an expert mountain climber, we made it to the top of the Matterhorn and the view was beautiful [1]. When I came back I was amazed to learn that the Matterhorn was the last major mountain in Europe to be climbed. I thought for the expert climbers, it must be child's play. Yet, it turns out that everyone had tried to climb the Matterhorn from the Italian side, which looks less formidable than the northeast side facing Switzerland, which appears to be a sheer wall of rock. Until 1865, when a British engraver, who's hobby was mountain climbing, and who had failed during this conventional approach, came over and with a telescope. He studied the northeast face and came to the conclusion that maybe it is not as difficult as people thought. So, with some companions in 1865, they tried the ascent, and it turned out to be remarkably friendly and there were many crevices which eased their ascent. In 1865, this was the first successful climb of the Matterhorn by trying an alternative approach, which other people had not tried.

3. Dr. Jensen's perspective on the discovery of estrogen action

3.1. Alternative Approach 1- Estradiol evokes responses by binding to a receptor, not through enzymatic conversion

Fortunately, the importance of considering alternative approaches stuck in my memory. I was working in Chicago with Charles Huggins, a urologist, awarded the Nobel prize for his work on androgens and prostate cancer. He was also interested in estrogens and their action. I was so impressed when Dr. Huggins showed me how an immature rat uterus, which is a tiny structure, grew by a factor of about 10, just by giving the animal small amounts of estradiol over the course of a few days. At the time, the widely accepted explanation for the action of estrogen was that within a cell the steroid could be oxidized to estrone by using one co-enzyme, and that product can be reduced to estradiol using another co-enzyme. Essentially, thereby, hydrogen is transferred from what is now called NADH to NADPH. These were known co-factors in many biochemical reactions, especially those producing growth. The one thing that disturbed me was that this mechanism would not explain the actions of diethylstilbestrol (DES), a synthetic estrogen that bears no aliphatic hydroxyl group and could not undergo that reversible oxidation/reduction. Despite that obvious dilemma, most investigators at that time were focused on the tissue-stimulating, biosynthetic processes in hormone-responsive tissue, rather than concerned over what happens to the hormone molecule itself. Now it is accepted that nothing of the sort (oxidoreduction) happens to estrogen. Yet 50 years ago, that was a surprise. Below I have described the alternative approaches to consider the actions of estrogen.

We proposed to find out what happens to a physiological dosage of estrogen that is administered to a female rat. The physiologic dose in this animal is very, very low, less than 0.1 μg . Clearly we would need a radioactive estrogen tracer with a very high specific activity indeed. Fortunately, a young organic chemist, Dr. Herbert Jacobson, had just joined my laboratory group. Dr. Jacobson, who now has been on the faculty at Albany Medical College for 44 years, was a co-discoverer of the ER. We thought that if we could radiolabel the sixth and seventh positions of the E_2 molecule with carrier-free tritium, by catalytic, hydrogenation, of the 6,7 dehydro compound with carrier free tritium gas, that the necessary specific activity would be obtainable. That procedure required using a tremendous amount of radioactivity- a catalytic "tritiumator" with the smallest gas reservoir that Dr. Jacobson and I could devise would require charging with sixty curies of carrier-free tritium. We tested our devise by performing several reductions of 6,7 dehydroestradiol with radioinert hydrogen gas, successfully producing high yields of E_2 , and were ready to work with tritium. Although at that time it was not tenable to work with sixty curies of tritium in the Chemistry Department at the University of Chicago, at nearby Argonne National Laboratory, appropriate facilities existed and were made available to us. Dr. Kenneth Wilzbach generously made his laboratory available to us. We transported our device and reagents there and did the catalytic reduction. In operation, as tritium (or hydrogen) is taken up, the mercury bead moves to the left, and one introduces, via a micrometer-operated syringe, the volume of mercury needed to restore it to its neutral position and maintain constant pressure. We had thought that tritium might react more slowly than radioinert hydrogen, but for some reason which we still do not know the chemical basis of, the reaction proceeded twice as fast with tritium as it did with hydrogen.

We now had tritium labeled estradiol with very high specific activity (about 200 microcuries/microgram) and could reliably measure as little as a trillionth of a gram [2]. We put the product to the use for which it was intended (Fig. 1). We administered a physiologic dose, 90 nanograms, to groups of immature female rats and examined the distribution of tritium taken up by their tissues over time. The uptake of radioactivity in all tissues was rapid, and in most of them peak concentrations was attained by fifteen minutes, and then it declined [2,3]. The highest concentrations were in liver and the kidneys, sites at which substantial steroid metabolism and degradation was found to have occurred. In contrast, in the uterus and vagina

(target organs for E_2), the uptake of tritium and its retention was prolonged, remaining high for one to two hours. Strikingly, during that interval, the concentration of tritium was 100 to 200 times that in the blood, but was nevertheless moving from blood to uterus against that substantial concentration gradient. When we first presented this at an international congress in Vienna, there was skepticism on two general points. First, how did we know this binding had anything to do with physiologic actions of estrogen? Second, what is the enzyme activity for the binding substance? Of course, we soon showed that one could not identify an enzyme for this as the tissue did not have one.

To address the question of physiologic actions, we administered tritiated hexestrol (a non-steroidal estrogen) to rats and we observed the same time course of tritium uptake by uteri and other tissues as we had seen with tritiated E_2 . When nafoxidine (which blocks estrogen-stimulated uterine growth) was administered in various dosages prior to tritiated E_2 , there we saw dose-related inhibition of E_2 -stimulated uterine growth concomitant with dose-related inhibition of tritium uptake [3]. Clearly, the two are related.

Among those holding to enzyme involvement in the estrogen response, the thinking was that E_2 underwent oxidoreduction of its 17β hydroxyl group, being oxidized to estrone with participation of one co-enzyme, and reduced back to E_2 with another co-enzyme. In that scenario, the hydrogen atom lost during the oxidation is not the same one that replaces it during reduction. To examine this, we synthesized E_2 labeled with tritium at position 17, mixed it with this 6,7-tritium labeled E_2 (our original product) and administered the mixture to rats, producing uterine growth. We isolated E_2 that had been taken up by the uteri of these rats and examined the ratio of the amount of tritium at the 17 position to that at 6 and 7, and found that the ratio was unchanged from that in the mixture injected. There had been no loss of tritium from position 17 in the E_2 molecule during E_2 -stimulated uterine growth. Taken together, the evidence suggested that there was an estrogen binding protein (estrophillin, it was called at that time), with which E_2 binds and causes the growth response, apparently without itself undergoing any chemical change.

We were among others in the field making great strides on uncovering E_2 's mechanisms of action at this time. For example, Dr. Jack Gorski made a very nice contribution by using sucrose density gradient centrifugation to show that in homogenized, solubilized uterine tissue, using radioactive estrogen as a marker, there was a very nice peak, indicating this was the ER with the hormone bound to it [4,5]. Further experiments with radioactivity demonstrated concentrated bound E_2 was in the nuclei of uterine cells, from which it could be extracted by aqueous potassium chloride solution. That extract, on sucrose density centrifugation displayed a radioactivity peak that sedimented more slowly than that previously described. Work from other laboratories subsequently showed that the more rapidly sedimenting form is a dimer of the slower form, and that the dimer participates in initiating transcription, and, hence, the estrogen response [6–7].

Notable work was done by Bert O'Malley [8] on the progestin receptor. He showed that similar to the case with the ER, that there existed inactive and active forms of progestin-receptor complexes, and that the active form stimulates transcription of specific RNAs. Repeatedly there has been discovery of this same overall pattern, of inactive transcription factors that become activated by a specific hormone. Dr. John Baxter demonstrated this for thyroid hormone [9]. Similar findings were obtained with glucocorticoids, and later with androgens. So sex steroid and thyroid hormones were prototypes, with estrogen as the *sine qua non*. These are the prototypes of nuclear receptors, which are converted to active transcription factors. At this date, forty nine nuclear receptors that have been identified and characterized. The action of nuclear receptors is a major branch of endocrinology, knowledge that stems from an alternative approach. Indeed, ask not what hormones do to the tissue, ask what tissues do to hormones.

3.2. Alternative Approach 2- Using a different strategy for antibody binding

Now that we had receptor proteins that could be tagged with radioligands, we wanted to produce antibodies to them [10–12]. Expert immunochemists attempted to make antibodies to ERs without success. Again we considered an alternative approach. Perhaps because estrogens are active in such low concentrations, conventional techniques of immunoprecipitation cannot yield a precipitate because it does not form. Our alternative approach? Typically, what has been done is that a receptor is bound to a radioactive hormone and then the antibody is introduced to produce a larger moiety because the antibody is a protein that is about the size of the receptor. What we tried was producing the antibody and the receptor complex first and then adding the hormone marker to form a radioactive immune complex (Fig. 2, top). You would expect then that the antibody would bind the immune complex labeled by the radioactive hormone and, obviously, this would be a larger moiety resulting from the two proteins together. On sucrose density centrifugation, that is what we saw, with an additional bonus that we had not counted on. Different proportions of monoclonal antibodies recognize different epitopes in the receptor molecule. With a monoclonal antibody to the receptor, the first side is occupied, producing a characteristic peak on the gradient. When a second antibody is added that recognizes a different epitope on the molecule, there is additional downward shift for this heavier multiple complex. Not only do monoclonal antibodies bind, but different monoclonal antibodies recognize different regions on the receptor of the molecule. This was of considerable value. It made possible many chemical techniques (i.e. ER enzyme immunoassay and immunocytochemistry; [13,14]) as well as the cloning of a receptor. We had no experience with cloning, so we collaborated with Dr. Pierre Chambon, who had cloning experience, but no receptor antibodies. Briefly, specific antibodies were needed for this. Fractions containing high levels of ER mRNA produced by sucrose gradient centrifugation were utilized to produce cDNA libraries, which were then screened with the specific antibodies to produce clones of the ER. Thus, Dr. Chambon carried out the first successful cloning of the ER with the four antibodies that we provided [15–17,18].

The receptors we identified, and the antibodies we made, have been very useful, not only for basic research, but also for clinical use. The premise of ELISA tests that use two antibodies to recognize different epitopes on the same analyte molecule enable quantitative measurement of that substance. We have produced the antibodies enabling ELISA analysis for ERs. The older method, measuring binding of radioactive hormone is far less convenient than quantitative evaluation by ELISA, or by histopathological evaluation, using our antibodies.

3.3. Alternative Approach 3- Blocking the ER, rather than estradiol production, for breast cancer therapy

Therapeutic treatment of breast cancer has also benefitted from consideration of alternative approaches. In the 1890's, before it was known what estrogens were, or how they were formed, it was observed in premenopausal women with breast cancer, that during the menstrual cycle there were changes in the size of the metastatic mass. Sir George Beatson, a Scottish surgeon who was knighted for his work on estrogens, removed the ovaries of young women with breast cancer. In 1896, he reported that, about one third of his pre-menopausal patients did have substantial diminution of the cancer even though they had metastatic diseases [19]. Later when estrogens were discovered, and it was found that estradiol was synthesized by the ovaries, it became clear that there was a relationship between the cancer and the hormone. Of course, most breast cancer is present, not in premenopausal women, but rather in postmenopausal women. In 1951, Dr. Charles Huggins removed the adrenal glands, a major source of E₂ in postmenopausal women with breast cancer. He saw a substantial number of remissions in his patients. In one striking case, a woman with rather extensive, global metastases had an almost complete remission of the metastases a year after adrenalectomy. Dr. Rolf Luft, a pituitary expert from the Karolinska Institute, removed the pituitary, which controls hormone secretory

activity in both the both the ovary and adrenal. A year after hypophysectomy, cancer metastases were improved.

In the 1950's and 1960's, treatments for breast cancer focused on removing the sources for hormones in premenopausal women, the ovaries, and among postmenopausal women, the adrenals and/or the pituitary controlling them. About one third of patients responded favorably, but the other two thirds that had undergone these serious operations were not helped by them. To address other possible treatments, I worked with Dr. Craig Jordan to investigate effects of anti-estrogens, such as tamoxifen. We used the "Huggins's method" with the Sprague Dawley rat for generating breast cancers, by administration of dimethylbenz[a]anthracene (DMBA) [20]. Tamoxifen was well tolerated by the rats and reduced tumors in these early studies. It was hard to get someone to do clinical studies in the seventies, but Craig Jordan was persistent [21]. We found in women with breast cancer that tamoxifen produced the kind of remission you get from adrenalectomy and/or ovariectomy. Here again we saw about one third of patients responding and without those major surgical procedures.

Next we considered the utility of putting the hormone, and/its organ sources, out of business. Aromatase inhibitors were developed by Dr. Brodie and others to prevent the synthesis of E₂. Arimidex, which is now in use, has fewer side-effects than tamoxifen. This approach blocked estrogen response, not by ablating organs sources, but by interfering with biosynthesis of the hormone at site or origin. To sum up the progress that has been made rather succinctly. "A lady with growth neo-plastic thought castration was just a bit drastic, she preferred that her ill could be cured with a pill, and today it is no longer fantastic."

In the early 1970's, Drs. Henrich Maas at Hamburg, and Edward Engelsman at Amsterdam, were doing adrenalectomy and ovariectomy, and measuring the amount of ER by its binding radioactive hormones. Fewer patients were available for these investigative procedures back then. A total of about 100 patients was recruited. Those whose cancer lesions had a substantial amount of ER experienced a much better response than did those with few ERs [22]. Studies of this nature really convinced people of the relationship between ER and response to hormone manipulation.

In the late 1970's, we completed a study of 160 women with breast cancer in whom we evaluated clinical response to surgical excision of estrogen source, versus quantitative ER concentration found in their cancer [23]. Remissions typically occurred among those women whose tumors had high levels of ER expression. We prefer to grade tumors as ER "rich" or ER "poor", rather than receptor "positive" or "negative". Figure 2 (Fig. 2, bottom) shows a tumor section stained for ER, and it appears that every cell is stain-positive. It is clearly ER rich. Others may have a small number of stained cells (that will respond to therapy), but the majority without receptor, are not estrogen dependent. This ER poor cancer will not benefit by endocrine surgery.

An important contribution to breast cancer management is the strategy of choosing the correct therapy for the individual patient (Fig. 3). A patient whose tumor is receptor poor, but is not completely negative, is still unlikely to experience an optimum therapeutic response. These women, who comprise two thirds of patients, should be put directly on chemotherapy rather than waiting six to eight months to see whether an endocrine modality works, while in the meantime, the cancer is growing [24]. The one third, who are ER rich, should directly have endocrine therapy [24]. Now we come down to the one part that is not really complete and still needs some work. Out of this one third, two thirds will experience remission [24]. So you observe remission produced by endocrine therapy in about 20 to 30% of all the patients. About 5 % of the women are ER rich, but it appears that the receptor binds hormone, is recognized by the antibody, but therapy somehow does not stop its growth [24]. We are working on that now, trying to identify the features of "non-responding" ERs. We need to get 100% of the 1/3

that are ER rich to respond optimally. Alternate approaches have enabled achieving very positive results in our basic studies on estrogen actions, which in turn have achieved the ultimate objective of helping diagnose and treat breast cancer patients.

4. A review of studies on a peptide, derived from α -fetoprotein, as a chemopreventive target of endocrine cancers

4.1. Alternative Approach 4- Secretion of α -fetoprotein, rather than only levels of estradiol and progesterone, influence breast cancer risk

From epidemiological studies, it is clear that a full-term pregnancy early in a woman's life reduces her breast cancer risk [25]. This is supported by animal studies in which carcinogen-induced mammary tumor development is reduced by pregnancy in rats [26–28]. Pregnancy is a complex hormonal state, in which many factors, may contribute to this reduction in breast cancer risk. One such factor that has been identified is α -fetoprotein (AFP) [29], which was discovered as a major fetal protein in the serum of mammals about fifty years ago [30,31]. AFP is 69 kD glycoprotein secreted primarily by the fetal liver [32–34]. Its presence in appreciable levels in the circulation of adults is limited to the maternal bloodstream, during pregnancy until parturition [32,35,36], and, to a lesser extent, production by some tumors. Over two decades ago, it was hypothesized that exposure levels of AFP underlie reduced risk of breast cancer in women [37], and that this was due to endocrine action of this protein on premalignant tissues that would have developed cancer many years after pregnancy [38]. Epidemiological studies [38], as well as those that measured α -fetoprotein levels [39], supported this hypothesis that AFP reduces cancer risk. There was an inverse correlation between the levels of AFP in maternal serum during pregnancy, and the risk of developing breast cancer twenty to thirty years later [39]. Moreover, basic studies substantiated further investigation of AFP for this potentially protective role. AFP reduced growth of transplantable rat mammary tumor [40], as well as human breast cancer xenografts to immunosuppressed mice [41]. As well, growth, of xenografts of estrogen-dependent tumors derived from humans (MCF-7) or rats (MTW9A) in mice, or MTW9A homografts to ovariectomized rats, is inhibited by exposure to AFP co-administered with E_2 [42]. Similar growth-suppressing effects were determined in follow-up studies comparing natural and recombinant human AFP in MCF-7 cells, but not in cells from an estrogen-independent tumor line (MDA-MB-231) [43]. In rats that were exposed to a chemical carcinogen that stimulates mammary tumors, methylnitrosourea (MNU), treatment with pregnancy-related hormones (i.e. E_2 , estriol, progesterone, or human chorionic gonadotropin) reduced tumor incidence and increased serum levels of AFP, compared to saline-administered rats [44]. Together, although these data were promising, we had to consider an alternative approach to harness the beneficial effects of AFP in estrogen-sensitive tissues as a chemopreventive agent.

4.2. Alternative Approach 5- A peptide derived from the active site of AFP can produce the benefits of the entire endogenous protein in endocrine cancers

AFP does not have all the required characteristics of suitable chemopreventive agents, such as small molecular size and ability to administer this agent easily, and ease of economically production and long-term storage stability. As well, the multiplicity of AFP's functions, including its immunosuppressive effects [45], reduce its potential as a useful clinical drug. As such, it was important to investigate small molecule analogs, or peptides, of AFP that would have these necessary characteristics of a suitable drug, low toxicity, and similar, or even greater, efficacy in reducing proliferation in estrogen-sensitive tissues. To this end, a rationale design process was utilized to identify, and/or synthesize, peptides derived from AFP and determine their potential for chemopreventive and/or chemotherapeutic effects in various models of breast cancer or other E_2 -dependent cancer models [46–48].

Recent studies from Dr. Herbert Jacobson's laboratory, and collaborators at Albany Medical College and elsewhere, supports the potential of a synthetic, cyclic 9-amino acid peptide Cyclo [EKTOVNOGN] (AFPep), which was derived from the active site of AFP, as an antiestrogenic and anticancer agent. This peptide was developed following the identification of an 8-amino acid peptide of the active site of AFP [46,49–51]. This peptide was then tested in different whole animal systems for its therapeutic efficacy in mice with E₂-dependent tumor xenografts, its cancer preventive effects in carcinogen-induced mammary tumorigenesis in rats, and its anti-E₂ effect in a mouse uterus growth inhibition model. Tumor burden in rat and mouse cancer models was reduced, and trophic effects of E₂ in the uterus, were inhibited by AFPep [52, 53]. Tumor volume of MNU-administered rats, or mice with MCF-7 cell xenografts, are lower at the end of the study following AFPep treatment compared to vehicle (Fig. 4) [53]. Similar growth-reducing effects of AFPep were demonstrated *in vitro* in E₂-exposed T47D human breast cancer cells, concomitant with reductions in phosphorylation of serine 118 of ERs (Fig. 4) [52]. Of particular clinical importance is that AFPep has been shown to be effective with oral administration, and similar to intraperitoneal and subcutaneous route of administration, to mice and rats [52]. A dose-response study demonstrated that chronic AFPep by oral administration is non-toxic in the animal models utilized [52], and does not produce uterine hypertrophy like other therapeutics used for E₂-sensitive cancers, such as tamoxifen [46]. Of further interest is that AFPep may have anticancer effects in tamoxifen-insensitive tumors. In summary, a molecule, such as AFPep, may be a chemopreventive agent, with little toxicity with long-term usage, or uterotrophic effects, characteristics of some of the most popular clinically-used anti-estrogen agents, such as tamoxifen, which have unwanted side effects and can be ineffective if tissues become unresponsive to this agent. Studies are continuing in Dr. Jacobson's laboratory, and others, to further characterize the novel actions of this and other AFP-derived peptides for their anticancer effects.

5. A review of estrogens' actions at subtypes of ERs for functional effects

5.1. Alternative Approach 6- Different distribution of ER subtypes in the body and brain may underlie specific effects of estrogens

As discussed thus far, some of estrogens' effects and implications involve its binding to the ER and/or interactions with AFP, with a focus on the clinical relevance of these effects in cancer treatment. Another intriguing aspect of estrogen research that has come into focus more recently is that the ER has different subtypes, which may confer tissue specificity for estrogen's actions in the body as well as the brain. Although the first “ER” discovered, now referred to as ER α , was characterized 50+ years ago, a second type, referred to as ER β , has been identified in 1996 [54], and there may be other subtypes yet to be characterized. Generally, ER α is widely expressed throughout most E₂-sensitive tissues, whereas ER β expression is more circumspect. In the periphery, organs, such as the bladder, lungs, and intestines, and glands, such as the prostate, have greater ER β expression than ER α [55–58]. In addition to differences in distribution between tissues, within tissues there can be circumspect expression patterns of ER α and ER β . As one example, the uterus primarily expressed ER α , but there is greater ER β than ER α expression in the glandular epithelium [54,59]. Bone is a second example of such a pattern, wherein there is greater expression of ER α throughout this tissue, but bone marrow has much greater expression of ER β [60]. Indeed, increased expression of ER α in the breast and uterus may account for trophic effects of E₂ in these tissues.

The brain is also a major target of estrogens for their trophic and other functional effects, which have been studied over the last 50 years in mammals. Elegant studies by Dr. Donald W. Pfaff were the first to show the uptake of tritiated E₂ into the rat brain as well as the localization of ER in the rat brain [61–63]. These studies led to the important discovery of the neural circuit for lordosis behavior, and E₂-dependent response that is critical for mating behavior of rodents and was the very first circuit for a mammalian behavior that was characterized [64]. Over

20 years ago, the first paper describing the important trophic actions of estrogens in the brain were described [65]. Since then, the potential mechanisms for these effects have been intensely investigated. Within the brain, there is great co-expression of ER α and ER β in some regions (e.g. medial amygdala, preoptic area, bed nucleus of the stria terminalis), but, in others, there is greater expression of ER α (ventromedial hypothalamus, pituitary) or ER β (anterior olfactory nucleus, cerebral cortex, hippocampus, dorsal raphe, substantia nigra, midbrain ventral tegmental area, cerebellum) [66–68]. This distribution of ER α and ER β , which is overlapping but also shows distinct differences in expression, has substantiated investigation of whether there are functional effects associated with ER α and ER β . There are clear effects of E₂ at ER α for reproduction-related function, such as that associated with motor activity/behavioral arousal, social memory, and lordosis, which are thought to involve ER α in the hypothalamus and projection sites [69–72]. Conversely, the role of ER β for its behavioral effects may be more focused on functions associated with forebrain and/or limbic structures. The investigation of actions of ER β for cortical and hippocampal processes by Drs. Frye and Walf and others is described as follows. Indeed, if beneficial effects of E₂ occur independent of actions at ER α , there is potential to exploit favorable actions of E₂, with minimal trophic effects on breast and/or uterus. To this end, this group, and others, has been investigating the relative role of E₂'s actions at ER α and ER β for its functional effects in rodent models.

5.2. Alternative Approach 7- Determining whether ER β may be sufficient for the trophic effects of estrogen in the brain, and ER α in the periphery may be the primary target of trophic effects in the body

As discussed in the sections describing Drs. Jensen and Jacobson's work, there is a clear need to understand estrogen action as it relates to cancers of the reproductive system (breast, uterus, ovary). For example, recent studies have further confirmed that the role of E₂ to increase risk for breast cancer is widely recognized [73], and greater breast cancer risk has been shown to be associated with high serum E₂ [74–77] and administration of E₂ to postmenopausal women [78,79]. Similar effects are found in recent studies using whole animal systems. As an example, in a recent study, Drs. Frye and Walf found that young adult, ovariectomized rats administered physiological dosages of subcutaneous E₂ had increased tumor incidence, which was potentiated with exposure to the chemical carcinogen, DMBA, which reliably induces mammary tumors [80,81]. In this model, E₂ increased uterine weight of rats, as well as had behavioral effects, irrespective of DMBA dosing [80,81]. Thus, E₂ can have trophic actions in ER α -associated tissues, and whole animal models may be used to further investigate the role and mechanisms of E₂ in these tissues as related to brain function, which is an ongoing question in this lab.

Key to eradication of breast cancer is, not only understanding its risk factors, but also the mechanisms of E₂ that may have a protective effect and/or reduce negative outcomes. Unlike what is observed with ER α and proliferation, the effects of ER β may be protective. Although ER β is typically not highly expressed in mammary glands, prognosis in women with breast cancer that have greater expression of ER β is more favorable [82]. In the animal model described above, whether there would be differences between ER α and ER β selective modulators (SERMs) for tumor incidence and uterine weight of ovx rats administered DMBA or not was investigated. The ER α -SERM, propyl pyrazole triol (PPT), increased tumor incidence and uterine weight, similar to that observed with E₂ (Fig. 5). Conversely, an ER β -SERM, diarylpropionitrile (DPN), did not have these effects (Fig. 5). Thus, ER β may not be associated with proliferative effects like ER α , but this is not entirely clear, and is being investigated.

Areas of interest in the central nervous system for E₂ action are the hippocampus and cortex, which are regions involved in cognition and affect. There is evidence that women can respond

favorably to estrogens with improved cognition and affect; however, there are some inconsistencies in these results, which require further investigation. Some of these limitations in the clinical studies of E₂ for its functional effects have been explored using animal models. A typical approach that is taken to model menopause and the effects of E₂ is to ovariectomized rats or mice and administered E₂ or E₂-mimetics. Compared to proestrous/sexually-receptive rats (with high E₂ levels), ovariectomy increases anxiety and depressive behavior, and decreases sexual responding (an E₂-dependent behavior) of rats and mice [83–87]. These effects can be reversed with administration of E₂, but the magnitude of the response is influenced by dosing and/or E₂ regimen utilized [84]. Research characterizing E₂'s actions at ER α and/or ER β for physiological and/or psychological effects is ongoing in our laboratory. Anxiety-like responding of ovariectomized rats is reduced with E₂ or DPN, but not PPT (Fig. 6a), concomitant, with the changes described above for tumorigenesis and uterine proliferation. To date, several approaches (e.g. knockout mice, targeted knockdown of ER β in the brain with antisense oligonucleotides, and studies comparing effects of compounds/SERMs with varying specificity for ER α and ER β) have been used to investigate the functional effects of steroids at ER β [86]. Together, these studies have shown that actions via ER β may be favorable for behaviors mediated by the hippocampus, such as affect.

In summary, actions at ER β may underlie favorable psychological effects in animal models, and that some of these effects may be parsed from their potential for proliferative effects in E₂-sensitive tissues in the periphery. Studies are ongoing to further characterize the psychological effects of estrogens, in relation to their trophic effects in the periphery.

How taking this alternative approach to investigations of estrogens' actions via ERs, and potential downstream modulators, has informed studies investigating the actions of progestogens and androgens is described below.

6. A review of the potential downstream (neuro)endocrine targets of ER β

6.1. Alternative Approach 9- Potential role of progestogens and androgens for E₂'s effects at ER β

6.1.1. Potential role of progestogens for E₂'s effects at ER β —These studies described thus far have demonstrated the clear role that ER β can have for some of its functional effects. A question is what are the potential neuroendocrine signaling mechanisms downstream of ER β that account for the trophic effects in the central nervous system and in the periphery. One recent focus of Dr. Frye's work has been the idea that estrogens may have actions through ER β to enhance progestogen formation, or interact with androgenic neurosteroids, which may underlie beneficial effects on psychological processes (anxiety, learning) and mitigate undesirable tumorigenic effects. Indeed, these effects may be related to not only what the hormones do to the receptor, but also what actions the receptor may have on the hormones themselves.

Separate lines of research on the non-classical effects of steroids for functional processes in Dr. Frye's laboratory have converged to suggest that a common denominator may be the progesterone (P₄) metabolite and neurosteroid, 3 α -hydroxy-5 α -pregnan-20-one (3 α ,5 α -THP). Ovarian P₄ is converted by 5 α -reductase to dihydroprogesterone, which is then metabolized to 3 α ,5 α -THP by 3 α -hydroxysteroid dehydrogenase. 3 α ,5 α -THP is also produced from central biosynthesis [87–89]. As with E₂, women experience variations in progestogen levels across the menstrual cycle, pregnancy, and with aging. During the luteal phase, compared to the follicular phase, levels of 3 α ,5 α -THP in circulation are 2–4 fold higher [90–94]. Throughout pregnancy, circulating levels of P₄ and 3 α ,5 α -THP increase with gestation, and peak late in the 3rd trimester, and 3 α ,5 α -THP levels decline precipitously within 1 hour of delivery. Levels of P₄ and 3 α ,5 α -THP in the brains menopausal women show the expected patterns of menstrual

variations, but lower levels post-menopause [95]. An analogous pattern occurs for female rats and mice [83,96–101]. Reproductive history is one of the strongest risk factors for breast cancer, in addition to genetics and age [102]. Although greater lifetime exposure to E₂ and progestogens increase risk for breast cancer in some women, women who have a first pregnancy in young adulthood have decreased risk to develop breast cancer, as described above [103]. As well, synthetic progestogens are typically given to non-hysterectomized women on E₂-based hormone therapy (HT) to counter unwanted proliferative effects. Progestogens are typically thought of as having negative prothrombotic and other growth-enhancing effects through actions at cognate progestin receptors (PRs). For example, unlike its role in the uterus, P₄ can have mitogenic effects in breast, primarily via acting at PRs [104]. However, as described in several studies from Dr. Frye's laboratory, the non-classical progestogen, 3 α ,5 α -THP, can have trophic actions and enhance cognition and affect, and protect against brain injury, via non-PR actions, such as working through neurotransmitter targets (e.g. GABA, glutamate, and/or dopamine receptors) [105,106]. The mechanisms, which may involve changes in cell cycle, of 3 α ,5 α -THP for these trophic effects in the brain vs. body, are of great interest.

Further investigation is needed to determine whether the beneficial effects of estrogens through ER β may be due, in part, to 3 α ,5 α -THP. Although it has long been known that E₂ can increase 3 α ,5 α -THP formation by enhancing activity of 5 α -reductase enzymes [107], emerging studies by Dr. Frye's laboratory suggest that these actions may have functional effects [108,109]. In ovariectomized rats, ER β -selective estrogens enhance cognitive and anti-anxiety behavior (Fig. 6a) [86,110], increase plasma 3 α ,5 α -THP levels (Fig. 6a), and expression of 5 α -reductase, an enzyme necessary for 3 α ,5 α -THP production (Fig. 6b). In these studies, the effects of treatment with E₂, which binds readily to ER α and ER β , to SERMs, which have greater affinity for ER α (PPT) or ER β (DPN), were compared. Mutant mice that do not express ER β (KO) have comparable E₂ levels as wildtype (WT) mice, but poorer cognitive performance, less anti-anxiety behavior [86,111–113], and lower hippocampus 3 α ,5 α -THP levels than WT mice when in proestrous, or when OVX and administered E₂ or DPN (Fig. 7).

6.1.2. Potential role of androgens for E₂'s effects at ER β —Given the data described above about the effects of estrogens at ER β altering 5 α -reductase activity, the role of androgens, such as testosterone (T), and its 5 α -reduced androgens, as well as its aromatized metabolite, E₂, are of interest. The presence of androgens early in development results in pervasive effects throughout the lifespan that include a pattern of androgen secretion. In older men, there is a pattern for increased anxiety and decreased performance in visuospatial tasks concomitant with reductions in T levels [114–116]. Administration of T to some of these men reinstates their affective and cognitive performance [114,117,118]. Of clinical importance, among older men, decline in androgens, and an increase in estrogens, may underlie risk for prostate proliferation. Although treatments for prostate cancer include those that alter androgen levels and/or actions, as prostate cancer progresses, carcinomas can become steroid-insensitive and metastasize, leaving no effective treatments. Thus, steroids clearly play a key role in the prognosis of prostate cancer; however, very little is known about the steroid-mediated mechanisms of prostate cancer prevention and/or progression in relation to androgens' effects in the central nervous system.

Androgens, such as T and its metabolites, may have specific receptor targets for their trophic effects in the brain for behavior and in peripheral tissues, such as the prostate, which contains high levels of androgen targets, such as androgen receptors (ARs) and ERs. Notably, ER β was first characterized in the rat prostate [54,55], and whether the role of androgens through ER β in the brain and periphery can be parsed out are of continued interest. In rodent models, T and its 5 α -reduced metabolite, dihydrotestosterone (DHT), can exert effects to reduce anxiety and enhance cognitive performance [119], as well as have proliferative effects in the prostate in part through their actions at ARs [120]. T and its aromatized metabolite, E₂, which binds both

ER α and ER β , can enhance prostate pathologies, but the role of ERs in these effects is of interest. The DHT metabolite, 3 α -Andostanediol (3 α -diol), which is sufficient to produce androgens' affective and cognitive-enhancing effects, binds to both ER β and γ -aminobutyric acid receptors, while the 3 α -diol metabolite, 3 β -Andostanediol (3 β -diol), can bind with high affinity to ER β [121–127]. SERMs and DHT metabolites, 3 α -diol and 3 β -diol, which all bind selectively to ER β , enhance learning and affect of young and aged male mice, but this effect is not observed in mice lacking ER β (β ERKO) [86,128], that also develop prostate hyperplasia [121]. Together, these data suggest that 3 α -diol may have actions at ER β in the brain that are favorable, and, with further study, these effects may be able to be dissociated from trophic actions in the prostate.

Given the important pleiotropic effects of progestogens and androgens, how beneficial effects are related to actions involving ER β and/or their capacity to enhance 3 α ,5 α -THP or 3 α -diol synthesis or action must be further examined. Further understanding of estrogens' actions via ERs, and other targets, will continue to elucidate the actions and implications of estrogens, progestogens, and androgens for their proliferative effects in the body and psychological effects.

6.2. Alternative Approach 10- Potential role of histones and other factors that may be downstream of ER β

In addition to further investigating the trophic effects involving ER β in the brain and in peripheral tissue, Dr. Walf has been concentrating on investigating other potential downstream mechanisms for these effects. One target of interest is histones (e.g. Histone 3) and their modifications, including acetylation and phosphorylation. One reason to begin to understand these effects further in the brain and body is because inhibitors of histone deacetylases (HDACs) are currently being investigated as treatments for breast, and other, cancers. HDACs target histone 3 and 4, in particular, by altering their acetylation, chromatin structure, and the cell's transcriptional activity, generally resulting in repression. As such, for cancer treatments, the ability to reduce repression of genes involved in cell cycle arrest, and cell differentiation and death, may be beneficial. A second reason to investigate these changes in histones is the increasingly clear involvement of epigenetic changes in expression of behaviors and other functional effects. Using the model described above in which female rats are exposed to DMBA, or not, and are administered SERMs and behavioral and other trophic effects are determined, recent studies have begun to characterize changes in histone modifications, as well as expression of other factors, such as cyclin D1 related to the cell cycle [129]. These experiments have begun to reveal a divergent pattern in histone modifications, as well as in other factors, for trophic processes in the central nervous system compared to peripheral tissues. Of interest is the clear role that estrogens, through ERs, may have on other molecules. As an example in the central nervous system, there is indication that activating ER α or ER β with SERMs produces divergent effects on the expression of synaptic proteins in the hippocampus, such as glutamate receptor subtypes [130]. Thus, investigations are continuing to further elucidate potential differences in regulation of histone 3 modifications, and other factors, associated with changes in E₂-sensitive central tissues, such as the hippocampus, as well as in peripheral tissues, such as uteri and tumors.

7. Summary

There have been many advances in the last fifty years on the study of estrogen action and its implications for health [131], with a focus on ER binding. Indeed, estrogens have clear effects that are independent of the mechanisms described within this review (as depicted in Fig. 8, and reviewed here and in: [86,1132–136]). This expert narrative review briefly summarizes perspectives and studies of persons who have been addressing this question for different times,

yet have each used alternative approaches to gain insight and inform future studies regarding effects and mechanisms of E₂.

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Fig. 1.

Top: A cartoon representation of the apparatus that was utilized to tritiate estradiol. Bottom: Graph below demonstrates the amount of radioactivity (dpm; as a measure of tissue sensitivity to estradiol) in various organs (uterus, vagina, liver, kidney, blood, muscle) up to 16 hours after injection of tritiated estradiol. Based upon results reported in [2,3].

Fig. 2. Top: Schematic of estrogen binding to antibody and receptor. Bottom: Immunocytochemistry examples of how estrogen receptor (ER) antibody was utilized to stain ER expression in mammary tumor tissue, with the sample on the left having low ER immunoreactivity ("ER poor") and the sample on the right have more ER immunoreactivity ("ER rich").

Fig. 3. Depiction of typical rates of estrogen receptor (ER) rich or ER poor breast cancer. This information was then used to put patient on endocrine and/or chemotherapy. A better outcome was demonstrated for those who had ER rich breast cancer with endocrine therapy. However, a question remained on how to treat the ~12% of patients who have ER rich tumors, but do not respond to endocrine therapy.

Fig. 4.

Tumor volumes following α -fetoprotein (AFP) peptide treatment (AFPep) in two animal models of breast cancer (carcinogen-induced cancer model in rats (A) and MCF-7 tumor xenograft model in mice (B)). In both cases, volumes of tumors across groups at the end of the experiment were reduced by administration of AFPep compared to vehicle (* $p < 0.05$). Based upon results reported in [52,53].

Fig. 5. Tumor (A) and uterine (B) wet weight (mean $\pm$ sem) of rats that were administered placebo vehicle, estradiol (E₂), or an ER α -SERM (propyl pyrazole triol- PPT), or an ER β -SERM (diarylpropionitrile- DPN) once a week for 14 weeks. E₂ and PPT increased these weights compared to vehicle (* p<0.05).

Fig. 6.

Anxiety behavior in the elevated plus maze (A) of rats administered placebo vehicle, estradiol (E₂), or an ERα-SERM (propyl pyrazole triol- PPT), or an ERβ-SERM (diarylpropionitrile- DPN). E₂ and DPN increased time spent on the open arms of the elevated plus maze compared to vehicle (* P<0.05). Circles indicate plasma levels of 3α,5α-THP (mean ± sem). Western blot showing representative bands (B) of the 5α-reductase type 1 enzyme, and the loading control β-actin, in the hippocampus of rats treated with vehicle, PPT, E₂, or DPN. E₂ and DPN increase expression of 5α-reductase in these samples.

Fig. 7.

Cognitive performance of adult female mice (genetic knockouts for ER β - KO; or their wildtype-WT counterparts) administered placebo vehicle, 17 β -estradiol (E₂), or diarylpropionitrile (DPN). E₂ and DPN enhanced performance in this task of WT, but not KO, mice, compared to vehicle administration (* P<0.05). Circles indicate hippocampus levels of 3 α ,5 α -THP in mice. KO mice have lower levels of 3 α ,5 α -THP in the hippocampus than do WT mice after administration of E₂ or DPN. Based upon results reported in [111].

Fig. 8.

Some of the potential mechanisms, in the cell itself (A) or via metabolism to other neuroactive steroids (B), of estradiol (E₂) for its functional effects. E₂ can have classic effects to bind to estrogen receptors (ERs), such as ER α and ER β , and have transcriptional activity. E₂ may interact with binding proteins, such as α -fetoprotein (AFP). E₂ may also interact with other membrane receptors, such as an ER (which may potentiate intracellular ER function), G-protein coupled receptors (GPCRs), growth factors, and/or neurotransmitter systems, which may involve activation of signal transduction pathways (e.g. adenosine 3',5'-monophosphate-cAMP, protein kinase A- PKA, phospholipase C- PLC, protein kinase C- PKC, mitogen activating protein kinase- MAPK, extracellular signal-regulated kinase- ERK1/2). E₂'s functional effects through ER β may be via altering steroid metabolism of progestogens and androgens.